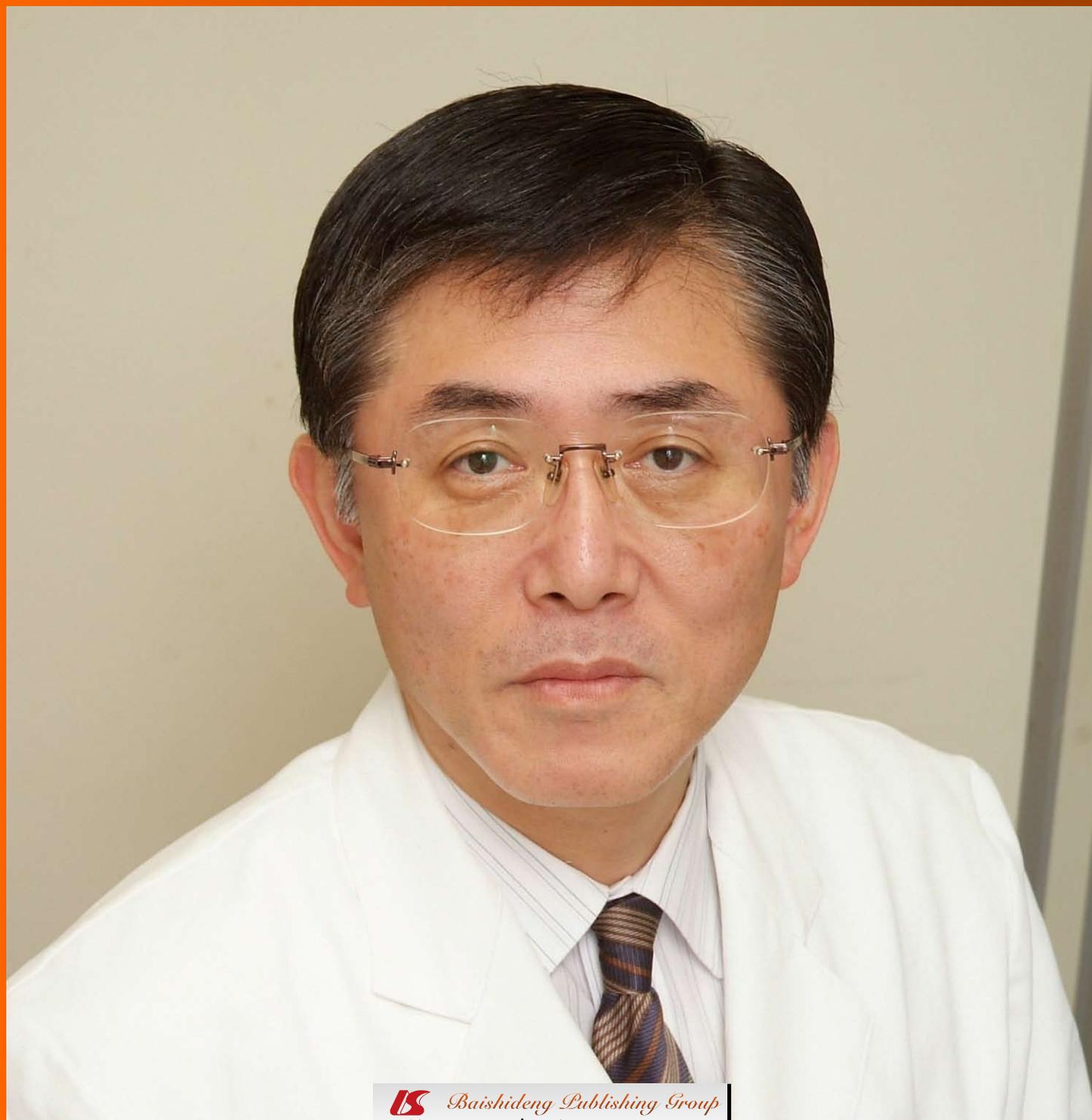


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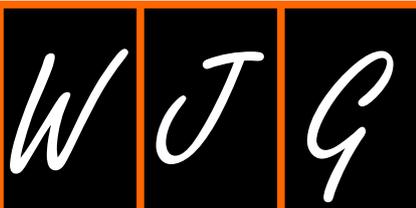
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Steroid-sparing strategies in the management of ulcerative colitis: Efficacy of leukocytapheresis

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Abstract

Active ulcerative colitis (UC) is frequently associated with infiltration of a large number of leukocytes into the bowel mucosa. Leukocytapheresis is a novel nonpharmacologic approach for active UC, in which leukocytes are mechanically removed from the circulatory system. Current data indicate that leukocytapheresis is efficacious in improving response and remission rates with excellent tolerability and safety in patients with UC. Corticosteroid therapy remains a mainstay in the treatment of active UC; however, long-term, high doses of corticosteroids usually produce predictable and potentially serious side effects. If leukocytapheresis can spare patients from exposure to corticosteroids, the risk of steroid-induced adverse events should be minimized. This may be of great benefit to patients because severe side effects of steroids seriously impair health-related quality of life. In this article, we reviewed current evidence on whether leukocytapheresis can avoid or reduce the use of corticosteroids in the management of patients with UC. Several studies have shown that leukocytapheresis was effective for steroid-naïve patients with active UC. Furthermore, both short-term and long-term studies have demonstrated the steroid-sparing effects of leukocytapheresis therapy in patients with UC. Although the evidence level is not striking, the

available data suggest that leukocytapheresis can avoid or reduce the use of corticosteroids in the management of UC. Large, well-designed clinical trials are necessary to more accurately evaluate the steroid-sparing effects of leukocytapheresis in the management of UC.

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Key words: Corticosteroid; Granulocyte and monocyte adsorptive apheresis; Leukocytapheresis; Steroid-naïve patients; Steroid-sparing effect; Ulcerative colitis

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INTRODUCTION

Active ulcerative colitis (UC) is frequently associated with infiltration of a large number of leukocytes into the bowel mucosa^[1]. The infiltrated leukocytes release degradative enzymes, oxygen derivatives and proinflammatory substances that can cause bowel injury and promote further inflammation^[2,3]. Removing excess and activated circulating leukocytes by apheresis has the potential to improve the condition of patients with inflamed bowels.

LEUKOCYTAPHERESIS

Leukocytapheresis is a novel nonpharmacologic approach

for active UC, in which leukocytes are mechanically removed from the circulatory system^[4-7]. Different apheresis techniques remove different types of leukocytes, and have different adsorption capacities. The two most common techniques involve drawing blood *via* a venous catheter, pumping it through a column containing cellulose acetate beads (Adacolumn) or a filter of nonwoven polyester fibers (Cellsorba), thereafter returning it to the circulatory system. As blood passes through the system, leukocytes adhere to the beads or filter. Leukocytapheresis appears to avoid and control an excess of cytokines by removing activated leukocytes from patient peripheral blood and inflamed bowels^[5,7]. However, the detailed biochemical mechanisms underlying the effects of leukocytapheresis remain largely unknown.

CORTICOSTEROIDS

Corticosteroid therapy remains a mainstay in the treatment of active UC^[8-11]. Patients frequently experience improvement in their symptoms within days of starting corticosteroids. During an acute severe exacerbation, approximately two-thirds of patients will respond to intravenous corticosteroid therapy. For steroid-refractory patients, options are limited to surgery or second-line agents, such as cyclosporine or infliximab, used in an attempt to avoid colectomy.

In the study by Faubion *et al*^[12], 63 patients with active UC were treated with corticosteroids. Short-term outcomes (30 d) were complete remission in 54% of patients, partial remission in 30%, and no response in 16%. One-year outcomes were prolonged response in 49% of patients, corticosteroid dependence in 22%, and operation in 29%. This study underlines the fact that most patients with UC initially respond to steroids, but after one year a significant proportion loses the response; this leads to steroid-dependency or the need for surgery, even among those who initially responded to the treatment. The pathophysiology of corticosteroid resistance and dependency in UC is poorly understood^[13]. Leukocytapheresis removes from the body blood cells that contribute to UC and, therefore, unlike corticosteroids, it is not expected to induce dependency or refractoriness.

EFFICACY AND SAFETY OF LEUKOCYTAPHERESIS: A SYSTEMATIC REVIEW AND META-ANALYSIS OF CLINICAL TRIALS

The data obtained from uncontrolled studies^[14-18] are generally quite consistent: a high response rate has been achieved in corticosteroid-naïve patients and a remission rate of approximately 50% has been achieved in patients with steroid-dependent or steroid-refractory UC. Additionally, leukocytapheresis is safe and well tolerated^[14-18]. The largest randomized, double-blind, sham-controlled study of Adacolumn leukocytapheresis therapy failed to

demonstrate efficacy for the induction of clinical remission or response in patients with moderate-to-severe UC^[19]. A number of meta-analyses^[20-22] were conducted to assess the safety and efficacy of leukocytapheresis compared with conventional pharmacotherapy in patients with UC. In the trials that compared leukocytapheresis and corticosteroids, side effects were much less frequent in patients treated with leukocytapheresis. Few severe adverse events were observed during leukocytapheresis therapy. Unlike corticosteroids, leukocytapheresis is associated with an excellent safety and tolerability profile. Furthermore, leukocytapheresis induces a clinical remission in a higher proportion of UC patients as compared to conventional medical therapy. However, many of the studies evaluated in the meta-analyses were conducted in Japanese patients, which may limit generalizability. High-quality randomized controlled trials (RCTs) comparing leukocytapheresis with conventional medical treatment or sham procedure in Western populations are required^[20-22].

POTENTIAL ADVANTAGES OF LEUKOCYTAPHERESIS OVER CORTICOSTEROIDS

Long-term, high doses of corticosteroids usually produce predictable and potentially serious side effects. If leukocytapheresis can spare patients from exposure to corticosteroids, the risk of steroid-induced adverse events should be minimized. This may be of great benefit to patients because severe side effects of steroids seriously impair health-related quality of life. In this article, we reviewed current evidence on whether leukocytapheresis can avoid or reduce the use of corticosteroids in the management of patients with UC.

FACTORS AFFECTING EFFICACY OF LEUKOCYTAPHERESIS

In a number of studies^[23,24], factors affecting the efficacy of leukocytapheresis were identified. We conducted a prospective study to identify factors affecting clinical and endoscopic efficacies of Adacolumn leukocytapheresis in patients with active UC^[23]. In the multivariate analysis, the dose of prednisolone administered at entry and the cumulative dose of prednisolone administered before entry were significant independent factors for both clinical and endoscopic remission, and negatively impacted the efficacy of leukocytapheresis. It appears that steroid-naïve patients and patients on low dose steroid and short duration of exposure respond to leukocytapheresis. Suzuki *et al*^[24], searched for predictors of clinical response to Adacolumn leukocytapheresis. First UC episode and short disease duration appeared to be good predictors of response to leukocytapheresis. From these data, leukocytapheresis may be a promising candidate therapy for steroid-naïve patients with active UC. Furthermore, leukocytapheresis can be an effective first-line treatment in

Table 1 Leukocytapheresis for steroid-naïve patients with active ulcerative colitis

Ref.	Patients (n)	Leukocytapheresis (sessions/wk)	Remission ¹ rate (%)
Hanai <i>et al</i> ^[14]	Steroid-naïve 8 (steroid-refractory 31)	Adacolumn 11/11	Naïve 88 (refractory 81)
Suzuki <i>et al</i> ^[15]	20	Adacolumn 5-10/2.5-5	85
Tanaka <i>et al</i> ^[25]	Steroid-naïve 26 (steroid-dependent 19)	Adacolumn 11/12	Naïve 85 (dependent 58)
Nishioka <i>et al</i> ^[26]	9	Cellsorba 10/10	33 (89 improved)
Umehara <i>et al</i> ^[27]	18	Cellsorba 5/5	61

¹Remission was defined as a clinical activity index (CAI) decrease to 4 or less and mucosal vascular patterns became at least partly visible in the studies by Hanai *et al*^[14], Suzuki *et al*^[15], and Nishioka *et al*^[26], a CAI decrease to 4 or less in the study by Tanaka *et al*^[25], and a CAI decrease to less than 4 in the study by Umehara *et al*^[27].

patients with active UC.

LEUKOCYTAPHERESIS FOR STEROID-NAÏVE UC

Leukocytapheresis has been mainly used for patients with steroid-dependent or steroid-refractory moderate-to-severe UC. About half of patients with steroid-dependent or steroid-refractory UC achieve clinical remission during a course of leukocytapheresis therapy^[20-22]. So far, five small-scale observational studies^[14,15,25-27] have evaluated the efficacy and safety of leukocytapheresis for steroid-naïve patients with active UC. Of these five studies, one study^[14] also included steroid-refractory patients, and one study^[25] steroid-dependent patients. Another study^[26] compared the outcomes of steroid-naïve patients treated with leukocytapheresis and corticosteroid therapy.

A brief summary of the five studies is presented in Table 1. In the short-term, the majority of patients achieved clinical improvement. The remission rate immediately after leukocytapheresis therapy ranged from 33% to 88%^[14,15,25-27]. Quantitative pooling of data was not feasible due to the diversity of interventions and outcome measures among the studies. In a prospective study by Hanai *et al*^[14], 81% of steroid-refractory and 88% of steroid-naïve patients achieved clinical remission one week after the last apheresis session. At 12 mo, 79% of patients had maintained their remission. In a prospective study by Suzuki *et al*^[15], 85% of patients achieved clinical remission during a course of leukocytapheresis therapy. At eight months, 60% of patients had maintained their remission. In the study by Tanaka *et al*^[25], the response rate was 85% in steroid-naïve patients and 58% in steroid-dependent patients. On average, remission was sustained with 5-aminosalicylic acid (5-ASA) for 7.8 mo in the responders. This is the first report showing a striking differ-

ence in clinical response to Adacolumn leukocytapheresis between steroid-naïve and steroid-dependent patients. In a controlled study by Nishioka *et al*^[26], 29 steroid-naïve patients were selected to be treated with Cellsorba leukocytapheresis ($n = 9$) or steroids ($n = 20$). In the steroid group, patients with moderately active disease received 0.5 mg/kg/d of prednisolone and those with severe disease 1.0 mg/kg/d. Eight patients (89%) in the apheresis group and 16 (80%) in the steroid group showed clinical improvement, and three (33%) in the apheresis group and seven (35%) in the steroid group achieved clinical remission. Three major adverse effects were observed in the steroid group, but none were observed in the apheresis group. The efficacy and safety of leukocytapheresis were equivalent, and in terms of severe adverse effects, superior to those of steroid therapy. In the study by Umehara *et al*^[27], 18 steroid-naïve patients with moderately active UC received weekly leukocytapheresis therapy with Cellsorba for five consecutive week. The remission rates at 8 and 48 wk after the last apheresis session were 61% and 28%, respectively. At 48 wk after achieving remission, the relapse rate was 55%, and the duration to relapse was 8.7 mo. In all studies^[14,15,25-27], leukocytapheresis was well tolerated, and no severe side effects were observed.

EFFICACY OF LEUKOCYTAPHERESIS WITHOUT CONCOMITANT STEROID THERAPY

In patients with moderately to severely active UC who failed to respond to optimal doses of 5-ASA compounds, systemic corticosteroids should be used. A few studies^[17,28] evaluated the efficacy and safety of leukocytapheresis without concomitant steroid therapy for patients who failed to respond to 5-ASA compounds. In our prospective study^[17], 30 consecutive patients with active distal UC were treated with weekly Adacolumn leukocytapheresis (a total of five sessions). During treatment, corticosteroid was not given. The median disease activity index score significantly decreased from six to two. Clinical remission was achieved in 21 patients (70%) after the last apheresis session. No serious side effects were observed. Ashida *et al*^[28] conducted a multicenter study to investigate the efficacy of leukocytapheresis without concomitant steroid therapy in patients with active UC. Twenty patients were treated with Cellsorba leukocytapheresis (twice a week for three weeks). The Lichtiger's clinical activity index score significantly decreased from 11.7 to 6.6 after the treatment. Of the 20 patients, 15 (75%) responded, and 7 (35%) achieved complete remission. No serious adverse reactions were observed.

In an RCT by Bresci *et al*^[29], 80 patients with active UC were randomly divided into two treatment groups: patients in the apheresis group received a five-session (one session per week) treatment with Adacolumn leukocytapheresis, and those in the steroid group were treated with methylprednisolone. Concomitant therapy with oral

5-ASA (2.4 g/d) was maintained in both groups. Patients who achieved remission were clinically and endoscopically followed for 12 mo after the end of leukocytapheresis or methylprednisolone therapy. Remission was achieved in 73% of patients in the apheresis group *vs* 50% in the steroid group. Leukocytapheresis was superior to methylprednisolone for the treatment of active UC, even though no statistically significant difference was observed. After a 12-mo follow up, a sustained remission was recorded in 40% of patients in the apheresis group *vs* 25% in the steroid group. Patients who had obtained remission after a course of leukocytapheresis showed fewer relapses during the follow up compared to those treated with methylprednisolone. During leukocytapheresis, only a transient mild headache was recorded in 10% of patients, whereas side effects were observed in 50% of those treated with methylprednisolone. The incidence of side effects in the apheresis group was significantly lower than that in the steroid group. Leukocytapheresis therapy seems able to maintain the condition of remission for a longer time after a flare.

STEROID-SPARING EFFECTS OF LEUKOCYTAPHERESIS

Leukocytapheresis could be an alternative treatment for steroid-dependent UC. A number of clinical trials^[30-34] evaluated the steroid-sparing effects of leukocytapheresis in patients with UC. Quantitative pooling of data was not feasible due to the diversity of interventions and outcome measures among the studies.

In an RCT by Hanai *et al*^[30], 69 patients with steroid-dependent UC were assigned to receive Adacolumn leukocytapheresis in addition to standard drug therapy (apheresis group, *n* = 46) or prednisolone (steroid group, *n* = 23). At week 12, 83% of patients in the apheresis group achieved remission *vs* 65% in the steroid group. During the 12 wk of treatment, the cumulative amount of prednisolone received per patient was significantly lower in the apheresis group than in the steroid group (1157 mg *vs* 1938 mg). Adacolumn leukocytapheresis therapy appeared to be an effective adjunct to standard drug therapy of moderately severe UC by promoting remission and sparing steroids.

The therapeutic benefit of leukocytapheresis in the maintenance of remission was additionally elucidated in a randomized pilot trial by Emmrich *et al*^[31]. Twenty patients with chronic active UC were treated weekly with CellSORBA leukocytapheresis for five weeks. A significant decrease in the activity index was observed. Fourteen patients achieved clinical remission, and mucosal healing was observed endoscopically in six patients. After randomization these 14 patients in remission entered a second period of either monthly leukocytapheresis (*n* = 8) or no further treatment (*n* = 6). In both groups, steroids were tapered down. After six months, five patients (63%) in the apheresis group remained in remission *vs* one patient (17%) in the control group. These results sug-

gest leukocytapheresis offers a therapeutic option in the induction and the maintenance of remission in chronic active UC.

In a prospective study by Cabriada *et al*^[32], 18 patients with steroid-dependent UC were treated with leukocytapheresis plus steroids after failure or intolerance to immunomodulators. Clinical and endoscopic examinations were conducted at one month after the last apheresis session and at 12 mo. The clinical, endoscopic remission and the relapse during the one-year follow-up were evaluated. Clinical remission was achieved in 10 patients (55%) after the treatment. At one year, sustained steroid-free clinical remission was observed in nine patients (50%). A tendency for sustained remission at one year was observed when initial endoscopic remission was achieved. These results suggest that initial remission can be maintained at one year in half of the patients without the need for additional steroids. Complete remission and endoscopic mucosal healing is proposed as an objective for achieving a lasting response.

Cabriada *et al*^[33] conducted a cohort study using a nationwide database in order to investigate short-term and long-term efficacies of leukocytapheresis for the management of steroid-dependent UC. One hundred and forty-two patients with steroid-dependent UC were treated with Adacolumn leukocytapheresis therapy. At one month after the last scheduled apheresis session, 68% of patients achieved clinical response, including 37% with steroid-free clinical remission. In the long-term, at six and 12 mo, 41% and 36% of patients were in clinical remission, respectively. Although this large-scale observational trial is uncontrolled, it clearly shows that Adacolumn leukocytapheresis allows long-term steroid-free clinical remission in up to one third of steroid-dependent UC patients.

Our recent study^[34] was conducted to determine if the introduction of Adacolumn leukocytapheresis at an early stage reduces corticosteroid administration and steroid dependency in the long-term. Twenty patients were treated with Adacolumn leukocytapheresis, with or without corticosteroids (apheresis group), and 20 patients were given corticosteroids without leukocytapheresis (steroid group). During a five-year follow-up period, five patients in the apheresis group did not require corticosteroids. The mean dose of steroid administered during the five years was significantly lower in the apheresis group than in the steroid group (2141 mg *vs* 5443 mg). Furthermore, the incidence of steroid-dependence was significantly lower in the apheresis group at the end of the study (5% *vs* 35%). In patients with first UC episode, Adacolumn leukocytapheresis therapy at an early stage significantly reduces steroid administration and steroid-dependency in the long-term.

TREATMENT COST OF LEUKOCYTAPHERESIS

When selecting a treatment option, the cost must be an

important factor. The cost of leukocytapheresis therapy (approximately \$ 1700 for one session with Adacolumn) is much higher as compared with corticosteroids. However, if leukocytapheresis can spare patients from corticosteroids, and reduce the incidence of steroid-dependency, hospitalization and surgery, it should be cost-effective.

CONCLUSION

Although the evidence level is not striking, the available data suggest that leukocytapheresis can avoid or reduce the use of corticosteroids in the management of UC. Large, well-designed clinical trials are necessary to more accurately evaluate the steroid-sparing effects of leukocytapheresis in the management of UC.

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Omega-3 fatty acids for the treatment of non-alcoholic fatty liver disease

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Abstract

Non-alcoholic fatty liver disease (NAFLD) has been recognized as a major health burden. It is the most important cause of chronic liver disease and a major independent cardiovascular risk factor. Lacking a definite treatment for NAFLD, a specific diet and an increase in physical activity represent the most commonly used therapeutic approaches. In this review, major literature data about the use of omega-3 polyunsaturated fatty acids (n-3 PUFAs) as a potential treatment of NAFLD have been described. n-3 PUFAs, besides having a beneficial impact on most of the cardio-metabolic risk factors (hypertension, hyperlipidemia, endothelial dysfunction and atherosclerosis) by regulating gene transcription factors

[i.e., peroxisome proliferator-activated receptor (PPAR) α , PPAR γ , sterol regulatory element-binding protein-1, carbohydrate responsive element-binding protein], impacts both lipid metabolism and on insulin sensitivity. In addition to an enhancement of hepatic beta oxidation and a decrease of the endogenous lipid production, n-3 PUFAs are able to determine a significant reduction of the expression of pro-inflammatory molecules (tumor necrosis factor- α and interleukin-6) and of oxygen reactive species. Further strengthening the results of the *in vitro* studies, both animal models and human intervention trials, showed a beneficial effect of n-3 PUFAs on the severity of NAFLD as expressed by laboratory parameters and imaging measurements. Despite available results provided encouraging data about the efficacy of n-3 PUFAs as a treatment of NAFLD in humans, well-designed randomized controlled trials of adequate size and duration, with histological endpoints, are needed to assess the long-term safety and efficacy of PUFA, as well as other therapies, for the treatment of NAFLD and non-alcoholic steatohepatitis patients. It is worthwhile to consider that n-3 PUFAs cannot be synthesized by the human body and must be derived from exogenous sources (fish oil, flaxseeds, olive oil) which are typical foods of the Mediterranean diet, known for its beneficial effects in preventing obesity, diabetes and, in turn, cardiovascular events. According to these data, it is important to consider that most of the beneficial effects of n-3 PUFAs can also be obtained by an equilibrate nutrition program.

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Key words: Hepatic steatosis; Non-alcoholic fatty liver disease; Omega-3 polyunsaturated fatty acids; Animal models

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is defined as pathological fat deposition in the liver cells of patients with minimal or no alcohol intake and without any other known cause. It encompasses a wide spectrum of liver damage stages ranging from isolated hepatic steatosis or simple fatty liver (FL), to non-alcoholic steatohepatitis (NASH) or even cryptogenic cirrhosis and hepatocellular carcinoma. In more detail, about 10%-29% of individuals with NASH develop cirrhosis within 10 years^[1], and 4%-27% of NASH-induced cirrhosis can ultimately progress to hepatocellular carcinoma^[2]. NAFLD affects 10%-35% of the adult population^[3] and, because of the increasing incidence of obesity and of type 2 diabetes mellitus, it has been recognized as a major health burden and as the most important cause of chronic liver disease^[4]. Overall, NAFLD is considered as the hepatic expression of metabolic syndrome^[5,6] and it is associated with an increased risk of cardiovascular disease^[7], along with venous^[8] and arterial thrombotic events^[9]. On the other hand, the impact of NAFLD on overall cardiovascular mortality is still widely challenged^[7]. Nowadays, there is no definite treatment for NAFLD and NASH, as their pathophysiology and natural history are not completely understood. Indeed, treatment is based on general approaches such as diet and physical activity^[10]. The aim of this review is to describe major literature data about clinical and pre-clinical studies evaluating the effects of omega-3 polyunsaturated fatty acid (n-3 PUFAs) supplementation on NAFLD.

MOLECULAR MECHANISMS

The pathophysiology of NAFLD is multifactorial and not completely understood. According to the "two-hit" hypothesis^[11], insulin resistance and visceral obesity promote the synthesis of fatty acids from glucose and inhibit β -oxidation of fatty acids. The excess of fatty acids leads to triglyceride synthesis and to their intrahepatic accumulation. Overall, these changes lead to FL (first hit), which is a relatively benign clinical condition^[12].

The increased levels of fatty acids and triglycerides are associated with the production of free radicals^[13,14], which, by causing lipid peroxidation and activating pro-inflammatory and fibrogenic cytokines^[15], lead to NASH establishment (second hit)^[16].

In particular, oxidative stress could be considered the

result of an imbalance between pro-oxidant and anti-oxidant processes. In fact, the excess of intra-hepatic triglyceride induces high rates of mitochondrial β -oxidation, with the consequent production of reactive oxygen species (ROS), such as superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). These reactive molecules, by inactivating the apoptotic caspase system, determine necrotic cell death^[17]. Moreover, the increase in pro-oxidant activity is associated with a decrease in the antioxidant potential (superoxide dismutase activity and glutathione content)^[13,18].

Following such an increase in pro-oxidant activity^[12], the progression from NAFLD to NASH is mediated by the activation of different transcription factors, such as sterol regulatory element binding protein 1c (SREBP-1c), peroxisome proliferator-activated receptor γ (PPAR γ) and carbohydrate responsive element-binding protein (ChREBP), which activate the expression of a series of genes essential for lipogenesis^[19-23].

Other mechanisms are involved in the pathogenesis of NASH, such as increased secretion by the adipose tissue of proinflammatory and prothrombotic adipocytokines [interleukin 6 (IL-6) and tumor necrosis factor α (TNF- α)] and the reduced production of adiponectin, a potent anti-inflammatory, insulin-sensitizing adipocytokine^[24,25]. Inflammation is a component of the wound healing process that leads to the deposition of extracellular matrix and fibrosis in the liver. There is much evidence supporting a central role for pro-inflammatory cytokines, particularly TNF- α and IL-6, in the development of NASH. In fact, increased cytokines levels are found in the liver and blood of patients with NASH^[26], and their inhibition improved NAFLD in animal^[27] and human models^[28].

Considering their beneficial impact on cardiometabolic clusters (hypertension, hyperlipidemia, endothelial dysfunction and atherosclerosis)^[29], n-3 PUFAs are emerging as a potential treatment of liver steatosis. They cannot be synthesized by the human body and, thus, must be derived from exogenous sources (fish oil, flax seeds, *etc.*).

n-3 PUFAs, especially eicosapentaenoic acid (C20:5n3, EPA) and docosahexaenoic acid (C22:6n3, DHA), by regulating gene transcription factors (i.e., PPAR α , PPAR γ , SREBP-1, ChREBP), can control key pathways involved in hepatic lipid metabolism^[30,31]. In more detail, n-3 PUFAs are potent activators of PPAR α , which up-regulates several genes involved in the stimulation of fatty acid oxidation^[32-35] and down-regulates pro-inflammatory genes, such as TNF- α and IL-6^[36]. Moreover, n-3 PUFAs activate PPAR γ , resulting in increased fat oxidation and improved insulin sensitivity^[37].

Besides enhancing hepatic beta oxidation, n-3 PUFAs can also decrease endogenous lipid production by inhibiting the expression and processing of SREBP-1, which, in response to increased glucose and insulin levels, stimulates the transcription of several lipogenic and glycolytic genes^[38-42]. Moreover n-3 PUFAs can inhibit hepatic glycolysis and lipogenesis and suppress the ac-

tivity of ChREBP, another regulator of glycolytic, and lipogenic genes, such as *L*-pyruvate kinase and fatty acid synthase^[43].

Forthcoming studies show a growing amount of other genes are involved in NAFLD pathophysiology and, in turn, in the effect of n-3 PUFAs^[44,45].

ANIMAL MODELS

A series of animal models have been used to study NAFLD. Most of them found that fat intake and obesity are strictly related to fatty liver development. In more detail, the Western lifestyle, with a high fat content diet and sedentary behavior, was found to lead to liver damage in animals^[46,47]. Further models showed that the “cafeteria diet” (a feeding regimen similar to fast food) is strictly associated with NAFLD development and subsequent hepatic necro-inflammatory changes in mice^[37]. By evaluating the mechanisms by which diet impacts NAFLD development, an increase in dietary cholesterol, sucrose or fructose was found to induce hepatic lipogenesis in mice through the up-regulation of SREBP-1 expression, which stimulates the transcription of a series of lipogenic genes^[48-51].

After proving the strict relation between diet and NAFLD, the effects of n-3 PUFAs were tested in a series of animal models.

The first interesting data were that n-3 PUFAs depletion was found to promote steatosis and insulin resistance in rodents. Thus, rats fed with a low n-3 PUFAs diet rapidly developed NAFLD^[52,53]. In a recent study^[54], a drastic drop in n-3 PUFAs was induced by feeding C57Bl/6J mice for 3 mo with a n-3 PUFAs depleted diet. The animals showed insulin resistance and hepatic steatosis, which was associated with a decrease in fatty acid oxidation. Compared to the animals following the control diet, which only differed in the n-3 PUFAs content, analysis of the liver tissue revealed higher expression of all enzymes involved in lipogenesis, as well as increased expression and activation of SREBP-1. On the contrary, supplementing the diet with n-3 PUFAs prevented or reversed hepatic steatosis in animals. Recently, it has been reported that rats fed with a high fat diet combined with n-3 PUFAs supplementation were protected against severe NAFLD development. In fact, significantly increased lipid peroxidation was seen in the group fed with the same diet without n-3 PUFAs supplementation^[55].

In a further experimental model to confirm the protective effect from NAFLD development in mice^[56], n-3 PUFA administration was also found to reverse already established hepatic steatosis in leptin deficient obese mice^[57].

Marsman *et al.*^[58] induced hepatic steatosis by a 3 wk methionine/choline deficient diet in rats, and then administered n-3 PUFAs, standard lipid solution, or NaCl for 2 wk. Compared with control animals receiving a standard diet, n-3 PUFAs treated animals showed histological evidence of mild macrovesicular steatosis. On the

contrary, severe macrovesicular steatosis was found in both standard lipid solution and saline diet groups. In the same study, liver ischemia/reperfusion injury was evaluated by clamping vessels for 40 min. At 6 and 24 h from reperfusion, n-3 PUFA treated rats showed lower alanine aminotransferase (ALT) serum levels, lower hepatic TNF- α levels and a higher anti-oxidative capacity compared with both standard lipid solution and saline diet groups. Overall, these findings suggest that n-3 PUFA treatment both reduces hepatic steatosis and attenuates hepatic ischemia/reperfusion injury in rats.

Other experimental studies analyzed further mechanisms by which n-3 PUFAs could impact on NAFLD. In particular, a diet enriched in n-3 PUFAs was shown to improve insulin sensitivity, and reduce intrahepatic triglyceride content and steatohepatitis, in both mice^[56,59] and rats^[60,61] with fatty liver. Sekiya *et al.*^[59] exposed ob/ob mice to a dietary supplementation of n-3 PUFAs, obtaining a down-regulation of the *SREBP-1* gene and a reduction of hepatic lipogenesis, with an improvement of insulin-dependent metabolism (reduction of glucose, insulin and free fatty acid serum levels).

Similarly, Levy *et al.*^[61] found that the “Quantitative Insulin Sensitivity Check Index” was higher in fish oil fed Fischer Rats than in the control animals. A possible explanation for these results comes from a murine model, in which n-3 PUFAs supplementation in obese mice induces an up-regulation of the genes involved in insulin sensitivity (PPAR γ), glucose transport (GLUT-2/GLUT-4), and insulin receptor signaling (IRS-1/IRS-2)^[37].

Apart from the effects on metabolic homeostasis, in experimental NAFLD murine models, by influencing the production of eicosanoids, prostaglandins, and of leukotrienes, n-3 PUFAs also showed anti-inflammatory properties^[62].

Overall, these results suggest that n-3 PUFAs improve insulin sensitivity and reduce markers of inflammation, all major events in NAFLD development^[37,62].

Moreover, n-3 PUFAs supplementation improves hepatic steatosis in obese animals by modifying the genetic expression of key enzymes^[63]. It has been shown that n-3 PUFAs are the natural ligands of PPAR α , which modulates lipid metabolism in hepatocytes^[64]. In fact, by inducing the expression of proteins with peroxisome proliferator response elements in their promoting region^[64], PPAR α regulates fatty acid binding and their export in very low density lipoprotein^[65,66]. In PPAR α (-/-) knockout animals, hepatic steatosis, which was minimal under normal conditions, drastically increased after a fasting period. The absence of PPAR α likely impaired mitochondrial β -oxidation in the liver during fasting, leading to hepatic steatosis development^[65]. Furthermore, there is evidence from other studies that n-3 PUFAs reduce hepatic ROS levels^[67]. n-3 PUFAs seem to improve the tolerance to oxidative stress, IRS-2 activity in the liver, brain and uterus of rats^[68]. Consequently, they may have a potential protective role against ROS-induced oxidative cellular damage in rat organs, especially in the liver. Re-

cently, using the methionine/choline deficient model of steatohepatitis, the effect of EPA, one of the most important long chain PUFAs, on hepatic fibrosis and ROS production was investigated in rat livers. For the study in question, steatosis was induced in 20 Wistar rats by a 20 wk methionine/choline deficient diet, followed by oral administration of EPA in 10/20 rats from week 12; a time at which hepatic fibrosis was already established. Control animals instead received a methionine/choline sufficient diet. At histology evaluation, EPA treatment was found to suppress hepatic fibrosis in liver sections, with repressed macronodular formation and decreased hepatic triglycerides content. EPA also suppressed the increase of hepatic fibrogenic factors, such as α -smooth muscle actin, TGF- β 1, procollagen, and connective tissue growth factor. The attenuation of hepatic fibrosis by EPA was significantly related to hepatic ROS levels. EPA also suppressed increases in hepatic ROS levels and reduced serum oxidative markers, such as 8-isoprostane and ferritin^[69].

All the aforementioned animal models show that omega-3 depletion can promote steatosis and insulin resistance. On the other hand, n-3 PUFAs supplementation, by inducing SREBP-1 up-regulation and lipogenic genes expression reduction, improving glycemic control, insulin levels and insulin sensitivity, reducing the oxidative stress, and exerting an anti-inflammatory effect, is able to prevent, or even at reverse, hepatic steatosis.

INTERVENTIONAL STUDIES

Although several clinical trials have been conducted, due to a wide variability in treatment duration, and the different n-3 PUFAs doses and preparations used, the efficacy of n-3 PUFAs in the treatment of NAFLD in humans has not yet wholly defined. The first clinical trial (Table 1) providing encouraging evidence about the efficacy of n-3 PUFAs in the treatment of NAFLD was performed by Capanni *et al*^[70]. They evaluated the efficacy of prolonged n-3 PUFAs supplementation in 56 patients with an ultrasonographic (US) diagnosis of NAFLD. 1 g/d of n-3 PUFAs was administered to 42 subjects for 12 mo. The 14 subjects refusing the same treatment served as controls. The primary outcome was the US appearance of the liver, including a quantitative measurement of fat storage on the basis of the Doppler perfusion index (DPI)^[71]. At the end of the treatment, subjects showed a significant ($P = 0.0001$) improvement of NAFLD compared with controls. A concomitant increase of DPI, proof of a hemodynamic improvement, was also reported in the treatment group, but not in the control group. In addition, n-3 PUFA supplementation was associated with a significant reduction of liver enzymes ($P = 0.003$), fasting glucose ($P = 0.02$) and triglyceride ($P = 0.02$) levels. However, it should be noted that this prospective study has some limitations, such as the absence of blindness and randomization. In a subsequent study^[72], the effectiveness of n-3 PUFAs supplementation was demon-

started on-top of a validated diet (Table 1). In this trial, 40 patients with NAFLD randomly received an American Heart Association (AHA) recommended diet^[73] plus n-3 PUFAs 2 g/d, or only the AHA diet, for 6 mo. Primary outcomes included: changes in fatty liver severity assessed by abdominal US, and liver ALT and triglyceride levels. Interestingly, inflammatory and metabolic markers such as TNF- α serum levels and insulin resistance assessed by homeostatic model assessment (HOMA) were also evaluated in this study. At the end of the treatment, patients who received diet plus n-3 PUFA supplementation had a significant reduction in ALT ($P < 0.01$), triglycerides ($P < 0.01$), TNF- α ($P < 0.05$), and HOMA ($P < 0.05$) levels. In addition, 33.4% of them showed a complete fatty liver regression. On the contrary, none of the patients receiving the diet alone showed a complete regression of the fatty liver. Indeed, this trial showed some design weaknesses, such as the lack of placebo and the lack of blindness of both participants and investigators. At variance with the latter reported studies, enrolling relatively few patients, Zhu *et al*^[74] performed a randomized clinical trial with a large sample size (Table 1). In 144 patients with NAFLD and mixed hyperlipidemia, the efficacy of n-3 PUFA from seal oils was evaluated. Patients were randomly assigned to two groups of treatment: Group A received an AHA recommended diet^[73] and 2 g of seal oils (rich in EPA, DHA, and DPA) \times 3/daily, while Group B received the recommended diet and 2 g placebo \times 3/daily. The treatment duration was 24 wk. Primary endpoints were changes in ALT and serum lipid levels, symptom scores (liver discomfort or pain, weakness, abdominal distention, and nausea) and modifications in fatty liver assessed by US. At the end of the treatment period, total symptom scores, ALT and triglycerides levels decreased more significantly ($P < 0.01$) in Group A than in Group B. At the abdominal US, a normal liver echo pattern and a significant liver steatosis improvement compared with the baseline was found in 19.7% and 53.03% of patients in Group A, respectively. On the other hand, in Group B only 7.35% of subjects achieved complete regression ($P = 0.04$) and 35.29% had some degree of liver steatosis improvement ($P = 0.04$), with no change being observed in the remaining 64.71% of patients in the group. It is noteworthy that some patients only on the diet ameliorated. Although only having a small sample size of the population, the results of a study performed by Sofi *et al*^[75] are of particular interest. It aimed to assess the efficacy of the administration of olive oil (rich in PUFAs) in patients with NAFLD. As many as 6 subjects received 5 mL/d of olive oil for 1 year, while 5 were selected as controls (Table 1). Outcome measurements were serum liver biochemistry, serum lipids, adiponectin levels, and the appearance of the liver with US and Doppler investigation. In this study, at variance with all the others, n-3 PUFAs were administered in olive oil instead of in capsules. Thus, this could be considered a “nutritional” rather than a “therapeutic” study. Since olive oil is one of the staples of the Mediterranean diet, it is interesting to note that, at the

Table 1 Summary of trials design and results

Ref.	Study design	Intervention	Population	Outcome measurements	Results	Comments
Capanni <i>et al</i> ^[70]	Open-label	Oral administration of n-3 PUFA, 1-g capsule/d for 12 mo	56 patients with NAFLD (42 subjects receiving therapy; 14 controls)	AST, ALT, GGT, TG, FG, n-6/n-3, liver echo texture by US and liver perfusion by DPI	↓AST ($P = 0.003$) and ALT ($P = 0.002$), ↓GGT ($P = 0.03$), ↓TG ($P = 0.02$) and FG ($P = 0.02$) in comparison with controls. Circulating arachidonate and n6:n3 ratio was reduced ($P = 0.0002$, and $P = 0.0001$ respectively) in treated patients. Improvement of liver echo texture ($P = 0.0001$), and increase of DPI ($P = 0.001$)	Limits of this study are the absence of blinding and randomization, and the use for comparison of a self-selected small group consisting of those patients who had been declined entry to the treatment arm
Spadaro <i>et al</i> ^[72]	Randomized; open-label	AHA diet + 2 g/d n-3 PUFA (Group DP) vs AHA diet (Group D) for 6 mo	40 patients with NAFLD (Group DP, $n = 20$; Group D, $n = 20$)	Liver fat assessed by abdominal US, ALT, AST, TNF- α serum levels, and HOMA	In DP group: ↓ALT ($P < 0.01$), TG ($P < 0.01$), serum TNF- α ($P < 0.05$) and HOMA (IR) ($P < 0.05$). Complete fatty liver regression in 33.4% of patients, and an overall reduction in 50%; In the D group: no significant modification of laboratory tests; no patient achieved complete regression of fatty liver, whereas some amount of reduction occurred in 27.7% of patients Group A vs Group B showed ↓ of total symptoms score, ALT, TG, LDL ($P < 0.05$); complete fatty liver regression in 19.7% vs 7.35% ($P = 0.004$); In both groups there was a tendency in improvement in AST, GGT, TCHO and HDL levels ($P < 0.05$)	Limits of the study are lack of placebo, and the non blinding of participants and investigators
Zhu <i>et al</i> ^[74]	Randomized	AHA diet + 2 g/d n-3 PUFA from seal oil (Group A) vs AHA diet + 2g of placebo (Group B) for 6 mo	144 patients with NAFLD and hyperlipidemia (Group A = 72; Group B = 72)	Liver fat assessed by symptom scores, ALT and serum lipid levels after 8, 12, 16, and 24 wk; fatty liver assessed by US at weeks 12 and 24 after treatment	↓ALT, AST, TG, TCHO, HOMA-IR, plasma thioredoxin; change in histological grade: steatosis: 2.4 (SD 0.5) vs 1.7 (SD 0.5); fibrosis: 1.7 (SD 1.1) vs 0.7 (SD 0.5); lobular inflammation: 2.1 (SD 0.7) vs 1.1 (SD 0.7); ballooning: 1.6 (SD 0.5) vs 0.9 (SD 0.4); NAS: 6.1 (SD 1.3) vs 3.7 (SD 1.4); Hepatic steatosis grade on the US changed from 2.1 ± 0.9 at baseline to 1.6 ± 1.1 after treatment ($P = 0.004$)	Limits of the study are the absence of a control group and small sample size
Tanaka <i>et al</i> ^[77]	Open label	EPA 2.7 g/d for 12 mo	23 patients with biopsy proven NAFLD	ALT, FFA, plasma soluble TNF receptor 1 and 2 levels, and serum ferritin and thioredoxin levels, body weight, blood glucose, insulin, and adiponectin concentrations; fatty liver infiltration assessed by histology	↓ALT, AST, TG, TCHO, HOMA-IR, plasma thioredoxin; change in histological grade: steatosis: 2.4 (SD 0.5) vs 1.7 (SD 0.5); fibrosis: 1.7 (SD 1.1) vs 0.7 (SD 0.5); lobular inflammation: 2.1 (SD 0.7) vs 1.1 (SD 0.7); ballooning: 1.6 (SD 0.5) vs 0.9 (SD 0.4); NAS: 6.1 (SD 1.3) vs 3.7 (SD 1.4); Hepatic steatosis grade on the US changed from 2.1 ± 0.9 at baseline to 1.6 ± 1.1 after treatment ($P = 0.004$)	Limits of the study are the absence of a control group and small sample size
Sofi <i>et al</i> ^[75]	Randomized	Dietary recommendation + 6.5 mL/d of olive oil enriched with n-3 PUFA (0.83 g n-3 PUFA, of which 0.47 g EPA and 0.24 g DHA) for 12 mo vs dietary recommendation alone	11 patients with NAFLD assessed by US (intervention group, $n = 6$; control group, $n = 5$)	Liver fat content assessed by B-mode US and DPI; liver enzymes, TG and adiponectin levels	Intervention group vs controls showed a ↓ of AST ($P = 0.02$), ALT ($P = 0.03$), GGT ($P = 0.03$), TG ($P = 0.04$) levels; ↑ of HDL ($P = 0.03$), adiponectin ($P = 0.04$). There was a significant ($P = 0.02$) improvement of DPI in the intervention group, while no change was observed in the control group. Improvement of liver steatosis on US in the intervention group (% of patients at T0 and T12): absent (from 0% to 16.7%); mild (from 16.7% to 50%); moderate (from 33% to 0%); severe (from 50% to 33%)	Limits of the study are the absence of a control group and small sample size

Nobili <i>et al</i> ^[78]	Randomized	DHA (250 and 500 mg/d) <i>vs</i> placebo for 6 mo	60 children with biopsy-proven NAFLD randomly assigned to receive DHA 250 mg/d (<i>n</i> = 20), DHA 500 mg/d (<i>n</i> = 20) or placebo (<i>n</i> = 20)	Primary: change in liver fat content as detected by US; secondary: changes in ISI, ALT, TG and BMI	DHA 250 mg <i>vs</i> placebo: odds of more severe <i>vs</i> less severe steatosis (OR = 0.01, robust 95% CI: 0.002 to 0.11, <i>P</i> < 0.001); ↑ of ISI (<i>P</i> < 0.01), ↓TG (<i>P</i> < 0.05); ALT and SDS of BMI; DHA 500 mg <i>vs</i> placebo: (OR = 0.04, 0.002 to 0.46; <i>P</i> = 0.01); ↑ of ISI (<i>P</i> < 0.01), ↓TG (<i>P</i> < 0.05); ALT and SDS of BMI; DHA 250 mg <i>vs</i> DHA 200 mg: NS	
Vega <i>et al</i> ^[79]	Open label	9 g/d of fish oil for 8 wk	22 patients with previous elevated liver fat on MRS (17 patients completed the trial)	Liver fat content assessed by B-mode US and DPI; liver enzymes, TG and adiponectin levels	↓ of plasma triglyceride level by 46% (<i>P</i> < 0.03), VLDL + IDL by 21% (<i>P</i> < 0.03), ApoB by 15% (<i>P</i> < 0.03). Liver fat content 7.9% pre-treatment; 8.0% after PUFA (NS)	Causes of liver disease other than NAFLD were not excluded and alcohol intake was not reported. It is unclear whether study participants received any other interventions such as diet or lifestyle advice

NAFLD: Non-alcoholic fatty liver disease; AST: Aspartate transaminase; ALT: Alanine transaminase; GGT: γ -glutamyl transpeptidase; TG: Triglycerides; FG: Fasting glucose; US: Ultrasonographic; DPI: Doppler perfusion index; AHA: American Heart Association; PUFA: Polyunsaturated fatty acid; TNF: Tumor necrosis factor; HOMA: Homeostatic model assessment; IR: Insulin resistance index; TCHO: Total cholesterol; HDL: High-density lipoprotein; MRS: Magnetic resonance spectroscopy; VLDL: Very low density lipoprotein; ISI: Insulin sensitivity index; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; DPI: Doppler perfusion index; BMI: Body mass index; SDS: Standard deviation score; NS: Not significant.

end of treatment, patients showed a significant (*P* < 0.05) improvement in liver echo-texture and DPI, a significant improvement of liver enzymes, and triglycerides (*P* = 0.04) and adiponectin levels (*P* = 0.04).

In the aforementioned studies, the lack of a liver biopsy for the diagnosis of NAFLD may hamper the relevance of their findings. However, in spite of its inherent operator-dependence, abdominal US analysis is currently thought to provide reliable, careful information about hepatic steatosis. This limits the need for liver biopsy for the diagnosis of NASH and to determine the severity of hepatic fibrosis^[76]. Moreover, despite its inherent limitations, US analysis has been validated against histopathological specimens, as well as other imaging methods, for the diagnosis of liver steatosis^[76]. In this regard, studies in which the effects of n-3 PUFAs on NAFLD are supported by histology or MRI findings have been also performed. Tanaka *et al*^[77] enrolled 23 patients with biopsy proven NASH. They received 2.7 g of EPA daily for 12 mo (Table 1). Outcome measurements were serum liver biochemistry, appearance on US, and liver histology (graded using the NAFLD activity score). All patients completed the trial and showed a significant improvement of laboratory markers of hepatic oxidative stress. The mean US steatosis degree improved significantly and, in 6 out of 7 patients who underwent repeated biopsy, steatosis, inflammation and fibrosis, resulted in significantly reduced levels. Although this was the first human study of n-3 PUFAs fatty acids to have histological data, which are the most valid outcome measurement, the absence of randomization, controls and blindness, along with the small sample size, do not allow us to draw

definitive conclusions. In another study^[78], in which the diagnosis of NAFLD was confirmed by biopsy, 60 children were randomly assigned to receive DHA 250 mg/d, DHA 500 mg/d or placebo (Table 1). The duration of treatment was 6 mo. The main outcome was the change in liver fat content as detected by US. After 6 mo, DHA supplementation was associated with lower odds of severe steatosis compared to a placebo. In addition, for the groups treated with DHA, where no effects on ALT values were found, there was an improvement of insulin sensitivity and triglycerides levels. Thus, this prospective study showed that, following this therapeutic regimen, both US and metabolic feature improvement occurred.

Therefore, Vega *et al*^[79] evaluated the efficacy of n-3 PUFAs on serum and hepatic triglycerides levels, the latter assessed by magnetic resonance spectroscopy (Table 1). Of the 22 patients enrolled, 17 completed the trial. They received a placebo for 4 wk, followed by an 8 wk treatment with 9 g/d of fish oil. Treatment with fish oil significantly reduced the levels of plasma triglycerides by 46% (*P* < 0.03), very low-density lipoprotein plus intermediate density lipoprotein cholesterol by 21% (*P* < 0.03), and total apolipoprotein B by 15% (*P* < 0.03). In contrast to the changes in plasma triglycerides, hepatic triglyceride content was not significantly reduced by fish oil treatment.

In conclusion, NAFLD may be considered the hepatic expression of metabolic syndrome^[5] which, in turn, predisposes to cardiovascular events. It is known that n-3 PUFAs have many beneficial effects on most of the metabolic syndrome features. In this regard, there is evidence suggesting that n-3 PUFAs are able to reduce

blood pressure^[80,81] and that they have favorable effects on plasma lipids levels^[82]. In addition, n-3 PUFAs also showed anti-platelet and anti-inflammatory properties which help to explain their cardio-protective effects^[29,82]. Most of the available clinical trials provide encouraging data about the efficacy of n-3 PUFAs as a treatment of NAFLD in humans^[9].

In keeping with this, in the era of poly-pills for coronary heart disease prevention, drugs with multifaceted mechanisms of action should be taken into serious consideration^[82]. On the other hand, it is worthwhile to consider that a significant amount of n-3 PUFA is contained in fish and in olive oil. All these are typical foods of the Mediterranean diet, which exhibits well known beneficial effects and is able to prevent obesity, diabetes and, in turn, cardiovascular events^[83]. For individuals eating low amounts of fish, a 500 mg/d EPA+DHA consumption is recommended in the absence of any type of cardiovascular disease, the suggested dosage being at least 800-1000 mg/d for those with coronary heart disease or congestive heart failure^[82].

According to these data, it is important to consider that most of the beneficial effects of n-3 PUFAs can also be obtained by an equilibrate nutrition program.

Well-designed randomized controlled trials of adequate size and duration, with histological endpoints, are needed to assess long-term safety and efficacy of PUFA, as well as other therapies for the treatment of NAFLD and NASH patients. Thus, while waiting for further data, current nutritional recommendations about daily intake should be strictly taken into consideration.

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Changes of the cytokine profile in inflammatory bowel diseases

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Abstract

Cytokines are indispensable signals of the mucosa-associated immune system for maintaining normal gut homeostasis. An imbalance of their profile in favour of inflammation initiation may lead to disease states, such as that is observed in inflammatory bowel diseases (IBD). Although Crohn's disease (CD) is often described as a prototype of T-helper 1-type diseases, and ulcerative colitis (UC) is traditionally viewed as a T-helper 2-mediated condition, the classic paradigm, which categorises cytokines into pro- and anti-inflammatory groups, has recently been changed. The inflammation regulatory pathways may not be mutually exclusive as individual cytokines can have diverse and even opposing functions in various clinical and immunological settings. None the less there are many common immunological responses in IBD that are mediated by cytokines. Although they regulate and influence the development, course and recurrence of the inflammatory process, the concrete pathogenic role of these small signaling molecules is sometimes not unambiguous in the subtypes of the disease. Our aim is to review the current information about pro- and anti-inflammatory effects of traditionally studied and recently discovered cytokines in the pathogenesis of UC and CD. The better

understanding of their production and functional activity may lead to the development of new therapeutic modalities.

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Key words: Ulcerative colitis; Crohn's disease; Interleukin-33; Tumor necrosis factor-like factor; Interleukin-8; Interleukin-35; Interleukin-25; Interleukin-4; Tumor necrosis factor ligand superfamily member 14

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INTRODUCTION

The balance of pro- and anti-inflammatory cytokines in the colonic mucosa is essential for normal gut homeostasis. A disturbance of the cytokine profile in favour of pro-inflammatory cytokine overproduction leads to disease states, such as that observed in inflammatory bowel diseases (IBD)^[1,2].

The concept that ulcerative colitis (UC) and Crohn's disease (CD) are two distinct forms of IBD has been changed recently. Instead, they are considered as a spectrum from mildly inflamed mucosa to severely active bowel inflammation with or without extraintestinal manifestations and different clinical behaviour.

CD is often described as a prototype of T-helper (Th) 1-mediated diseases because the primary inflamma-

tory mediators are the Th1 cytokines such as interleukin (IL)-12, interferon (IFN)- γ , and tumor necrosis factor (TNF)- α ^[3,4]. However, UC is usually viewed as a Th2-type condition because of the increased intestinal expression of the Th2-associated cytokine IL-5 and IL-13, although a clear association with IL-4, another definitive Th2 cytokine, has never been established^[3,4]. The role of pro-inflammatory cytokines such as IL-1 α , IL-1 β , IL-2, -6, -8, -12, -17, -23, IFN, or TNF in IBD is associated with the initiation and progression of UC and CD. Cytokines with anti-inflammatory effects, like IL-4, -10, and partly IL-13 also contribute to the pathogenesis of IBD, decreasing the inflammatory process by down-regulating pro-inflammatory cytokine production.

However, this classic paradigm has recently been changed. These pathways may not be mutually exclusive as individual cytokines can have diverse and even opposing functions in various clinical and immunological settings^[4].

Although many common immunological responses in IBD are mediated by cytokines, the concrete pathogenic role of these small peptide molecules is sometime not unambiguous in the subtypes of the disease. Therefore we aimed to review the current information about pro- and anti-inflammatory effects of traditionally studied and recently discovered cytokines in the pathogenesis of UC and CD. Controlling their expression, production and functional activity is an approach that may allow the development of more efficient and less harmful therapeutic strategies.

CYTOKINES WITH PRO-INFLAMMATORY EFFECTS IN ULCERATIVE COLITIS

The IL-1 family

The cytokines of the IL-1 family play a major role in several autoimmune inflammatory diseases, including IBD^[5]. IL-1 represents two structurally distinct forms: IL-1 α and IL-1 β . For both IL-1 α and - β , the most significant and relevant properties are the initiation of cyclooxygenase type 2, inducible nitric oxide synthase and phospholipase A2, which are produced by various cell types^[6]. Endogenous IL-1 receptor antagonist (IL-1Ra), a natural occurring antagonist of IL-1, regulates normal immune homeostasis in the gut^[1]. The increase of the IL-1/IL-1Ra ratio is parallel with the activity of colitis, while the IL-1/IL-1Ra ratio remains constant in the non-affected part of the colon and in non-IBD inflammatory controls^[7]. The main source of IL-1 in IBD is the monocyte/macrophage system as it can activate the IL-1 converting enzyme, therefore active IL-1 β is released into the colonic mucosa^[8].

IL-33, as known as IL-F11, is the newest identified member of the IL-1 family^[9,10]. It has been detected in several different cell types such as myofibroblasts, adipocytes, smooth muscle cells, endothelial cells bronchial and intestinal epithelial cells, macrophages and dendritic

cells^[10-12]. For the expression of the biological effect of IL-33 the binding to its receptor, IL-1 receptor like 1 (also known as ST2), is required^[10]. IL-33 has a pathogenic role in allergy^[13], airway inflammation^[14] and rheumatic diseases^[15]. Based on the results of Schmitz *et al.*^[10], IL-33 appears to reconstitute mucosal barrier defences against luminal pathogens, increasing epithelial protection by mucus secretion and augmenting immune response *via* type 2 helper T cell (Th2)-related cytokines, such as IL-5 and IL-13. In 2010, elevated expression of IL-33 in UC was reported by four independent groups^[16-19]. In active UC, the expression of the full-length, biologically active form of IL-33 is markedly increased in epithelial cells and in the infiltrating macrophages and B-cells of the lamina propria, while in the serum only the cleaved form of IL-33 is detectable^[18]. This latter possesses reduced biological activity^[20] therefore leading to the speculation that the presence of extracellular proteases has the ability to inactivate full-length IL-33 preventing possible harmful effects (i.e., anaphylactic shock) triggered by high levels of circulating IL-33^[21].

Similarly to IL-33, the expression of its receptor, ST2, was shown to be increased in both colonic wall and serum of IBD patients^[16]. Although the epithelial-derived ST2 expression is decreased and redistributed in IBD^[20], a marked infiltration of ST2 expressing antigen presenting cells and Th cells is present in the lamina propria and perivisceral adipose tissue^[18]. The same epithelial expression of ST2 was not detected in non-IBD colitis samples, such as diverticulitis or infectious colitis^[18]. Regarding the colon, the IL-33/ST2 axis could have a dual and perhaps dichotomous role in the pathogenesis of IBD. Pro-inflammatory cytokine stimuli, such as TNF- α and IL-1 β , and signals from pathogen-associated molecular patterns result in an increased IL-33 level in epithelial cells. After epithelial damage the released IL-33 may enhance the immune responses *via* ST2 expressing immune cells, therefore exacerbating the severity of inflammation^[20,22]. Thus, it is tempting to speculate that the blockade of IL-33 during UC may help to reduce the severity of the disease.

In line with the activation of inflammation IL-33, partly come from endothelial cells, may also act on ST2 expressing epithelial cells and myofibroblasts, promoting wound healing and angiogenesis^[20,23].

The TNF superfamily

The TNF protein superfamily consists of 18 type 2 proteins that exist in either membrane-bound or soluble forms^[24]. Receptors for these ligands are type 1 transmembrane proteins^[25]. Binding of TNF-like ligands to their receptors triggers intracellular pathways that are directly involved in cell proliferation, differentiation, and survival^[26]. Most members of the TNF/TNF-receptor protein superfamilies are expressed on immune cells and play a critical role in multiple components of the immune response, including defence against microor-

ganisms, inflammation, programmed cell death, and the development of the immune system^[24-26].

TNF- α is a master cytokine in the pathogenesis of IBD^[27]. It exerts its pleiotropic effects through the expression of adhesion molecules, fibroblast proliferation, procoagulant factors, as well as the initiation of cytotoxic, apoptotic, and acute-phase responses^[28]. It also has the ability to increase IL-1 β , IL-6, and IL-33 production as well as modulate ST2 expression in epithelial cells^[18,29]. The source of TNF- α in IBD is partly the innate immune cells, such as macrophages or monocytes, and also differentiated Th1 cells^[30]. The serum levels of TNF- α correlate with the clinical activity of UC and CD^[31]. Its orchestrating role in colonic inflammation established the basis of anti-TNF- α antibody therapy in IBD.

Tumor necrosis factor-like factor (TL1A), another newly discovered member of the TNF family, stimulates IFN- γ secretion by binding to death receptor 3 (DR3)^[32]. DR3 is expressed by a high percentage of cells from mucosal biopsies of UC and CD, and an increase of IFN- γ level has been observed with disease activity in IBD patients^[32]. Although TL1A seems to be involved in intestinal epithelial cell apoptosis in IBD^[30], its concrete role in UC pathogenesis still remains unknown.

The IL-6 family

IL-6, IL-11, IL-31, leukemia inhibitory factor, oncostatin M, cardiotrophin-1, ciliary neurotrophic factor, and cardiotrophin-like cytokine belong to the IL-6 family of cytokines.

IL-6 is an immunoregulatory cytokine that activates a cell surface signaling assembly composed of IL-6, soluble IL-6 receptor (sIL-6R), and the shared signaling receptor gp130^[33-35]. The combination of IL-6 and sIL-6R only stimulates gp130 expressing cells, a mechanism that is called trans-signalling. IL-6 signaling *via* signal transducer and activator of transcription-3 (STAT3) plays an important role in UC pathogenesis, moreover in carcinogenesis of UC-associated colorectal cancers^[36].

Mitsuyama *et al.*^[37] found that sIL-6R levels were significantly increased in patients with active UC and CD compared with inactive disease. Thereby, serum IL-6 and sIL-6R levels correlated strongly with C-reactive protein levels.

Besides mononuclear cells, intestinal epithelial cells are supposed to contribute to IL-6 production in the lamina propria^[38,39]. Recent data have shown interesting new aspects of epithelial function^[40]. It was demonstrated in Caco2 cells that IL-6 induces NF-kappaB activation and then enhanced expression of the intercellular adhesion molecule 1, which is important in IBD pathogenesis and most likely in extraintestinal manifestations of the disease^[41,42].

Based on these data, the blockade of IL-6/STAT3 signaling and the use of anti-IL-6R antibodies have been suggested as promising therapeutic approaches for the future.

IL-8

IL-8, a small basic heparin-binding protein, is a member of the cysteine-amino acid-cysteine chemokine family (2 cysteines are separated by a single amino acid in the first 2 of the 4 conserved cysteine residues)^[43]. It primarily mediates the activation and migration of neutrophils into tissue from peripheral blood. In a recent study^[44], the tissue level of IL-8 was found to be higher in active UC compared to normal colonic tissue, and its serum concentration was also related to endoscopic and histological severity of UC. Based on these results, IL-8 seems to be a reliable biomarker, closely related to disease activity, but its pathogenic role in the initiation and maintain of colitis needs to be further studied.

The IL-12 family

IL-12, IL-23, IL-27 and IL-35 belong to the IL-12 family of pro-inflammatory heterodimeric cytokines and comprise IL-12p40/IL-12p35, IL-12p40/IL-23p19, Epstein-Barr virus-induced gene 3 (*EBI3*)/IL27p28(IL-30) and IL12p35/*EBI3* subunits^[45-48].

IL-12 and IL-23 are mainly produced by antigen presenting cells, dendritic cells and phagocytes^[49]. Their receptors are also heterodimeric^[49].

IL-12 receptor (IL-12R) is expressed mainly on T cells, natural killer (NK) cells and natural killer T (NKT) cells^[49]. IL-12 expression is elevated in the mucosa of UC patients and it correlates with disease activity^[50]. Recently, the basic leucine zipper protein, NFIL3, was shown to be a regulator of IL-12p40 in macrophages and mucosal immunity^[51]. Interactions of macrophages with the colonic microbiota induce NFIL3 to limit their inflammatory capacity.

IL-23 promotes the differentiation of naïve CD4+ T cells into Th17 cells^[52]. The production of IL-23 by the cells of innate immunity is a response to pattern-recognition-receptor (toll-like- and nucleotide oligomerization domain-like receptors) stimulation or endogenous signals, indicating a potential role for T cells in reinforcement of the IL-23 response^[53]. The pathogenic role of IL-23 receptor (IL-23R) polymorphisms in UC may result in part from its wide distribution among other immune cells. IL-23R is expressed by NK cells, NKT cells, CD4+ T cells and CD8+ T cells^[54]. It is possible that some of the disease-associated polymorphisms observed in the *IL-23R* gene region may influence IL-12RB2 expression, given their adjacent location on the genome^[55]. The regulation of IL-23R and IL-12RB2 expression has a key role in the regulation of T cell differentiation.

Although in most colitis mouse models IL-23 plays a pro-inflammatory role, in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis, which is T cell mediated, IL-23 functions as an anti-inflammatory cytokine because it suppresses IL-12 production^[56,57].

There is a strong link between IL-23 and Th17 response *in vivo*. It appears that IL-23 is not necessary

for the initiation of Th17 cell differentiation but it is required at a crucial point in controlling the Th17 response^[57]. IL-23 signaling is primarily mediated through the adapter molecule, STAT3. STAT3 was also shown to be essential in the Th17 response, as it binds to the *IL-17a* gene promoter and mediates IL-23-regulated expression of IL-17A, the main effector cytokine of Th17 cells. In lack of IL-23, a decrease in the accumulation of Th17 cells appears in response to inflammatory stimuli, suggesting a regulatory role of IL-23 in Th17 cell response^[57,58]. Besides Th17 cell differentiation, IL-23 also influences the development of regulatory T cells (T_{regs}) by suppressing Foxp3 expression^[59,60]. IL-23 reduces the frequency of Foxp3+ T_{regs} in the colon and is dispensable for the pathogenesis of mucosal inflammation in the lack of T_{regs}^[61].

The main source of the newly discovered IL-35 is the T_{regs}^[48]. Recently, it has been shown^[62] that IL-35 controls the development of T-cell-dependent colitis in mice models, suggesting the potential in targeting IL-35 for patients with chronic intestinal inflammation. The role of IL-35 in the pathogenesis of human IBD needs to be further investigated.

IL-13

UC has been traditionally considered as a Th2 mediated disease, in which IL-13 was identified as an important effector cytokine^[63]. The mRNA expression of IL-13 in UC mucosa is increased^[64], and *ex vivo* cultured lamina propria mononuclear cells from UC patients secrete significantly higher amounts of IL-13 upon stimulation than those from both healthy controls and CD patients^[63]. The critical cell population for IL-13 secretion is CD161+ NKT cells, producing IL-13 in response to stimulation by CD1d+ antigen presenting cells in UC^[63].

The functional importance of NKT cell-derived IL-13 in UC has been studied in detail. It was shown that both receptors of IL-13, IL-4R α and IL-13R α 2, were expressed in colonic epithelial cells, which proves the ability for functional IL-13 signaling in UC^[65]. The UC-specific CD161+ NKT cells show cytotoxic activity against colon epithelium, which effect is, at least partially, dependent upon functional IL-13^[63]. IL-13 was also shown to exert pernicious effects on epithelial barrier function by increasing epithelial cell apoptosis, unmaking tight junction integrity, and decreasing restitution velocity^[65]. Based on these results, it was hypothesized by Fuss *et al*^[66] that stimuli from commensal flora-derived microbial products stimulate CD161+ NKT cells to produce IL-13 in the colonic mucosa. Then, the downstream effects, such as the cytotoxic activity of NKT cells, the IL-13 induced epithelial cell apoptosis, and the disruption of tight junctions, culminate to epithelial injury. In active UC, the suppression of IL-13 production by interferon- β 1 administration or the inhibition of STAT6, a key adaptor molecule in IL-13 signaling, by small interfering RNA or a histone deacetylase inhibi-

tor result in significant epithelial healing, supporting the aforementioned hypothesis^[67-69].

Recently, it was also shown that IL-13 signaling through IL-13R α 2 led to the increase of transforming growth factor (TGF)- β 1 production, which favours to the progression of colonic wall fibrosis^[70,71].

The IL-17 family

IL-17, which is mainly produced by Th17 cells, is acting as a key mediator in delayed-type immune reactions by increasing chemokine production and recruiting monocytes and neutrophils to the inflammatory site^[72]. After sequencing the human genome 6 structurally related isoforms of the IL-17 family were described: IL-17A (also known as IL-17), IL-17B, IL-17C, IL-17D, IL-17E (also known as IL-25), and IL-17F^[73]. IL-17A and IL-17F share 55% homology, which is the highest amongst the family members, but IL-17F has significantly weaker activity than IL-17A^[57]. There are more receptors for IL-17 (i.e., IL-17RA, IL-17RC), widely expressed by epithelial cells, endothelium or fibroblasts, and it is supposed that the different receptors show different affinity for IL-17s and different signaling pathways^[74].

Although the main source of IL-17A is the Th17 cells, CD8+ T cells are also able to produce this cytokine during chronic inflammation^[57]. However, a causative relationship between UC and IL-17A remains controversial.

In most of IL-17A knock out mice dextrane sulfate sodium (DSS) treatment failed to result in a typical acute colitis^[75], and after the administration of TNBS, the TNBS-induced colitis was attenuated in the IL-17RA knock out animals^[76]. These data support the pro-inflammatory role of IL-17 in colitis models.

On the other hand, O'Connor *et al*^[77] demonstrated IL-17A-mediated protection in the CD45RB^{hi} transfer model of colitis. An accelerated wasting disease elicited by IL-17A^{-/-} CD45RB^{hi} CD4+ T cells correlated with higher expression of genes encoding Th1 type cytokines in colon tissue. Furthermore, IL-17RA^{-/-} T cells elicited an accelerated wasting disease in Rag1^{-/-} recipients. Their findings support the observation that surprisingly IL-17A can mediate protective function rather than pathology in experimental colitis. Additionally, they have also identified T cells as not only the source but also a target of IL-17 *in vivo*.

In humans, it was recently shown that IL-17 levels were increased in UC compared to healthy colonic mucosa, but in the most reliable studies in which protein rather than messenger RNA was measured this increase was found to be far less than that found in CD^[78].

Since different results were obtained from different studies, it will be important to clarify the source and function of IL-17A in the pathogenesis of UC.

IL-25 was shown to inhibit CD14+ cell-derived cytokines, mainly IL-12 production and Th1 cell-driven experimental colitis in mice, suggesting its potential therapeutic role in both UC and CD^[79].

IL-5 and IL-21

IL-5, as known as eosinophil differentiation factor, is a selective eosinophil activating growth hormone and a member of the common β -chain-dependent cytokine family. The source of mucosal IL-5 is the mononuclear cells, which produce a high amount of this cytokine in active UC but not in CD^[3,65]. IL-5 together with IL-13 and granulocyte/monocyte colony stimulating factor have been recognized as activators of eosinophil function, including migration to the site of inflammation^[80]. Though IL-5 seems to have a regulatory role in eosinophil recruitment in UC mucosa, the role of this cytokine in priming of the blood eosinophils is not as obvious^[81,82]. There is no enhanced IL-5 production of circulating lymphocytes in UC, which indicates that in addition to IL-5 other factors may be involved in the priming of blood eosinophils in IBD.

IL-21 is a T cell derived member of the common γ -chain-dependent cytokine family, acting as a maintainer of the Th1 mediated inflammation in the colonic epithelium by inducing IFN- γ production^[83]. In IBD, IL-21 is mostly produced by CD4+ lamina propria T cells coexpressing IFN- γ and follicular T cells^[84,85]. The number of these cells is higher in CD than UC^[84]. Based on the recent results, IL-21 inhibits T_{reg} differentiation and leads to the resistance of CD4+ T cells to T_{reg}-mediated immune-suppression, therefore enhances the inflammatory process^[85].

CYTOKINES WITH ANTI-INFLAMMATORY EFFECTS IN ULCERATIVE COLITIS

The IL-10 family

IL-10 may be considered the most important anti-inflammatory cytokine in humans, secreted by CD4+ Th2 cells^[86]. During the last two decades, a range of cytokines related to IL-10 were discovered, making IL-10 the founding member of the type II cytokine family that includes IL-19, IL-20, IL-22, IL-24, IL-26, IL-28 and IL-29^[87].

IL-10 inhibits antigen presentation and the release of pro-inflammatory cytokines, hereby attenuates the inflammatory process in the mucosa. It is expressed by many cells of the innate and adaptive immune system. The former triggers IL-10 expression in a toll-like receptor (TLR)-dependent and a TLR-independent way. The major sources of IL-10 are macrophages and dendritic cells^[88]. Regarding the adaptive immune system, Th2 cells primarily promote humoral immunity express IL-10^[88].

The key role of IL-10 within the colonic mucosal immune system has been extensively studied in IL-10 knockout mice models^[89]. In UC, IL-10 mRNA expression was found to be highly increased in mucosal T cells, and the IL-10 production of T_{regs} is also important in the pathogenesis of IBD^[59,90]. A subset of IL-10 and TGF- β 1 producing B cells, namely the regulatory B cells (B_{regs}), are involved in UC pathogenesis as well^[91].

The mutations of the IL-10 pathway genes such as *IL-10* 1q32 or *Stat3* 17q21 have also been shown to be associated with UC^[92,93].

IL-19 is associated with the pathogenesis of both Th1 and Th2 mediated diseases^[94]. IL-19 produced by lipopolysaccharide activated macrophages suppresses pro-inflammatory cytokine release, especially the secretion of TNF- α , IL-6 and IL-12 by an IL-10 independent way^[95]. IL-19 deficient mice are susceptible to DSS-induced colitis^[94]. In a recent study, it was shown that IL-19 polymorphisms (rs2243188 and rs2243193) might have a protective role in the development of UC^[96]. Although these results are promising, the exact role of IL-19 in IBD needs to be further studied.

IL-22 has elevated levels in both serum and mucosa of active CD^[97], but it has been recently proven that it has a protective role in DSS-colitis murine model of UC by inducing mucin membrane bound production by goblet cells^[98]. In humans, the mucosal level of IL-22 was found to be elevated in active UC compared to inactive disease or healthy control samples^[99]. It was also recently published, that after *Trichuris trichiura* therapeutic self-infection, the active UC went into remission, and IL-22-producing Th cells accumulated in the mucosa^[100]. It seems that this kind of helminthiasis may reduce symptomatic colitis by promoting goblet cell hyperplasia and mucus production through Th2 cytokines and IL-22.

TGF- β and IL-4

TGF- β has multiple biological effects on both hematopoietic and nonhematopoietic cells^[101]. Binding of TGF- β to its receptor, TGF- β RII, phosphorylates smad and mothers against decapentaplegic-related protein transcription factors that have primarily immunosuppressive function^[101]. Genetic mutations in TGF- β RII are linked to UC and UC-associated cancer in humans^[102], and mice lacking TGF- β responsiveness in epithelial cells or T lymphocytes develop severe intestinal inflammation^[103,104].

In human UC patients, IL-33 expression is highly up-regulated within the colonic mucosa and IL-33-deficient mice are protected from DSS-induced colitis^[10,17,19]. Recent data^[105] show that CD68TGF- β DNRII mice, lacking normal TGF- β signaling, produce high levels of IgE and IL-33 within the colon following oral DSS administration. One source of IL-33 in these mice was the intestinal macrophages, which demonstrates that TGF- β serves as an anti-inflammatory factor *via* suppressing the production of IL-33. This may be an important mechanism that could partially explain the reason how mutations in TGF- β RII in humans are associated with increased risk for UC and UC-associated neoplasias^[106].

IL-4, an anti-inflammatory cytokine, is a stimulatory factor for B and T cells, and has an immunosuppressive effect in the colon^[107,108]. IL-4 and IL-10 are able to down-regulate inflammatory mediators including TNF- α and IL-1 and favour a humoral immune response^[109]. In

proctitis, the combined effects of IL-4 and IL-10 were shown to shift the Th1/Th2 cell activation in favour of a Th2-type response^[109], which eventually ameliorated mucosal healing.

In T-cell receptor- α chain-deficient (TCR- α -/-) mice, anti-IL-4 monoclonal antibody treatment altered the cytokine profile of CD4+ $\beta\beta$ T cells (a subset of CD4+ Th2-type cells) from dominant Th2 to Th1 type, resulting in the prevention of mucosal inflammation in TCR- α -/- mice^[110]. The treatment of peripheral blood mononuclear cells from active UC and CD patients with IL-4 *in vitro* resulted in significant decrease of the vascular endothelial growth factor (VEGF) production of these cells, which suggests that the known defective immunosuppressive role of IL-4 in IBD may contribute to the pathogenesis of inflammation by VEGF mediated mechanisms^[111].

CYTOKINES WITH PRO-INFLAMMATORY EFFECTS IN CROHN'S DISEASE

The IL-1 family

Similarly to UC, the IL-1 system plays an important role in the pathogenesis of CD. The IL-1/IL-1Ra ratio is in line with the activity of CD^[7]. In a recent study using phage display technology, a short peptide (TCP-353) was identified from the blood mononuclear cells of CD patients which specifically binds to CD sera and stimulates the pro-inflammatory responses (IL-1 β , IL-6 and TNF- α) of CD mononuclear cells^[112]. This novelty may have diagnostic, pathogenic and therapeutic significance with regard to the treatment of CD.

IL-18, another member of the IL-1 family, was originally described as an important Th1 cell polarizing cytokine^[113]. The level of IL-18 is increased in the inflamed mucosa of a subgroup of CD patients^[114,115]. The balance between this pleiotropic pro-inflammatory cytokine and its natural inhibitor, IL-18-binding protein (IL-18BP), may contribute to the pathogenesis of IBD^[116]. IL-18 is localized to lamina propria cells and intestinal epithelial cells, suggesting that both groups of cells may be involved in the complex events occurring in CD^[114]. In the presence of IL-18, mucosal T cells from active CD have been shown to produce less IL-10 than control tissue^[117]. Recombinant IL-18 alone is able to induce a significant proliferative response in mucosal lymphocytes of active CD, moreover a synergy between IL-18 and IL-12 in macrophages may regulate driving of mucosal lymphocytes toward a Th1 response^[118,119].

Leach *et al.*^[116] found that IL-18, produced in the colons of children with CD, contributes to the local inflammatory changes. They showed that systemic IL-18 level is a possible and useful indicator of disease activity. Furthermore, free IL-18 was found to be greatly elevated in CD children, suggesting that compensatory increases in IL-18BP are insufficient. Further exploration of the role of IL-18 in the pathogenesis of CD is now required.

The TNF superfamily

The role of TNF- α in CD has been widely investigated^[120-122]. Binding TNF- α to serum soluble TNF receptor 1 and 2 (sTNFR1 and 2) initiates pro-inflammatory signaling. The levels of sTNFR1 and 2 are elevated in CD sera compared to both UC and normal controls, hence it can be used as a marker for disease activity and discriminatory factor between the two subtypes of IBD^[123,124]. It was recently demonstrated that TNFR1-signaling cascade in colonic myeloid lineage cells contributes to the suppression of acute damage-associated mortality presumably by controlling colonic epithelial cell homeostasis^[125].

The central pro-inflammatory role of TNF- α has substantiated the use of anti-TNF- α antibodies in the treatment of CD^[126].

The TL1A/DR3 system is also involved in the pathogenesis of CD^[32]. The macrophages of the lamina propria are a major producer of TL1A, which expression is markedly enhanced in CD compared with UC or normal colon^[127]. Kamada *et al.*^[127] found that TL1A and IL-23 synergistically promotes the production of IFN- γ and IL-17 by mucosal T cells, while TL1A alone does not induce cytokine production. Furthermore, they have also shown that TL1A promotes Th17 differentiation from naïve T cells by mucosal macrophages; however, IL-23 did not show any synergistic effects on Th17 differentiation.

TNF ligand superfamily, member 14 (TNFSF14, also known as LIGHT) is a type II membrane protein that forms a biologically active homotrimer, which can be cleaved into a soluble form or exist in an intracellular form with deleted transmembrane region and not displayed on the cell surface^[128,129]. The human intestinal mucosa may be a primary site for LIGHT-mediated pro-inflammatory activity, which shows a correlation with disease activity^[128]. In CD, it was shown that IFN-producing CD4+ lamina propria T cells express LIGHT mediating a Th1 response^[128]. As several data from transgenic mouse models^[130,131] indicated that LIGHT-dependent inflammation selectively targeted the intestine, the mucosal specificity of LIGHT-mediated inflammation could have significant pathological implications in human CD, which needs further investigation.

The IL-6 family

The IL-6/STAT3 signaling system plays a key role in the pathogenesis of CD. The circulating levels of IL-6 and sIL-6R is in correlation with the activity of the disease^[132]. The pathogenic role of IL-6-sIL-6R system in mediating the resistance of T cells to apoptosis in CD was proved by blocking IL-6 trans-signaling^[133].

Subepithelial myofibroblasts can also be a source of mucosal IL-6 in CD. It was recently demonstrated that the increased production of IL-6 synthesis related to the oxidative state, suggesting redox regulation with the involvement of extracellular signal-regulated kinase 1/2

and p38 mitogen-activated protein kinase activation^[134]. Based on these data, IL-6 may have an influence not just on the chronic inflammatory process, but on relapses occurring in the pathology of CD.

The IL-12 family

As it is in UC, the expression of IL-12 is up-regulated in active CD mucosa as well, and its level is in correlation with disease activity^[50]. Because of the elevated levels of IL-12p40 and IL-12Rβ2 in the early phase of CD, it is suggested that IL-12 is primarily involved in the early induction of Th1 polarization of naïve T cells^[135]. However, the expansion and maintenance of Th1 cell response in the colon requires additional signals. The IL-12-dependent synthesis of IFN-γ of the mucosal T cells can be enhanced by cytokines that signal through the common γ-chain receptor (i.e., IL-7, IL-15, IL-21)^[136].

IL-23 also has an elevated mucosal level in CD^[137]. Based on the results from mouse studies with targeted deletion of either the IL-12/p35 or IL-23/p19 subunit, it is suggested that IL-23 and not IL-12 is essential for manifestation of intestinal inflammation occurring in IL-10-deficient mice^[138]. The IL-23-driven intestinal inflammation seems to be mediated by IL-17 and IL-6 production. It needs to be further investigated whether the harmful effect of IL-23 on the ongoing mucosal inflammation occurs only in the absence of IL-10-related regulatory effects.

IL-27 is a newly described, heterodimeric member of the IL-12 family^[47]. It was proved by *in vitro* studies that IL-27 is mainly produced by activated monocytes and dendritic cells, it induces the proliferation of naïve CD4+ T cells and synergizes with IL-12 for IFN-γ production^[47].

The mucosal expression of IL-27p28 was shown to correlate with the activity of disease in both UC and CD^[137]. Particularly, IL-27p28 and EB13 transcripts have shown to be significantly elevated only in active CD^[137].

The IL-17 family

In humans, the mucosal level of IL-17 levels is highly elevated in active CD^[78]. Recently, it has been shown that in CD patients increased numbers of circulating IL-17 and IFN-γ-producing CD161+ memory cells are present, and these cells constitute a high percentage of colonic mucosal cells^[139]. In addition, CD patients have increased numbers of circulating IL-23R expressing T cells, which respond to IL-23 with increased production of IL-17, IL-22 and IFN-γ, which is further increased by the presence of IL-1β. Moreover, these cells express gut homing receptors CCR6 and β7-integrin, which makes them to be programmed to recruit into the lamina propria during inflammation^[140]. Based on these results, Th17 cells producing both IL-17 and IFN-γ are identified as important elements in the inflammatory response in CD.

Dendritic cells are crucial in inducing acquired im-

munity. In CD, dendritic cells of myeloid origin were found to produce a higher amount of IL-23 and a lower amount of IL-10, when stimulated with exogenous bacterial derivative, moreover they induced a dysregulated Th1/Th17 immune response in mixed lymphocyte reaction than it is in UC and normal control^[78].

Similarly to UC, different results were obtained from different studies; therefore it will be important to clarify the source and function of IL-17 in the pathogenesis of CD.

IL-21

IL-21 is significantly overexpressed in CD mucosa^[141]. IL-21 is generated mainly by CD4+IFN-γ-producing T cells^[84]. In contrast, only a small fraction of IL-21 producing CD4+ T-cells co-express IL-17A, thus indicating that, in humans, IL-21 is produced preferentially by Th1 rather than Th17 cells. Activation of CD4+ T-lymphocytes from normal colon with anti-CD3 antibody and exogenous IL-12 increases the proportion of IL-21-secreting Th1 cells, whereas blockade of endogenous IL-12 in CD mucosal cell cultures significantly reduces IL-21 production^[142]. On the other hand, blocking IL-21 in cells from CD with antibodies or soluble receptor fusion proteins inhibits IL-17A and IFN-γ production^[142].

It was also found that intestinal epithelial cells and subepithelial fibroblasts constitutively express IL-21R and respond to IL-21 by inflammatory molecule secretion. Following IL-21 stimulation, colonic fibroblasts secrete large amounts of matrix metalloproteinase 1 and 3, enzymes involved in mucosal injury of CD^[143,144].

CYTOKINES WITH ANTI-INFLAMMATORY EFFECTS IN CROHN'S DISEASE

The IL-10 family

IL-10 plays a pivotal anti-inflammatory role in CD. An inactivation of IL-10 resulted in an increased production of the pro-inflammatory IL-12 and IFN-γ in mice^[145]. In humans, the inflamed mucosa and granulomas of CD show low IL-10 levels^[146]. It was also recently described that endogenous IL-10 constrains Th17 cell development through the control of dendritic cells' IL-1 production, which reaffirms the crucial anti-inflammatory role of IL-10 in patients with CD^[147].

On the contrary, the level of IL-22 is elevated in CD mucosa and serum^[98]. It was shown that IL-23R genotypes have an effect the serum concentrations of IL-22, which links genetic CD susceptibility to Th17 cell function^[97].

Regarding IL-22, a new regulatory pathway was recently described in CD^[148]. The aryl hydrocarbon receptor (AhR) may represent a link between the environment and the mucosal immune system. AhR is a transcription factor which is activated by a large number of environmental factors^[148]. It has been recently shown that muco-

Table 1 The disease-related immunological and pathological effects of cytokines

		Ulcerative colitis	Crohn's disease
Interleukin-1 family	IL-1	Inflammation induction	
	IL-18	NI	Mucosal T cell IL-10 secretion ↓ Th1 response ↑
	IL-33	Reconstitute mucosal barrier defence Epithelial mucus secretion ↑ Th2-response ↑ Wound healing and angiogenesis ↑	NI
Tumor necrosis factor superfamily	TNF- α	Adhesion molecules expression ↑ Fibroblast proliferation ↑ Procoagulant factors level ↑ Initiation of cytotoxic, apoptotic, and acute-phase responses IL-1 β , IL-6, IL-33 production ↑ Modulates epithelial cell ST2 expression	Initiates pro-inflammatory signaling
	TL1A	IFN- γ secretion ↑ Modulates epithelial cell apoptosis	IFN- γ , IL-17 production ↑ Th17 differentiation ↑
	LIGHT	NI	Mediates Th1 response and mucosa specific inflammation
	IL-6	Involved in colitis-associated carcinogenesis Possible role in extraintestinal manifestations	Mediates T cell resistance to apoptosis Influences of disease relapse
Interleukin-8	IL-8	Mediates the activation and migration of neutrophils	NI
Interleukin-12 family	IL-12	Modulates macrophage activity	Early induction of Th1 polarization of naïve T cells IFN- γ of the mucosal T cells
	IL-23	Promotes Th17 cell differentiation Controlling Th17 response Influences T _{reg} cell development Number of mucosal T _{reg} cells ↓	IL-17 and IL-6 mediated intestinal inflammation
	IL-27		Proliferation of naïve CD4+ T cells ↑ IFN- γ production ↑
Interleukin-13	IL-35	Possible controlling of T-cell dependent inflammation	NI
	IL-13	Induces cellular cytotoxicity against colonic epithelium Epithelial cell apoptosis ↑ Tight junction integrity ↓ Epithelial restitution velocity ↓ Colonic wall fibrosis ↑	NI
Interleukin 17 family	IL-17	Pro- and anti-inflammatory effects	IL-17, IL-22, IFN- γ production ↑ Enhance T cell recruitment into the lamina propria
	IL-25	Possible inhibition of IL-12 secretion Possible promotion of Th1-driven inflammation	NI
Interleukin-21	IL-21	Maintainer of Th1-mediated inflammation Inhibits T _{reg} cell differentiation CD4+ T cell resistance to T _{reg} -suppression ↑	Required for IL-17A and IFN- γ production Fibroblasts MMP secretion ↑
Interleukin-5	IL-5	Activates eosinophil function and migration	NI
Interleukin-10 family	IL-10	Inhibits antigen presentation Pro-inflammatory cytokine release ↓	Constrains Th17 cell development
	IL-19	TNF- α , IL-6, IL-12 secretion ↓	NI
	IL-22	Goblet cell hyperplasia ↑ Mucus production ↑	Promotes mucosal healing
Interleukin-4	IL-4	TNF- α , IL-1 production ↓ Humoral immune response ↑ Mucosal healing ↑ Monocyte/macrophage VEGF production ↓	NI
Transforming growth factor- β	TGF- β	Possible suppression of IL-33 production	Collagen synthesis ↑ Regulates the balance between matrix-degrading MMPs and their inhibitors IL-13 expression ↑ EMT ↑

IL: Interleukin; TNF: Tumor necrosis factor; IFN: Interferon; TL1A: TNF-like factor; TGF- β : Transforming growth factor- β ; LIGHT: Lymphotoxins, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for herpesvirus entry mediator, a receptor expressed by T lymphocytes; MMP: Matrix metalloproteinase; VEGF: Vascular-endothelial growth factor; EMT: Epithelial-to-mesenchymal transition; NI: (possibly) Not involved in pathogenesis.

sal T cells and NK cells isolated from active CD biopsies express low levels of AhR and respond to AhR ligands with decreasing pro-inflammatory cytokine production and up-regulating IL-22^[49]. Hereby, the changing mucosal cytokine profile promotes mucosal healing.

TGF- β

TGF- β is thought to be an inhibitory key cytokine of immunological homeostasis and inflammatory responses. On the other hand, TGF- β is also a potent profibrogenic agent inducing collagen synthesis and regulating the bal-

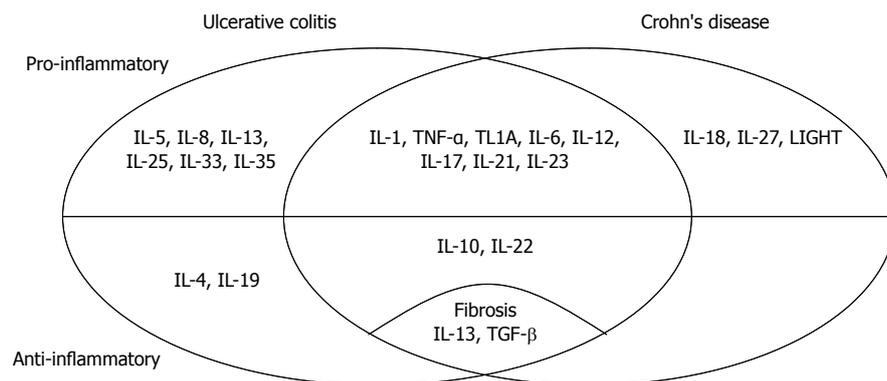


Figure 1 The disease-related pathogenic role of cytokines with pro-inflammatory, anti-inflammatory and pro-fibrogenic effects in ulcerative colitis and Crohn's disease. LIGHT: Lymphotoxins, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for herpesvirus entry mediator, a receptor expressed by T lymphocytes; IL: Interleukin; TNF: Tumor necrosis factor; TL1A: TNF-like factor; TGF-β: Transforming growth factor-β.

ance between matrix-degrading matrix metalloproteinases and their inhibitors^[150]. It has a role in CD-related fibrosis, as changes in TGF-β signaling and matrix metalloproteinase production were identified in the mucosa overlying strictures^[150].

It was also shown that TGF-β induces IL-13 expression and epithelial-to-mesenchymal transition of intestinal epithelial cells, while IL-13 promotes the expression of genes associated with cell invasion^[151]. Based on these data, it seems that TGF-β and IL-13 play a synergistic role in the pathogenesis of CD-associated fistulae^[151], which has therapeutic consequences.

CONCLUSION

Cytokines have important and complex role in the pathogenesis of IBD (Table 1 and Figure 1). There are several different biologic therapies directed to cytokines or their receptors which have possibilities in the treatment of IBD.

Some anti-TNF-α antibodies are currently being used to treat CD and UC. Although these molecules dramatically improved the treatment of patients, sometimes severe side effects or the development of anti-drug antibodies limits their application.

Neutralizing antibodies targeting other pathways of the immune response have been developed and tested^[152]. Antibodies targeting the IL-12 and IL-23 pathways, or pro-inflammatory cytokines (i.e., IFN-γ, IL-2, IL-6, IL-17A) initially showed a promising result, but for none of their efficacy has undoubtedly been established^[153]. Administration of the regulatory cytokines, namely IL-10 and IL-11, also failed to induce reproducible clinical effects^[152].

Accordingly to the complex effects and regulation of cytokines in IBD, the cytokine-based therapies of the future must have higher specificity and lower toxicity.

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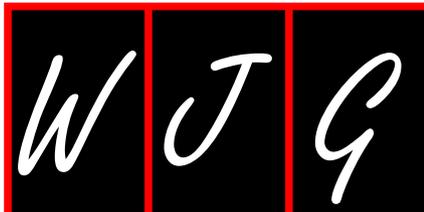
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Crohn's and colitis in children and adolescents

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Abstract

Crohn's disease and ulcerative colitis can be grouped as the inflammatory bowel diseases (IBD). These conditions have become increasingly common in recent years, including in children and young people. Although much is known about aspects of the pathogenesis of these diseases, the precise aetiology is not yet understood, and there remains no cure. Recent data has illustrated the importance of a number of genes—several of these are important in the onset of IBD in early life, including in infancy. Pain, diarrhoea and weight loss are typical symptoms of paediatric Crohn's disease whereas bloody diarrhoea is more typical of colitis in children. However, atypical symptoms may occur in both conditions: these include isolated impairment of linear growth or presentation with extra-intestinal manifestations such as erythema nodosum. Growth and nutrition are commonly compromised at diagnosis in both Crohn's disease and colitis. Consideration of possible IBD and completion of appropriate investi-

gations are essential to ensure prompt diagnosis, thereby avoiding the consequences of diagnostic delay. Patterns of disease including location and progression of IBD in childhood differ substantially from adult-onset disease. Various treatment options are available for children and adolescents with IBD. Exclusive enteral nutrition plays a central role in the induction of remission of active Crohn's disease. Medical and surgical therapies need to be considered within the context of a growing and developing child. The overall management of these chronic conditions in children should include multi-disciplinary expertise, with focus upon maintaining control of gut inflammation, optimising nutrition, growth and quality of life, whilst preventing disease or treatment-related complications.

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) comprise the inflammatory bowel diseases (IBD). These chronic conditions affecting the gastrointestinal tract are becoming increasingly common. At present there is an incomplete understanding of the causation of CD and UC. Although children and adolescents can be diagnosed with IBD at any age, the second decade of life is the most common period. From diagnosis these children face a lifetime of

illness, with many potential consequences and effects.

CROHN'S AND COLITIS

IBD is characterised by chronic inflammation of the intestinal tract with variable periods of remission and exacerbation. Traditionally IBD is thought of as the two major clinical subtypes (CD and UC). However, it can also be seen as a heterogeneous group of disorders of intestinal inflammation^[1].

Classically, UC involves disease that extends proximally for a variable distance from the rectum, with involvement of the superficial layers of the colonic mucosa. Pediatric cohort studies show that pancolitis is the most frequent presentation of UC in childhood, with few children having isolated proctitis^[2,3]. This finding contrasts greatly with the disease patterns seen in adults with UC^[2]. Furthermore, those children without pancolitis at diagnosis commonly have extension of disease to involve the whole colon over the subsequent years.

In contrast to UC, CD is characterised by transmural inflammation in a non-contiguous pattern (so-called skip lesions), anywhere from the mouth to the anus. Disease distribution of CD differs between children and adults^[2]. In paediatric CD, the ileocolonic region is the most common location of disease. Disease limited to the colon is less frequently seen, and isolated terminal ileal disease is uncommon, occurring in less than 10% of children^[2]. Involvement of the gut proximal to the terminal ileum occurs in more than half of children with CD, with common areas being the stomach and duodenum^[2,4]. Aphthoid or serpiginous ulceration are particular endoscopic features of CD: other features such as friability, oedema, granularity and loss of vascular markings, may be seen in both UC and CD.

One particular histological feature of CD is non-caseating granuloma located in the inflamed mucosa. Perianal disease, including multiple large anal tags, perianal abscesses, non-healing deep fissures or fistulas, is a feature of CD, but not of UC. The inflammatory changes in CD may be complicated by stricturing or fistulising disease, with progression in many patients towards these phenotypes over time^[5].

The term IBD-unclassified (IBDU) refers to those patients with chronic bowel inflammation whose pattern of disease is not clearly able to be classified as CD or UC. Over the course of the disease, IBDU is often reclassified as either CD or UC as the pattern and features of inflammation evolve. IBDU is more commonly reclassified as UC than CD^[6]. The term indeterminate colitis, however, should be reserved for the situation where, following colectomy and histopathological examination of the colon, the distinction between UC and CD remains unclear^[6].

EPIDEMIOLOGY

IBD can present at any age, with the peak age range of

diagnosis in the second and third decades of life^[7]. In childhood, rates of IBD increase from the first year of life, with highest rates in teenage years. Around 25% of all diagnoses of IBD are made in the first two decades of life^[8,9]. A family history of IBD is more commonly elicited in children with IBD than in adults^[7].

Generally UC is found to be more common than CD in the preschool age group, whilst CD is three times more frequent than UC in older children in many case series^[10,11]. There is also a slight male preponderance (1.5:1) in prepubescent patients with CD as opposed to a slight female preponderance in adults^[2].

Although the incidence and prevalence of IBD varies, there is overwhelming data showing increasing rates in many areas of the world^[12,13]. In more recent years, an increasing incidence has been observed in countries that traditionally did not report IBD, such as Taiwan, China and other Eastern countries^[14]. In addition, children of families migrating from the developing world to the developed world have increased rates of IBD^[15]. There is also clear evidence that the incidence of IBD in the paediatric population is increasing, especially for CD. Benchimol *et al*^[16] observed an increased incidence rate of paediatric CD in the Canadian province of Ontario from 9.5 to 11.4 per 100 000 per annum over an 11 year period to 2005; however the incidence of UC in this period remained unchanged (4.1 to 4.2 per 100 000). In Australia, recent Victorian studies clearly show increasing rates in children, with a greater than 10-fold increase in CD over the 30 year period to 2001^[17]. In addition, an eleven-fold increase in paediatric UC was seen in the same area, with particular increases over the most recent two decades^[18]. It is unclear why IBD has become more common over the last decades: suggested factors include changes in lifestyle, diet, urbanisation and other environmental changes.

PATHOGENESIS OF IBD

The most accepted hypothesis for the pathogenesis of IBD is that interactions between the gut luminal contents (especially the intestinal microflora) and the mucosa lead to dysregulated inflammation in a genetically-predisposed host. A wide range of microorganisms have been considered as potential causative agents for IBD. These include *Mycobacterium paratuberculosis*, *Listeria monocytogenes*, Novel Burkholderiales and *Escherichia coli* subtypes^[19,20]. It is also speculated that viral agents may play roles in the development of IBD^[21]. Recently, a small study conducted in Finland focused on faecal detection of viral agents in a group of 50 children being evaluated for possible IBD (33 were diagnosed with IBD whilst 17 were shown to not have IBD)^[22]. Viral agents were not detected in the IBD group-but were present in 3 of the control group.

There is not yet clear data to support a role for any one of these organisms as the primary factor in the aetiology of IBD. Our recent work has focused upon several mucous-associated organisms, including members of the

Helicobacter and *Campylobacter* families^[23,24]. Although these studies show that such organisms are commonly present at the time of diagnosis of IBD, it is unclear if they have a causative role.

Some of the most exciting recent developments in our understanding of the pathogenesis of IBD have been in the field of genetics. A decade ago, *NOD2/CARD15* was identified as the first susceptibility gene for CD^[25]. *NOD2* is a member of a family of intracellular proteins that respond to bacterial proteins and contribute to host defence^[26,27]. In one large study 50% of patients with CD were found to have at least one *NOD2* gene mutation, with 17% having a double mutation^[28]. Those patients with 2 mutations were characterised as having a younger age of onset, more frequent stricturing disease, and less frequent colonic involvement, suggesting a link with earlier onset of disease. *NOD2* mutations are present at the same rates in patients with UC as in controls and are also not seen in non-European populations, such as in Japan, India and South Korea^[29,31]. Furthermore, *NOD2* mutations are not associated with early onset of disease in children of Ashkenazi background^[32]. Tumour-necrosis factor (TNF)- α promoter gene mutations were, however, associated with early onset in this group of children.

In more recent years, a number of other genes have been shown to be important for IBD—most in CD but some in UC. A recent transatlantic collaboration scanned a cohort of 3426 childhood-onset IBD patients and identified 5 new loci associated with paediatric IBD^[33]. In 2010, a multi-national collaboration identified many further loci implicated in CD, bringing the total of loci identified to 71^[34]. Mutations in the interleukin (IL)-10 receptor were recently shown in a group of infants with very early onset of severe and treatment resistant disease. Mutations in the coding for one of chains of the IL-10 receptor were identified: this change renders the patients' cells unresponsive to the anti-inflammatory effect of IL-10^[35]. A recent review article highlighted the findings of two paediatric gene wide association studies^[36]. Although emphasising key genetic pathways common to adult-onset disease, these studies also identified novel regions associated with early-onset disease, including genes encoding IL-27. The relevance of these potential links was recently outlined in a hypothesis article^[37]. In addition, a current prospective study (www.neopics.org) focusing on genetic influences on children aged less than 6 years of age should further define key aspects in this group.

PATTERNS OF PRESENTATION OF IBD IN CHILDREN

Children with IBD may present with a range of symptoms, depending on the location, severity and chronicity of inflammation. Classically, CD most commonly presents with pain, diarrhoea and weight loss, whilst UC most commonly starts with bloody diarrhoea^[38]. Children with distinct disease locations may present with other defined

gastrointestinal symptoms. For instance, oesophageal involvement may lead to odynophagia and dysphagia whilst perianal presentation may include pain, discharge or a mass. Recent studies suggest that fewer children have the so-called classical symptoms, and that children may have a range of presenting features (including atypical symptoms) including abdominal pain, diarrhoea, short stature or weight loss^[2,38]. Some children presenting with atypical or non-gastrointestinal symptoms may have delayed recognition and diagnosis. Although many of the gastrointestinal symptoms seen in paediatric IBD are similar to those reported in adults, particular features in children include linear growth failure and pubertal delay.

Despite its name, IBD is not limited to the bowel. Up to 30% of patients will develop an extra-intestinal manifestation (EIM) at some point during their lifetime^[39]. The most common EIM in children are arthritis (axial or peripheral), cutaneous changes (e.g., erythema nodosum and pyoderma gangrenosum), eye diseases (such as episcleritis and uveitis that occur in approximately 1% of patients with IBD) and liver disease^[40]. Hepatobiliary complications can take the form of primary sclerosing cholangitis, autoimmune hepatitis or overlap syndrome^[40].

IMPACT OF IBD UPON GROWTH AND NUTRITION IN CHILDREN

Weight loss, or lack of weight gain, is a presenting feature in 85% of children with CD and at least 65% of children with UC^[7]. This impairment of weight is predominantly a result of decreased oral intake due to anorexia, early satiety, nausea or pain. In addition to compromised weight, linear growth may also be impaired at diagnosis or subsequently^[41]. These consequences are primarily related to the systemic circulation of pro-inflammatory cytokines, such as TNF- α and IL-6. IL-6 influences the activity of key proteins, including insulin-like growth factor (IGF)-1, and interferes with the effects of growth hormone and other key pathways^[42].

An additional consequence of nutritional impairment and elevated levels of cytokines is delayed pubertal development. Given that many children present in the pre-pubertal or peri-pubertal period, pubertal delay can be of significant concern and importance. Failure to adequately induce disease remission at this crucial stage can have significant consequences such as missed or delayed pubertal growth spurt and reduced final height, abnormal bone mineralisation, and maintenance of prepubertal sex hormone levels^[38].

Children with IBD can also have micronutrient deficiencies. The most common of these are iron, vitamin D, vitamin B12, calcium and zinc. In a cohort of children with IBD from Sydney, Australia, only 40% had normal Vitamin D status^[43]. Lack of Vitamin D along with inadequate calcium intake (and also vitamin K deficiency) contributes adversely to bone health. Since 90% of peak bone mass is attained during childhood and adolescence, failure to attain maximal potential may increase future

fracture risk^[44]. Underlying systemic inflammation is an independent detrimental influence on bone health^[44]. Sylvester *et al*^[45] have shown low mean bone mineral density (BMD) scores in children with IBD and also demonstrated that BMD scores are associated with body mass index and IL-6 levels.

APPROACH TO POSSIBLE IBD IN CHILDREN

Diagnostic pathways begin with the consideration of possible IBD as an important first step. A suggestive history of gut symptoms may be present, but children may present with atypical symptoms. Examination findings of weight loss, chronic disease (e.g., clubbing) or extra-intestinal features of IBD (e.g., erythema nodosum) may be detected. Weight and height should be accurately measured and plotted on an appropriate growth chart. Previous growth data should be obtained from the child's health records and parental heights should be recorded to calculate mid-parental height.

Exclusion of other potential pathologies, especially enteric infections, is important. Several stool cultures should be requested to exclude an enteric infectious cause in children presenting with diarrhoea and/or abdominal pain, with inclusion of less common organisms such as *Yersinia* and *Aeromonas*. Stool can also be sent for faecal markers of inflammation—these include the presence of faecal white cells, stool α -1-antitrypsin, lactoferrin and calprotectin (where available). S100A12, another non-invasive marker of gut inflammation shows high sensitivity and specificity in differentiating between children with IBD and non-IBD conditions^[46]. Non-invasive tests such as calprotectin and S100A12 may also have roles in disease monitoring after diagnosis^[47].

Blood tests should be requested for full blood count (especially Hb, platelets, and white count), erythrocyte sedimentation rate (ESR), C-reactive protein, albumin and liver chemistry. Further baseline assessment should include iron studies, B12/folate levels and vitamin D. Serum based markers of systemic inflammation may be helpful in children with IBD, but exclusion of the diagnosis can not be made with normal tests. A recent North American study suggests that normal bloods (platelets, ESR, albumin or Haemoglobin) may be seen in 21% of mild CD, 54% of mild UC and 4% of more severe CD or UC^[48]. The addition of specific serological tests (ASCA, ANCA and pANCA) to a standard diagnostic approach is shown to improve and enhance diagnostic yield^[49].

If IBD is suspected on the basis of history, examination findings and/or the results of preliminary tests, then further investigations should be arranged. Definitive diagnosis relies on endoscopic and histologic findings, often supported by radiologic findings. Upper gastrointestinal endoscopy and ileo-colonoscopy should both be undertaken in any child or adolescent with suspected IBD, along with multiple mucosal biopsies^[50]. As an upper gut location of IBD is present in at least two

thirds of children with CD, findings in this region may be sufficient firstly to make a diagnosis of IBD or secondly assist in differentiating between CD and UC^[4].

Baseline investigations should also include an assessment of the small bowel^[50]. The vast length of the small bowel is not accessible to standard endoscopy. An increasingly preferred method to view the small bowel is a small bowel series magnetic resonance imaging, which can provide detail of the extent of inflammatory changes through the mucous without radiation exposure^[51]. This has largely supplanted the small bowel meal and follow-through as a tool to assess the small bowel. Capsule endoscopy also has an increasing role, with this modality able to identify superficial and smaller mucosal lesions^[52]. Other potential modalities include white-blood cell scans, positron emission tomography scans and ultrasound scanning^[53-55]. CT scanning, however, is rarely required in children and adolescents (and is generally discouraged due to potential cumulative radiation exposure).

MANAGEMENT OF IBD IN CHILDREN

Although the key concept in the management of IBD is inducing and maintaining remission, the pervasive effects of IBD in children mean that holistic care is essential, with consideration of multiple aspects of the condition and its complications. Provision of these management aspects in a child (and family) focused multi-disciplinary team setting is optimal to ensure superior outcomes.

In terms of control of gut inflammation, the management principles are to induce remission (control inflammation) and to then maintain remission. Although remission can be considered at clinical (relief of symptoms) and biochemical levels (normalisation of systemic markers of inflammation), histological remission (normalisation of histologic changes or mucosal healing) is seen as the ideal goal of therapy. Therapies to induce remission (e.g., corticosteroids or exclusive enteral nutrition) can be considered separately to those utilised to maintain remission [e.g., amino-salicylates (ASA) or immunomodulators such as thiopurines].

Whilst corticosteroids have traditionally been utilised to induce remission in active IBD, there is increasing support and rationale for exclusive enteral nutrition (EEN) in paediatric CD. EEN involves the sole administration of a nutritional formula, with exclusion of normal diet, for a period of up to 8 wk^[56,57]. EEN has remission rates equivalent to those of CS, but has numerous advantages such as avoiding steroid-related side-effects and in addition leads to superior rates of mucosal healing^[58]. Antibiotics (especially metronidazole and/or ciprofloxacin) may have roles in mild luminal or perianal CD. Aminosalicylates may have particular roles in inducing remission in mild to moderate active UC. Tacrolimus^[59] or cyclosporin may have a role in the management of severe colitis, whilst biologic drugs (such as infliximab) have roles in the induction of remission of severe disease.

ASA drugs have roles in the maintenance of remission

of UC, and although often also used for maintenance in CD, they are not as well supported for this by available evidence. Steroids and antibiotics do not have roles in the maintenance of remission of IBD in children. The immunosuppressive drugs have defined roles in the maintenance of remission of IBD in children. Thiopurines (azathioprine or 6-mercaptopurine) are typically used first: methotrexate tending to be used in the setting of thiopurine failure or intolerance^[60]. Early commencement of thiopurines in moderate-severe disease leads to less steroid requirement, more prolonged remission and better growth^[61]. Other drugs (such as thalidomide, tacrolimus or mycophenolate) may play a role in maintenance of remission. Supplementary nutrition can also have a role in maintaining remission in CD, but the subgroup most likely to benefit from this approach has not yet clearly been defined^[57].

Biological therapies have clear roles in the induction of remission in severe disease and in the subsequent maintenance of disease with ongoing dosing. The efficacy and safety of both infliximab^[62] and adalimumab^[63] has been considered in children and adolescents.

In addition to the current standard therapies, numerous other therapies are being developed or considered for roles in IBD. Many of these are biologic therapies that are able to be considered consequent to improved understanding of the complex inflammatory events in IBD. Other therapies that may play adjunctive roles include fish oils^[64] and probiotics^[65]. Additional novel therapies reported recently include low dose naltrexone^[66] and pig whip-worm therapy^[67]. The definitive roles for these therapies in children have not yet been proven.

One important factor in achieving optimal outcomes for children of any age with medical therapies is adherence. Recent work highlights an important relationship between adherence and disease severity^[68].

As well as medical therapies, many children with IBD require surgical intervention. Common indications in children with CD include the management of perianal disease, resection of disease unresponsive to medical therapy, or resection of a fibrotic stricture. In children with UC the indications for colectomy include fulminant UC unresponsive to medical therapy, severe colitis complicated by toxic megacolon and/or perforation, chronic colitis unresponsive to medical agents and following the development of pre-cancerous changes.

The cumulative risk of surgery in a series of 404 children with CD was 20% at 3 years and 34% at 5 years^[3]. A lower rate of resective surgery was seen in a Scottish series, with 20.2% having undergone surgery by 5 years^[2]. In this series, the authors demonstrated that the median time to first surgery was longer in their group of children with CD than a comparative adult group (13.7 years from diagnosis compared to 7.8 years; $P < 0.01$). In contrast, the reverse was seen in the individuals with UC (11.1 years from diagnosis in children contrasting to > 50 years in adults; $P = 0.38$)^[2].

The various therapeutic options need to be considered within the context of the individual patient and their disease pattern/location. Clearly the potential side-effects of an individual therapy need to be outlined in candid discussions with the patient and parents: these aspects need to be considered in the context of the potential benefits and the relative risk of the adverse effects.

In addition to the use of specific nutritional therapies to induce or maintain remission, the overall management of paediatric IBD requires close attention to growth and nutrition. Weight and height should be monitored regularly, with calculation of height velocity and assessment of pubertal development. Successful growth can be considered as an indicator of the success of therapy for IBD. Provision of a full well-balanced diet, with inclusion of adequate macronutrients (protein, fat, carbohydrates) and micronutrients (e.g., calcium and iron), should be reviewed by a paediatric dietitian regularly, with at least annual review. Monitoring of micro-nutrients is also important. Levels of iron, B12, folate and vitamin D should be reviewed on an annual basis.

The psychosocial aspects and consequences of IBD also require attention. IBD can impact greatly upon the quality of life of young patients^[69]. Disruption to schooling and social activities is common, especially in those with unstable or severe disease. Attention to coping and provision of supports, may require psychological intervention. Peer-support activities and supports also play important roles in the overall management of children with IBD.

PROGNOSIS AND OUTCOMES OF IBD IN CHILDREN

Given diagnosis in the first decades of life, infants and children have many decades of disease in front of them. Several recent cohorts have illustrated key aspects of the natural history and outcomes of IBD in children, with emphasis of key differences from adult-onset cohorts^[2,3,70].

Immune reactivity based upon a series of specific serological responses, has been shown to associate with disease outcome in children^[71]. In this group of 796 children with CD, an increased number of serological responses were linked with more aggressive disease pattern and earlier progression of disease. Subsequently, Siegel *et al*^[72] have developed a tool to outline predicted disease course in children with CD, incorporating serologic responses, along with patient and disease factors. The need for surgery has also been linked with NOD2 mutations in children with CD^[73]. Risk scores have also been considered in paediatric UC: Moore *et al*^[74] showed that white blood count and haematocrit values at diagnosis were associated with colectomy at 3 years in a cohort of 135 children with UC.

CONCLUSION

Crohn's and colitis has become an increasingly common diagnosis in children of all ages. These conditions have particular features and patterns in children, compared to adults. Early consideration of the diagnosis is important to avoid additional adverse impact upon growth, nutrition and normal functioning. Nutritional aspects are critical in the overall management of IBD. Whilst EEN is the therapy of choice to induce remission in CD, overall monitoring of growth and nutrition are key elements of ongoing management. Further work on the utility of drugs, such as antibiotics, will likely proceed in conjunction recognition of the importance of the intestinal microflora in the pathogenesis of IBD. The care of children and adolescents with IBD needs to be considered within a multi-disciplinary focus, with many different health professionals playing important roles.

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Effectiveness of impedance monitoring during radiofrequency ablation for predicting popping

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Abstract

AIM: To retrospectively evaluate the effectiveness of impedance monitoring for predicting popping during radiofrequency ablation (RFA) using internally cooled electrodes.

METHODS: We reviewed 140 patients (94 males, 46 females; age range 73.0 ± 11.1 year) who underwent RFA between February 2006 and November 2008 with a modified protocol using a limited power delivery rather than a conventional one to avoid popping. All the patients provided their written informed consent, and the study was approved by the institutional review board. Intraprocedural impedances were measured for the study subjects, and the tumors were classified into three types according to the characteristics of their impedance curves: increasing, flat, or decreasing. The tumors were further sorted into seven subtypes (A-G) depending on the curvature of the impedance curve's increase or decrease. Relative popping rates were determined for the three types and seven subtypes. A chi-square test was performed to estimate statistical significance.

RESULTS: A total of 148 nodules treated by RFA were analyzed. The study samples included 132 nodules of hepatocellular carcinoma, 14 nodules of metastatic liver cancer, and two nodules of intrahepatic cholangiocarcinoma. The numbers of nodules with each impedance curve type were as follows: 37 increasing-type nodules, 43 flat-type nodules, and 68 decreasing-type nodules. Popping occurrence rates were 24.3%, 46.5% and 64.7%, respectively. Flat-type nodules exhibited a significantly higher rate of popping compared to increasing-type nodules ($P = 0.039$). Decreasing-type nodules exhibited a significantly higher rate of popping compared to increasing-type nodules ($P < 0.0001$). Notably, nodules that showed a sharp decrease in impedance in the latter ablation period (subtype E) exhibited a significantly higher rate of popping compared to other subtypes.

CONCLUSION: Intraprocedural impedance monitoring can be a useful tool to predict the occurrence of popping during liver tumor RFA performed with internally cooled electrodes.

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Key words: Radiofrequency ablation; Internally cooled electrode; Popping; Liver; Complication

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INTRODUCTION

In addition to resection, radiofrequency ablation (RFA) is one of the most effective local treatments applied to liver tumors along with resection^[1-10]. The procedure is effective for relatively small tumors or tumors that recur after resection^[11-13]. In addition, it can be performed safely in elderly patients and cirrhotic patients^[14,15]. The RFA process involves inserting an electrode into the tumor and exciting it with a radio frequency current. This leads to a temperature increase in, and subsequent coagulation necrosis of, the tissue surrounding the electrode. However, complications arising from liver tumor RFA have been reported in numerous cases. Among those complications are intraperitoneal bleeding, subcapsular hematoma, biliary tract damage, portal vein thrombosis, peritoneal dissemination and gastrointestinal tract damage^[16-21].

There is a phenomenon called “popping” that refers to a form of explosive tissue disruption caused by a rapid elevation of intra-tissue pressure^[22-26]. When intra-tissue fluid vaporizes due to elevated tissue temperature, the tissue volume expands to approximately 1700 times that of the initial volume. The mechanism underlying this degree of tissue volume expansion is as follows: when 1 mol (18 mL) of water turns into gas at a standard temperature, pressure and dry, its volume increases to 22.4 L, which is 1244.4 times the volume of water. Assuming that the steam temperature is 373 K, the volume can be estimated by accounting for the increase in volume due to temperature with the following formula: $1244.4 \text{ times} \times 373 \text{ K} / 273 \text{ K} = 1700 \text{ times}$. Protein coagulation occurs at a temperature of approximately 60 °C, while vaporization occurs above 100 °C. Popping that occurs close to the subcapsular or main vessel has been thought to raise the risk of complications such as bleeding and dissemination.

In RFA procedures, two types of devices with different kinds of tips, the “internally cooled electrode” needle and the “expandable electrode” needle, are commercially available for ablation. It has been reported that the probability of popping is higher when using the internally cooled electrode due to the likelihood of intra-tissue pressure increasing more rapidly^[27]. According to the literature, there is a higher probability of scattered recurrence with the internally cool electrode^[28]. The conventional RFA protocol involves power delivery starting from 40 W followed by a power increase of 10 W every minute when using a 2-cm exposed tip; power delivery is started at 60 W, then increased by 20 W every minute when using a 3-cm exposed tip. Power output is increased with no limitations until a break occurs. A modified protocol to address the concern of complications from RFA has been introduced in recent years, and the authors reported that the likelihood of popping during RFA may be reduced by limiting power delivery^[24]. We

have applied these findings to our RFA procedure, limiting power delivery for the treatment of nodules on the surface of the liver or close to main vessels; however, a challenge still remained with respect to implementation of the modified protocol: in our hospital, popping occurred during 73 out of 148 sessions.

In our search for a safer RFA procedure, we reviewed 305 consecutive cases in which RFA was performed using the standard conventional protocol between June 2004 and January 2006. Of these 305 cases, major complications occurred in three cases (0.98%): a subcapsular hemorrhage, an intraperitoneal hemorrhage and a case of hemobilia (Figure 1). Steam popping had occurred in all three cases during RFA and it was therefore deemed as a potential contributor to the complications listed above.

In the present study, we retrospectively analyzed our RFA cases in which the modified protocol was applied between February 2006 and November 2008. The purpose of our study is to assess intraprocedural impedance monitoring to predict the likelihood of popping as hypothesized based on the aforementioned popping mechanism.

MATERIALS AND METHODS

Patients

Between February 2006 and November 2008, 280 patients in our hospital underwent RFA using internally cooled electrodes (Covidien) according to the modified protocol with limited power delivery to prevent popping. Among these individuals, 140 patients (94 males, 46 females; age range 73.0 ± 11.1 year) and 148 sessions were retrospectively analyzed in this study, excluding the 54 patients who showed unstable impedance curves and the 86 patients who did not reach the break point during RFA. In our hospital, RFA is indicated for tumors that are 3 cm or less in the largest dimension, and for patients with no more than three tumors. Patients with impaired liver function could also be candidates for RFA if they are free of ascites. Among the 140 patients analyzed, 35 had undergone transcatheter arterial chemoembolization (TACE) prior to RFA. All patients provided their written informed consent before treatment, and the study was approved by the institutional review board.

Ablation method for preventing popping

RFA was performed by three surgeons who specialize in liver surgery with 10, 20 and 25 year of experience, respectively. Midazolam (Dormicum; Astellas, Tokyo, Japan) was used for sedation at a dose of 0.03-0.06 mg/kg, and lidocaine (Xylocaine; Fujisawa, Tokyo, Japan) was used for local anesthesia. Cefazolin (Cefamezin; Astellas, Tokyo, Japan) was administered prophylactically against infection for 1-2 d following RFA. Abdominal ultrasound (Nemio; Toshiba, Tokyo, Japan) was used to

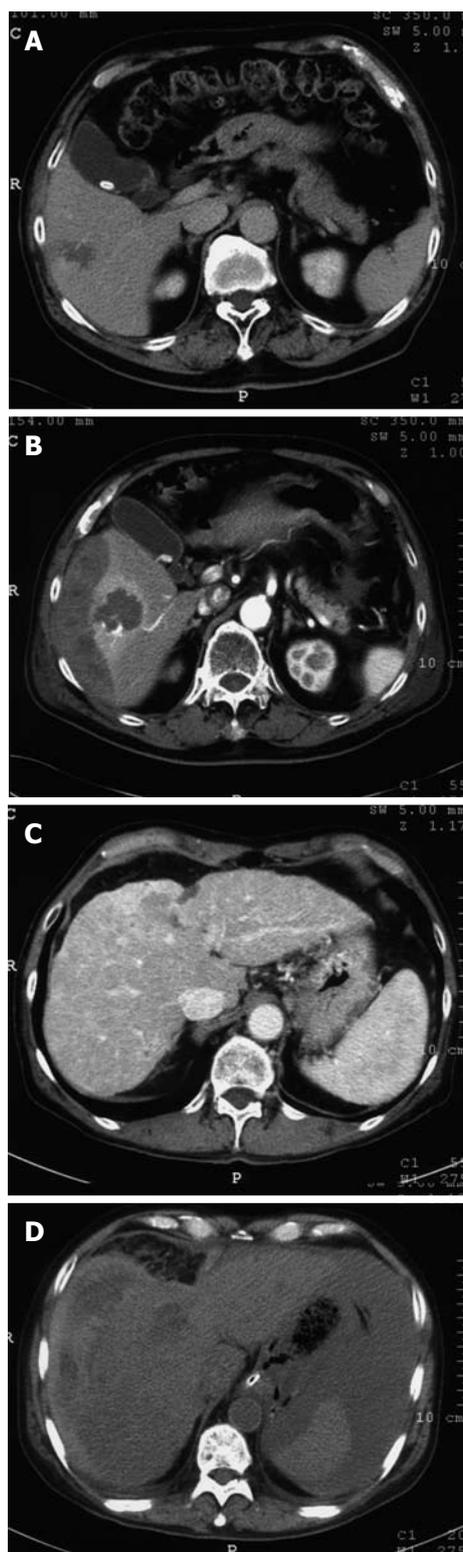


Figure 1 A case showing subcapsular (A, B) and intraperitoneal (C, D) hemorrhage after popping, which occurred during radiofrequency ablation for hepatocellular carcinoma. A, C: Before ablation; B, D: After ablation. RFA: Radiofrequency ablation; HCC: Hepatocellular carcinoma.

place the radiofrequency (RF) electrode in the tumors. A 17-gauge internally cooled electrode with either a 2-cm or 3-cm exposed tip was used, depending on the tumor

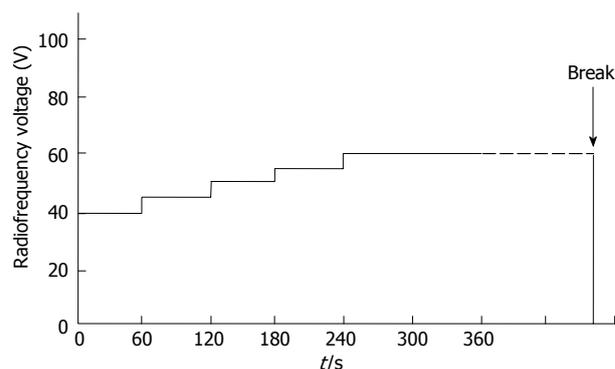


Figure 2 The ablation protocol to avoid popping involves the initiation of power delivery from 40 V with increases of 5 V every minute to a maximum of 60 V until a break is observed.

size as observed during the study period. For tumors smaller than 2 cm in diameter, a 2-cm exposed tip was used, while for tumors 2 cm or larger in diameter, a 3-cm exposed tip was used. Overlapping ablation was performed in three cases in which the tumors were greater than 3 cm in diameter. The electrodes were then connected to a generator (Series CC-1, Radionics: Covidien at present). Power was delivered using an impedance control mode to avoid popping. The RF voltage was initially 40 V and was increased by 5 V every minute to a maximum of 60 V, with no limitations on ablation time (Figure 2). This protocol was applied consistently whether using a 2-cm or 3-cm exposed tip.

The occurrence of a break was considered as one of the reasons for terminating ablation. During RFA, patients were monitored for popping before reaching the first break; the impedance curve reflects the data collected prior to popping. The time that elapsed prior to the break was recorded by the performing surgeon. When it was determined that coagulation necrosis was obtained after review of the echogram or by measuring the temperature in the ablated site after the first break, the procedure was terminated; otherwise, it continued even after reaching a break multiple times. The equipment was configured so that a break automatically occurred when the impedance increased to 25 Ω before the start of RFA. Thereafter the RF power was automatically returned to 0 W. The occurrence of popping is defined as the audible explosion sound confirmed by the rapidly expanding, highly echoic area.

RF system

During each procedure, a computer with monitoring software was connected to the main unit of the generator to record the RF power (W), RF current (mA), RF voltage (V), temperature ($^{\circ}$ C), and impedance (Ω) simultaneously (Figure 3).

Statistical analysis

All statistical analyses were conducted using JMP 8.0.2

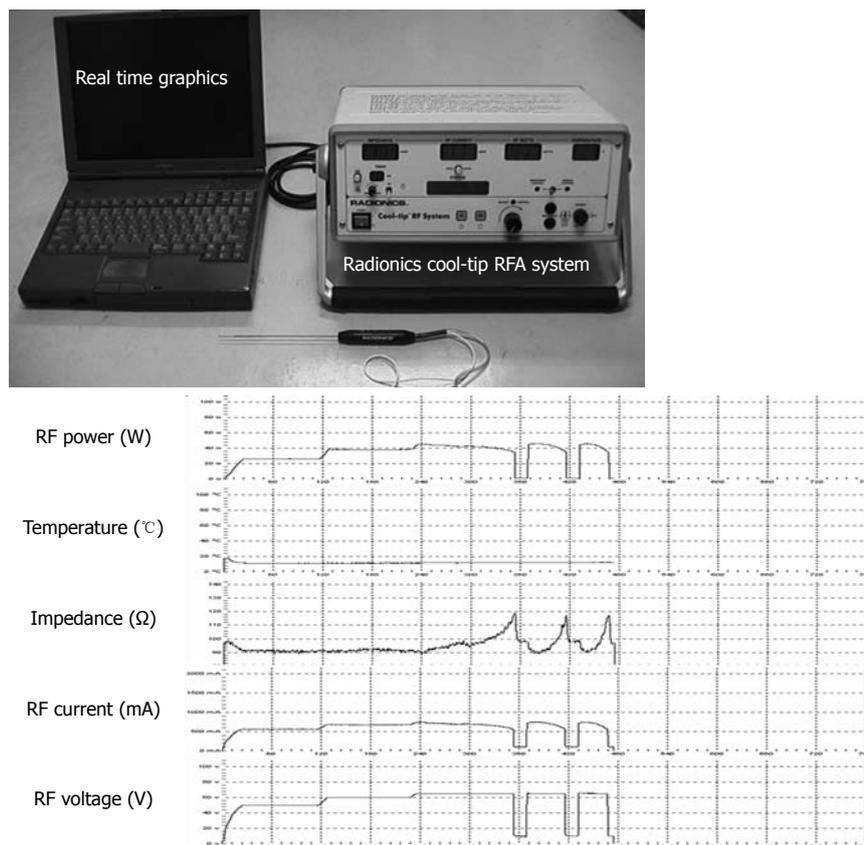


Figure 3 Radio frequency power, radio frequency current, radio frequency voltage, temperature and impedance were intraoperatively monitored using the equipment as shown in the diagram. RF: Radio frequency.

Table 1 Patient characteristics (140 patients, 148 nodules)

Characteristics	Value
Male/female (n/n)	94/46
Age (yr)	73.0 ± 11.1
ICGR 15 (%)	22.6 ± 14.4
Maximum tumor diameters (mm)	21.9 ± 7.1
Hepatocellular carcinomas	132
Metastatic liver cancers	14
Intrahepatic cholangiocarcinomas	2
Tumor locations	
Segment 1	3
Segment 2	11
Segment 3	23
Segment 4	13
Segment 5	20
Segment 6	22
Segment 7	23
Segment 8	33
RFA needle lengths (2 cm/3 cm)	79/69
Ablation time (s)	862 ± 613

Data are presented by mean ± SD or *n*. RFA: Radiofrequency ablation; ICGR 15: Indocyanine green retention rate at 15 min.

software (Macintosh; SAS institute Japan). A χ^2 test was performed to identify any significant differences among the three types and among the seven subtypes. Differences were considered statistically significant at $P < 0.05$.

RESULTS

A total of 140 patients and 148 nodules treated by RFA were analyzed. The study samples included 132 nodules of hepatocellular carcinoma, 14 nodules of metastatic liver cancer, and two nodules of intrahepatic cholangiocarcinoma. Popping occurred in 73 out of the 148 RFA sessions (Table 1).

All 148 nodules were classified into three types according to the characteristics of their impedance curves up to the point where the first break took place (Figure 4): increasing-type nodules showed an increase in impedance; flat-type nodules showed a flat impedance curve; and decreasing-type nodules showed a decrease in impedance. Popping rates were determined for each type. The nodules were further sorted into seven subtypes, A to G, depending on the curvature of each impedance curve's increase or decrease up to the first break point. Likewise, popping rates were determined for each subtype (Figure 4).

Though there was no significant difference among subtypes in terms of maximum tumor diameters, type B exhibited significantly higher ICGR 15 levels than type C ($P = 0.028$). There was no distinct difference among subtypes in terms of tumor location (Table 2).

The nodule distribution was as follows: 37, 43 and

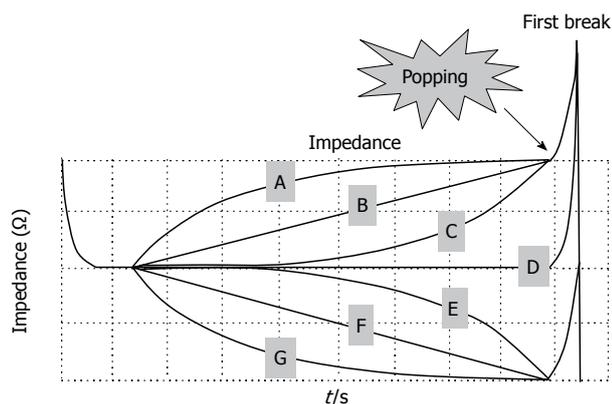


Figure 4 The graph showing the characteristic impedance curve shapes leading to the break point. The graph showing the seven characteristic impedance curves leading to the break point, which dictated the subgroups into which the previously classified nodules were further sorted. A-C: Increasing type; D: Flat type; E-G: Decreasing type; B, D, F: Three characteristic impedance curve shapes leading to the first break point, into which the 148 nodules were classified: increasing, flat and decreasing.

68 for increasing, flat and decreasing types, respectively. Popping occurred with nine increasing-type nodules (24.3%), 20 flat-type nodules (46.5%), and 44 decreasing-type nodules (64.7%) (Figure 5). Flat-type nodules exhibited a significantly higher rate of popping compared to increasing-type nodules ($P = 0.039$). Decreasing-type nodules exhibited a significantly higher rate of popping compared to increasing-type nodules ($P < 0.0001$). Regarding the subtype analysis, the popping occurrence rates were 1/2 (50%) for nodule subtype A, 3/9 (33.3%) for B, 5/26 (19.2%) for C, 20/43 (46.5%) for D, 19/23 (86.4%) for E, 14/21 (66.7%) for F and 11/24 (45.8%) for G (Figure 5). Notably, subtype E showed a rapid decrease in impedance during the latter half of the ablation period and exhibited a significantly higher rate of popping compared to subtypes B ($P = 0.006$), C ($P < 0.0001$), D ($P = 0.004$) and G ($P = 0.008$). The results for subtype A could not be examined statistically because of the small number of samples.

With respect to the lengths of the needles that were used, popping occurred during 36 out of the 79 (45.6%) RFA sessions using a 2-cm exposed tip, and 37 out of the 69 (53.6%) RFAs using a 3-cm exposed tip ($P = 0.328$). The distribution of nodules treated by a 2-cm exposed tip was as follows: A ($n = 2$), B ($n = 5$), C ($n = 14$), D ($n = 23$), E ($n = 11$), F ($n = 12$) and G ($n = 12$). The distribution of those treated using a 3-cm exposed tip was as follows: A ($n = 0$), B ($n = 4$), C ($n = 12$), D ($n = 20$), E ($n = 12$), F ($n = 9$) and G ($n = 12$). Popping was most common in the subtype E nodules, which showed a rapid decrease in impedance during the latter half of ablation, regardless of the exposed tip length (2 cm: 9/11 = 81.8%; 3 cm: 10/12 = 83.3%). Among 14 metastatic liver cancer samples, popping occurred in seven cases with the highest occurrence in subtypes E

Table 2 Tumor characteristics for each impedance type (148 nodules)

Impedance subtype	Maximum tumor diameter (mm) ¹	ICGR 15 (%) ¹	Tumor location (segments 1,2,3,4,5,6,7,8) ²
A	20.3 ± 6.0	17.8 ± 13.9	(0,1,0,0,0,0,1)
B	20.4 ± 5.3	29.8 ± 10.7 ^a	(1,1,1,1,1,2,1,1)
C	21.2 ± 8.1	19.9 ± 11.1	(0,3,3,3,5,3,3,6)
D	22.7 ± 6.7	24.2 ± 17.9	(0,2,8,4,8,6,4,11)
E	22.7 ± 6.3	21.4 ± 10.2	(1,2,1,2,1,3,8,5)
F	22.3 ± 7.8	24.1 ± 15.9	(1,1,4,1,3,5,3,3)
G	20.9 ± 8.1	20.2 ± 14.0	(0,1,5,2,2,4,4,6)

^a $P = 0.028$ vs type C ($P = 0.028$). ¹Data are means ± SD; ²Data are numbers of nodules. ICGR 15: Indocyanine green retention rate at 15 min.

(2/3 = 66.7%) and F (2/3 = 66.7%). Popping occurred in neither of the two intrahepatic cholangiocarcinomas. TACE was performed prior to RFA in 35 nodules; in these TACE cases, popping was found in subtypes C (2/6 = 33.3%), D (4/8 = 50.0%), E (3/3 = 100%), F (3/9 = 66.6%) and G (3/9 = 66.6%). No nodule of TACE was classified as subtype A or B. We also evaluated 48 nodules located on the surface of the liver and 100 nodules close to major vessels. Among 48 nodules that were on the surface of the liver, popping was observed in subtypes A (1/1 = 100%), B (1/3 = 33.3%), C (2/10 = 20.0%), D (4/10 = 40.0%), E (9/11 = 81.8%), F (4/7 = 57.1%) and G (4/6 = 66.7%). Among 100 nodules close to major vessels, popping occurred in subtypes A (0/1 = 0%), B (2/6 = 33.3%), C (3/16 = 18.8%), D (16/33 = 48.5%), E (10/12 = 83.3%), F (10/14 = 71.4%) and G (7/18 = 38.9%). Among the samples in which TACE was performed prior to RFA, popping was observed in subtype E most frequently, regardless of tumor location.

DISCUSSION

Although the rate of complications caused by RFA varies among hospitals, it is generally between 2.2% and 9.5%^[29-33]. Peritoneal dissemination is one of the most serious complications that should be avoided and for which certain types of tumors are reportedly at a high risk, particularly subcapsular liver tumors and hepatocellular carcinomas that are poorly differentiated or that have high levels of alpha fetoprotein^[34,35]. Several studies have also shown that tumors abutting the main portal vein are at a high risk of rapid intrahepatic dissemination^[36,37]. Consequently, RFA should be performed with special caution when treating tumors that have developed close to the subcapsular or main vessel. In our hospital, because of these risks, liver tumors in these regions have been treated by RFA with limited power delivery to avoid complications.

Our results showed that decreases in impedance, particularly a rapid decrease in impedance during the latter half of the ablation period, strongly predict popping. We

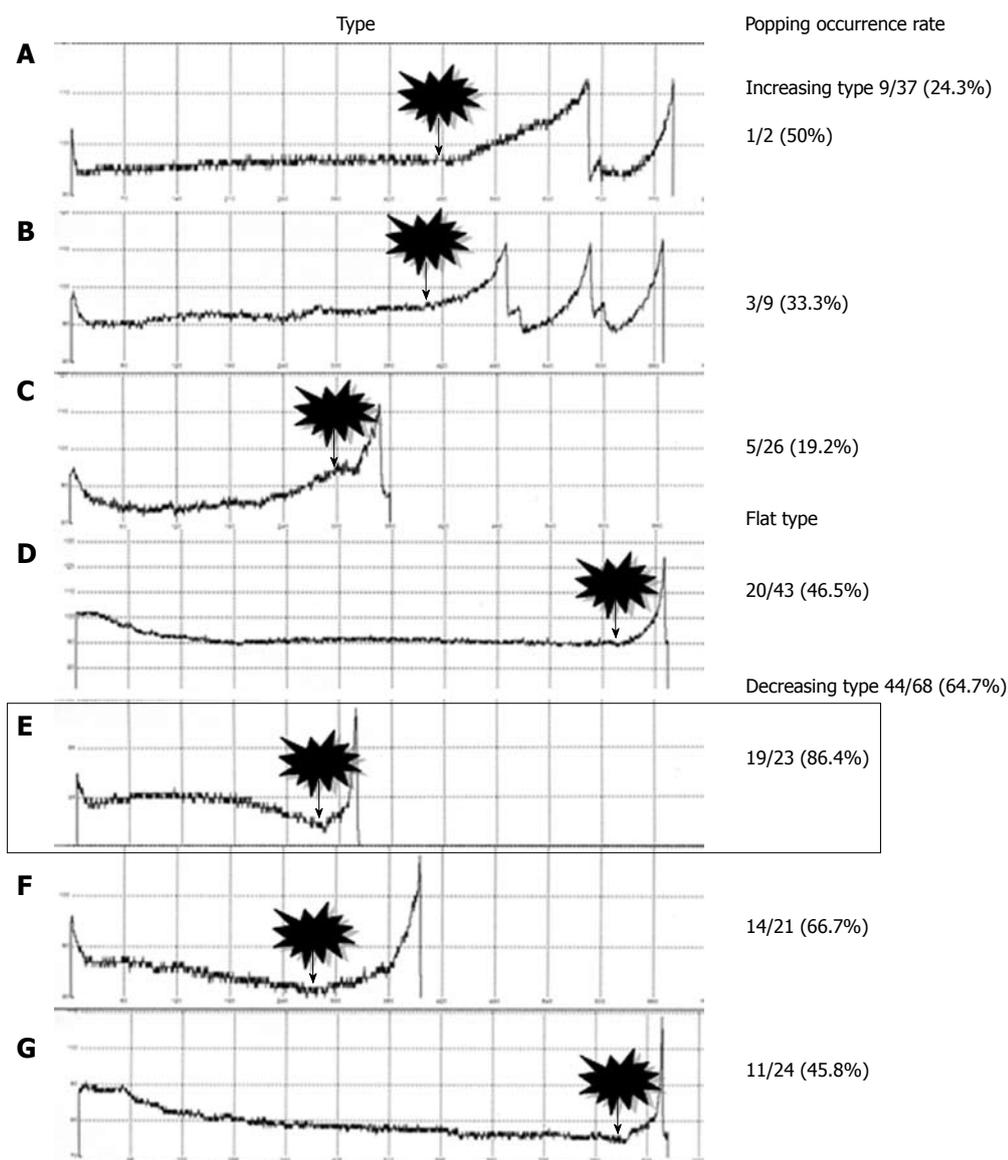


Figure 5 Subtype E (nodules that showed a rapid decrease in impedance during the latter half of the ablation) exhibited a significantly higher rate of popping compared to subtypes B, C, D and G ($P < 0.05$). Decreasing-type nodules exhibited a significantly higher rate of popping compared to increasing-type nodules ($P < 0.0001$). Flat-type nodules exhibited a significantly higher rate of popping compared to increasing-type nodules ($P = 0.039$).

investigated tumor location, diameter, and hepatic function reserve in each subtype of impedance. We used an ICGR value of 15 min for the hepatic functional reserve test. The principle is that indocyanine green (ICG) is transported to the liver in association with lipoprotein in the blood and then ingested by hepatocytes. We calculated the retention rate of ICG after 15 min of injection. If hepatic functional reserve is impaired, the retention rate is high. The result achieved with an ICGR of 15 min showed that the rate of popping was slightly higher in subtype B than in subtype C ($P = 0.028$). There were no significant differences among subgroups. We did not find any relationship between the impedance curve and the hepatic function reserve. Tumor location and diameter were also similar among the subtypes.

The reason for the drop in impedance just before the occurrence of popping is that the elevation of intra-tissue temperature activates intra-tissue molecular movement, which results in higher electrical conductivity. Electrical conductivity is the amount of electricity that a substance can conduct, and it varies for different substances, e.g., 5% NaCl has an electrical conductivity of 67 mS/cm, and 5% HCl has an electrical conductivity of 395 mS/cm. The electrical conductivity of a substance increases as the temperature rises, and it can be estimated with the formula $k_T = k_{25} [1 + 0.02 (T - 25)]$, where k_T is the electrical conductivity at temperature T ($^{\circ}\text{C}$), and k_{25} is the electrical conductivity at 25 $^{\circ}\text{C}$. Because tissue impedance is inversely related to electrical conductivity, tissue temperature increases as tissue impedance de-

creases. Thus, an abrupt decrease in impedance can be an indication of a rapid increase in intra-tissue temperature, possibly leading to steam popping following the vaporization of intra-tissue fluid.

The most favorable pattern of impedance curvature exhibited during the ablation period is the increasing type, which indicates that tissue coagulation occurs in parallel with an elevation in tissue temperature alongside a gradual increase in tissue impedance. If the temperature of intra-tissue fluid rises above 100 °C and starts to vaporize, the tissue impedance decreases, resulting in popping. As for the reason for the variation in impedance curves obtained with the same RFA protocol, we believe that fluid produced through coagulation may have played a part in some way.

Between June 2004 and January 2006, when we implemented RFA in the conventional protocol, major complications associated with RFA were observed in three out of 305 cases (0.98%). It was determined that popping had occurred during all three cases. Following this outcome, we opted for a lower power output when treating tumors on the surface of the liver or in close proximity to major vessels. Although no significant differences were found, this modified protocol may have contributed to a decrease in complications; hemobilia as a postoperative complication occurred in only one out of 473 cases (0.21%). The rates of local recurrence were equivalent in the conventional and modified protocols: 42 out of 305 cases (13.8%) between June 2004 and January 2006, and 48 out of 473 cases (10.1%) between February 2006 and November 2008. Based on the present study findings, we reduce the power delivery if we observe impedance curvatures that signal imminent popping. Between December 2008 and November 2011, we performed RFA on 731 nodules using the modified protocol; popping occurred for only 44 nodules (6.0%). No severe complication was observed in any of these cases.

There were several limitations on our analysis that have to be acknowledged. The first limitation concerns the patients excluded: for the 54 patients who showed unstable impedance curves, popping remained unpredictable; for the 86 patients who did not reach the break point during RFA (meaning that complete coagulation necrosis had not been achieved according to the present consensus on break points), there was a relatively higher risk of tumor recurrence. Second, due to a limitation resulting from the nature of our retrospective study design, a further prospective study will be necessary to confirm whether using the modified method actually improves the complication rate among patients whose tumors are close to major vessels or located subcapsularly.

In conclusion, by monitoring intraprocedural impedance during the RFA procedure, it is possible to predict popping in certain cases.

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COMMENTS

Background

Radiofrequency ablation (RFA) is one of the most effective and safe treatments administered to patients with liver tumors. Certain complications may be induced in association with RFA, such as liver abscess, gastrointestinal tract injury, bleeding, subcapsular hematoma, biliary tract injury, portal vein thrombosis, and peritoneal seeding. A modified protocol used to address the concern of complications related to RFA has been introduced in recent years. This procedure reportedly decreased the frequency of popping by limiting power delivery.

Research frontiers

Popping is a phenomenon of vapor explosion that can occur during RFA. Popping occurs if water vaporizes prior to tumor coagulation and may pose a risk of complications. RFA using low power delivery has been advocated to avoid this phenomenon. The authors applied this procedure for the treatment of tumors near Glisson's capsule or the surface of the liver.

Innovations and breakthroughs

The authors monitored and analyzed the impedance curves to identify the characteristic warning signs that precede popping. It was found that popping was most frequent in nodules that exhibited a rapid decrease in impedance during the latter half of the ablation. This is the first such investigation.

Applications

By monitoring the intraprocedural impedance during RFA, it is possible in certain cases to predict popping. To avoid popping, power delivery should be limited when an impedance curve displays the characteristic warning signs.

Terminology

Popping is a phenomenon that refers to a form of explosive tissue disruption caused by a rapid elevation of intra-tissue pressure. The occurrence of popping should be avoided during RFA due to the risk of complications. During RFA, tumor necrosis is caused by protein coagulation, which occurs at a temperature of approximately 60 °C. However, if the temperature rises above 100 °C prior to achieving protein coagulation, intra-tissue fluid vaporizes and the tissue volume expands to approximately 1700 times that of the initial volume. This is the mechanism underlying popping, which can be anticipated through the use of intraprocedural impedance monitoring.

Peer review

The topic deals with the issue of RFA efficacy control, and the ideas presented seem to be of interest for those involved in liver tumor management. The manuscript is well structured. Materials and methods are appropriately characterized and results with tables and pictures provide evidence to draw conclusions.

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Several factors including *ITPA* polymorphism influence ribavirin-induced anemia in chronic hepatitis C

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Abstract

AIM: To construct formulae for predicting the likelihood of ribavirin-induced anemia in pegylated interferon α plus ribavirin for chronic hepatitis C.

METHODS: Five hundred and sixty-one Japanese patients with hepatitis C virus genotype 1b who had

received combination treatment were enrolled and assigned randomly to the derivation and confirmatory groups. Single nucleotide polymorphisms at or nearby *ITPA* were genotyped by real-time detection polymerase chain reaction. Factors influencing significant anemia (hemoglobin concentration < 10.0 g/dL at week 4 of treatment) and significant hemoglobin decline (declining concentrations > 3.0 g/dL at week 4) were analyzed using multiple regression analyses. Prediction formulae were constructed by significantly independent factors.

RESULTS: Multivariate analysis for the derivation group identified four independent factors associated with significant hemoglobin decline: hemoglobin decline at week 2 [$P = 3.29 \times 10^{-17}$, odds ratio (OR) = 7.54 (g/dL)], estimated glomerular filtration rate [$P = 2.16 \times 10^{-4}$, OR = 0.962 (mL/min/1.73 m²)], rs1127354 [$P = 5.75 \times 10^{-4}$, OR = 10.94] and baseline hemoglobin [$P = 7.86 \times 10^{-4}$, OR = 1.50 (g/dL)]. Using the model constructed by these factors, positive and negative predictive values and predictive accuracy were 79.8%, 88.8% and 86.2%, respectively. For the confirmatory group, they were 83.3%, 91.0% and 88.3%. These factors were closely correlated with significant anemia. However, the model could not be constructed, because no patients with rs1127354 minor genotype CA/AA had significant anemia.

CONCLUSION: Reliable formulae for predicting the likelihood of ribavirin-induced anemia were constructed. Such modeling may be useful in developing individual tailoring and optimization of ribavirin dosage.

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Key words: Chronic hepatitis C virus infection; Ribavirin; Pegylated interferon α ; Prediction model; Hemolytic anemia; Single nucleotide polymorphism

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Tsubota A, Shimada N, Abe H, Yoshizawa K, Agata R, Yumoto Y, Ika M, Namiki Y, Nagatsuma K, Matsudaira H, Fujise K, Tada N, Aizawa Y. Several factors including *ITPA* polymorphism influence ribavirin-induced anemia in chronic hepatitis C. *World J Gastroenterol* 2012; 18(41): 5879-5888 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i41/5879.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i41.5879>

INTRODUCTION

Development and availability of nonstructural (NS) 3 serine protease inhibitors (PIs), such as telaprevir and boceprevir, further improve treatment outcome in combination with pegylated interferon (peg-IFN) α and ribavirin (RBV) for chronic hepatitis C virus (HCV) genotype 1 infection, while the addition of novel antiviral agents increases the frequency and severity of adverse effects (including anemia), medication costs and the complexity of treatment regimens^[1-3]. Triple combination therapy with PI, RBV and peg-IFN α will be the first-line treatment for the HCV genotype 1 infection until the establishment of combination with NS3/4A PIs and NS5B polymerase or NS5A inhibitors^[4]. Meanwhile, conventional peg-IFN α plus RBV combination will be in demand for easy-to-treat patients who are infected with HCV genotype 2 or 3 or low viral loads and those who contraindicate or are intolerant of triple combination therapy. Accordingly, peg-IFN α plus RBV combination will assume a crucial role in the treatment of HCV infection for the foreseeable future.

In RBV-based treatment, hemolytic anemia is common and one of the major critical adverse effects^[1-3,5-7] and therefore makes it difficult for patients to tolerate treatment continuation, resulting in early dose reduction or premature withdrawal that may diminish the treatment efficacy. So far, many factors have been reported to be significantly associated with the significant anemia that could necessitate dose reduction or discontinuation^[8-20]. Specifically, host genetic variants at the inosine triphosphatase (*ITPA*) gene located on chromosome 20 (20p13 region) that lead to *ITPA* deficiency or low activity have an overwhelming impact on protection against RBV-induced hemolytic anemia, and decrease the need for RBV dose reduction at week 4 of treatment and throughout the treatment course^[15-18]. However, there are few reports that provide a convenient prediction model or scoring system for pretreatment screening or early identification of clinically significant anemia that has been defined previously and used generally^[15].

To modify RBV dose prior to treatment or during the early treatment phase and continue treatment as long

as possible, the present study focused on the construction of a convenient and useful model for predicting the likelihood of clinically significant anemia and quantitative decline in the hemoglobin (Hb) concentration from baseline at week 4 of treatment in peg-IFN α plus RBV treatment for chronic hepatitis C patients infected with HCV genotype 1b. Easy identification of candidates at a high risk for clinically significant anemia may facilitate intensive safety monitoring in combination treatment.

MATERIALS AND METHODS

Study population and protocol

Between 2006 and 2010, 561 chronic hepatitis C patients infected with HCV genotype 1b were consecutively enrolled in this study at Katsushika Medical Center and Kashiwa Hospital, The Jikei University School of Medicine, and Shinmatsudo Central General Hospital. Patients received peg-IFN α -2b at a dose of 1.5 μ g/kg or peg-IFN α -2a at a dose of 180 μ g once weekly and RBV at a dose of 600-1000 mg twice daily for 48 wk. The dose of RBV was adjusted according to body weight (BW); 600 mg for \leq 60 kg, 800 mg for $>$ 60 kg to \leq 80 kg, and 1000 mg for $>$ 80 kg. Leading inclusion criteria were chronic hepatitis C that were diagnosed by laboratory, virology and histology; HCV genotype 1b confirmed by the conventional polymerase chain reaction (PCR)-based method; acquisition of written informed consent to the provision of genetic material; availability of genetic DNA for genotyping single nucleotide polymorphisms (SNPs); absence of liver cancer, liver failure or other forms of liver disease; and lack of concurrent treatment with any other antiviral or immunomodulatory agent. The study protocol was conducted in accordance with the provisions of the Declaration of Helsinki and Good Clinical Practice guidelines and was approved by the Institutional Review Boards of all participating sites.

Clinical and laboratory data were assessed at baseline, once weekly during the first 4 wk, and thereafter every 4 wk. As described previously^[15], significant anemia was provisionally defined as Hb concentrations of $<$ 10.0 g/dL at week 4 of treatment, and significant Hb decline was defined as a decline in Hb concentration of $>$ 3.0 g/dL at week 4 of treatment. The reasons for choosing this time point (the end of 4 wk after treatment initiation) were as follows: (1) dose of RBV or peg-IFN was not reduced in most patients, and thus Hb dynamics would not be affected by treatment modification and could be evaluated in an unbiased manner; and (2) Hb decline within the first 4 wk is most prominent throughout the treatment period and reaches a nadir after approximately 4-6 wk^[6,11].

At baseline, creatinine clearance (Ccr; mL/min) was estimated by using the Cockcroft-Gault formula^[21]: Ccr (for male) = [(140 - age) \times BW (kg)] / (72 \times Scr) (Scr, serum creatinine; Ccr \times 0.85 for female). Estimated glomerular filtration rate (GFR, mL/min/1.73 m²) was

Table 1 Baseline profiles of the study population (mean \pm SD)

Variable	Overall cohort (<i>n</i> = 561)	Derivation group (<i>n</i> = 374)	Confirmatory group (<i>n</i> = 187)
Demographic feature			
Age (yr)	59.1 \pm 10.9	59.1 \pm 10.8	59.5 \pm 11.3
Sex (female/male)	302/259	201/173	101/86
Weight (kg)	59.8 \pm 11.4	59.9 \pm 11.5	58.5 \pm 10.9
BMI (kg/m ²)	23.2 \pm 3.3	23.2 \pm 3.4	23.2 \pm 3.2
Height (cm)	160.3 \pm 9.0	160.4 \pm 9.1	158.5 \pm 8.7
BSA (m ²)	1.62 \pm 0.18	1.62 \pm 0.18	1.59 \pm 0.18
Laboratory data			
ALT (IU/L)	63 \pm 54	63 \pm 56	58 \pm 47
GGT (IU/L)	58 \pm 63	56 \pm 60	58 \pm 72
Albumin (g/dL)	4.1 \pm 0.4	4.1 \pm 0.4	4.1 \pm 0.3
Creatinine (mg/dL)	0.70 \pm 0.16	0.70 \pm 0.17	0.70 \pm 0.17
WBC count ($\times 10^3$ /mL)	5.0 \pm 1.5	5.0 \pm 1.5	5.1 \pm 1.4
Hemoglobin (g/dL)	13.7 \pm 1.5	13.7 \pm 1.5	13.8 \pm 1.3
Platelet count ($\times 10^4$ /mL)	16.7 \pm 5.7	16.5 \pm 5.7	17.0 \pm 6.2
Estimated calculation value			
Ccr (mL/min)	91.8 \pm 27.9	91.6 \pm 28.0	87.9 \pm 25.6
GFR (mL/ min/1.73 m ²)	77.5 \pm 16.8	79.6 \pm 17.0	78.2 \pm 15.9
CL/F (L/h)	11.3 \pm 5.4	11.2 \pm 5.3	10.9 \pm 5.1
Liver histopathology			
Stage of fibrosis	221/131/161	146/89/107	75/42/54
0-1/2/3-4			
Grade of inflammation	267/222/19	170/156/14	97/66/5
1/2/3			
SNP genotype			
rs1127354 CC/ CA/AA	431/114/16	289/75/10	142/39/6
rs6051702 AA/ AC/CC	388/158/15	260/104/10	128/54/5
Treatment			
Ribavirin dosage (mg/kg/d)	11.4 \pm 1.5	11.3 \pm 1.9	11.4 \pm 1.9
Peg-IFN α -2a/-2b	82/479	52/322	30/157
Virology			
Viral load (log ₁₀ IU/mL)	6.2 \pm 0.8	6.2 \pm 0.8	6.2 \pm 0.8

BMI: Body mass index; BSA: Body surface area; ALT: Alanine transaminase; GGT: γ glutamyl transpeptidase; WBC: White blood cell; GFR: Glomerular filtration rate; SNP: Single nucleotide polymorphism; Peg-IFN: Pegylated interferon.

calculated according to the formula proposed by the Japanese Society of Nephrology: GFR (for male) = $194 \times \text{Scr}^{-1.094} \times \text{Age}^{-0.287}$ (GFR $\times 0.739$ for female). Apparent clearance of ribavirin (CL/F, L/h) was determined as follows¹⁹: CL/F = $32.3 \times \text{BW} \times (1 - 0.0094 \times \text{age}) \times (1 - 0.42 \times \text{gender}) / \text{Scr}$ (gender = 0 for male and 1 for female; Scr is in $\mu\text{mol/L}$). All liver biopsy specimens were reviewed by using the established ranking system for staging of fibrosis and grading of necroinflammation activity with some modification²².

Virological data were assessed by monitoring serum HCV RNA levels every 4 wk during and off treatment. Viral loads were measured using a quantitative PCR assay (Amplicor HCV Monitor version 2.0 or Amplicor HCV

version 2.0; Roche Diagnostics, Basel, Switzerland). The presence or absence of serum HCV RNA was assessed using a qualitative PCR assay (Amplicor HCV version 2.0). Virological response (VR) was defined as undetectable HCV RNA by the end of treatment. Rapid virological response and slow virological response (SVR) were defined as undetectable HCV RNA at week 4 of treatment and 24 wk post-treatment. VR with relapse was defined as VR during treatment but reappearance of HCV RNA during the follow-up period. Nonvirological response (NVR) was defined as persistent presence of HCV RNA throughout the treatment.

SNP genotyping of *ITPA* and *C20orf194*

Genomic DNA was extracted from whole blood using the MagNA Pure LC and the DNA Isolation Kit (Roche Diagnostics). Genetic polymorphisms, rs1127354 at the *ITPA* exon 2^[15,17,18] and rs6051702 at the *C20orf194*^[15,18], were genotyped by real-time detection PCR using the TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA, United States). Another functional (splicing variant-related) SNP at the *ITPA* intron 2, rs727010, was not examined because no polymorphisms were observed in the Asian genetic population, as registered in the HapMap Project database and reported previously^[17,18,23].

Statistical analysis

Mantel-Haenszel, Pearson χ^2 test or Mann-Whitney test was used to compare frequencies in categorical data or differences in continuous data between two groups, respectively. Time-course changes in Hb decline from baseline were evaluated by using repeated measures analysis of variance. Possible variables influencing significant anemia and significant Hb decline included baseline characteristics (Table 1). Variables that reached statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) in univariate comparisons were subsequently entered into multiple logistic regression analysis using forward and backward stepwise selection method to identify significantly independent factors associated with each anemic event. Based on the final-step results, score (S) was constructed by the exposure of some set of independent factors (x_1, x_2, \dots, x_p):

$$S = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \dots + \beta_p x_p \quad (\beta_0: \text{Intercept}, \beta_1, \beta_2, \dots, \beta_p: \text{Regression coefficients}).$$

The model could be expressed as: $P = 1/[1 + \exp(-S)]$, where $P > 0.5$ was development of anemic events and $P < 0.5$ was non-development of anemic events.

Hosmer-Lemeshow goodness of fit test and likelihood-ratio χ^2 test were used and positive/negative predictive values and predictive accuracy were calculated to evaluate the fitness of the model. Split-group validation was used to develop and validate the best fitness of the model. Patients were randomly divided into two groups in the ratio of 2:1 by using a computer-generated random number list: 66.7% of the patients (374 patients) were as-

Table 2 Time-course changes in hemoglobin concentration from baseline

	Week 2 of treatment	Week 4 of treatment
Overall cohort		
mean (SD), g/dL	-1.12 (1.13)	-2.31 (1.39)
Median (25th–75th quartile), g/dL	-1.05 (-1.8 to -0.3)	-2.3 (-3.2 to -1.3)
Derivation group		
mean (SD), g/dL	-1.09 (1.11)	-2.27 (1.40)
Median (25th–75th quartile), g/dL	-1.0 (-1.8 to -0.3)	-2.3 (-3.1 to -1.3)
Confirmatory group		
mean (SD), g/dL	-1.18 (1.17)	-2.33 (1.37)
Median (25th–75th quartile), g/dL	-1.1 (-1.95 to -0.4)	-2.3 (-3.3 to -1.35)

signed to the derivation group and 33.3% (187 patients) to the confirmatory group. The reproducibility of the resulting model based on data from the derivation group was assessed with data from the validation group. Receiver operating characteristic (ROC) curves were generated with every cut-off point of predicted probability of significant Hb decline corresponding to each Hb decline at week 2. For a balanced optimization of both sensitivity and false-positive rate [= (1 - specificity)], an optimal cut-off point value was determined by maximizing Youden's index (= sensitivity + specificity - 1). The area under the ROC curve (AUC) was calculated to assess the degree of discrimination provided by the two parameters. To formulate a predictive value of quantitative Hb decline at weeks 2 and 4, the association between Hb decline and baseline variables was also analyzed using multiple linear regression analysis. The fitness of the model was evaluated by using values of *R* and *R*² and Durbin-Watson test. The correlation between predictive and measured values in Hb decline was assessed by Spearman's ρ . All *P* values for statistical tests were two tailed and values < 0.05 denoted the presence of a statistically significant difference. All data analyses were performed using the SPSS statistical package for Windows, version 17.0 (SPSS, Chicago, IL, United States).

RESULTS

Patient profiles and treatment-induced anemia

Baseline characteristics of the study population are summarized in Table 1. There were no significant differences in the patient profiles between the groups. The mean (SD) and median (25th to 75th quartiles) of Hb decline from baseline at week 2 and 4 of treatment are shown in Table 2. The changes at each time point were not statistically different between the groups. Significant Hb decline was observed in 113 of 374 (30%) derivation group patients and 58 of 187 (31%) confirmatory group patients. Significant anemia was observed in 51 of 374 (14%) patients and 30 of 187 (16%) patients, respectively. Incidence of these anemic events was similar between

Table 3 Pretreatment variables influencing significant hemoglobin decline in the derivation group

Variable	<i>P</i> value		OR (95% CI)
	Univariate analysis	Multivariate analysis	
Age (yr)	0.110		
Sex (female vs male)	0.0163		
Weight (kg)	5.18×10^{-3}		
BMI	5.93×10^{-3}		
Height (cm)	0.153		
BSA (m ²)	0.0139		
ALT (IU/L)	0.114		
GGT (IU/L)	0.118		
Albumin (g/dL)	6.88×10^{-3}		
Creatinine (mg/dL)	4.71×10^{-4}		
WBC count ($\times 10^3$ /mL)	0.147		
Hemoglobin (g/dL)	7.75×10^{-8}	1.29×10^{-9}	1.89 (1.54-2.32)
Platelet count ($\times 10^4$ /mL)	0.558		
Ccr (mL/min)	0.140		
GFR (mL/min/1.73 m ²)	5.69×10^{-4}	6.46×10^{-4}	0.959 (0.942-0.977)
CL/F (L/h)	0.814		
Stage of fibrosis	0.641		
Grade of inflammation	0.570		
rs1127354 (CC vs CA/AA)	8.04×10^{-10}	1.60×10^{-7}	28.26 (8.10-98.62)
rs6051702 (AA vs AC/CC)	0.372		
RBV dosage (mg/kg/d)	0.419		
Peg-IFN α (2a vs 2b)	0.360		
Viral load (log ₁₀ IU/mL)	0.355		

BMI: Body mass index; BSA: Body surface area; ALT: Alanine transaminase; GGT: γ glutamyl transpeptidase; WBC: White blood cell; GFR: Glomerular filtration rate; SNP: Single nucleotide polymorphism; Peg-IFN: Pegylated interferon; OR: Odds ratio; CI: Confidence interval.

the groups. Most of the patients complained of dyspnea on effort, easy fatigability or lightheadedness. None received erythropoiesis-stimulating agents throughout the treatment period.

Of the overall patients, 255 (45%) achieved SVR, 165 (29%) had VR with relapse, and 141 (25%) showed NVR. Of the 374 derivation group patients, SVR was 45% (167 patients), VR with relapse was 30% (111 patients) and NVR was 26% (96 patients). Of the 187 confirmatory group patients, they were 47% (88 patients), 29% (54 patients) and 24% (45 patients), respectively. Treatment outcome was almost equal among the overall cohort and split groups.

Baseline factors associated with significant Hb decline

To construct the prediction model for significant Hb decline, baseline variables were statistically analyzed in the derivation group (Table 3). Patients who showed significant Hb decline were more likely to be male (*P* = 0.0163), have higher BW (*P* = 0.00518), higher body mass index (BMI; *P* = 0.00593), larger body surface area (BSA; *P* = 0.0139), higher albumin (*P* = 0.00688), higher creatinine (*P* = 4.71×10^{-4}), higher Hb (*P* = 7.75×10^{-8}), lower GFR (*P* = 5.69×10^{-4}), and SNP rs1127354 major

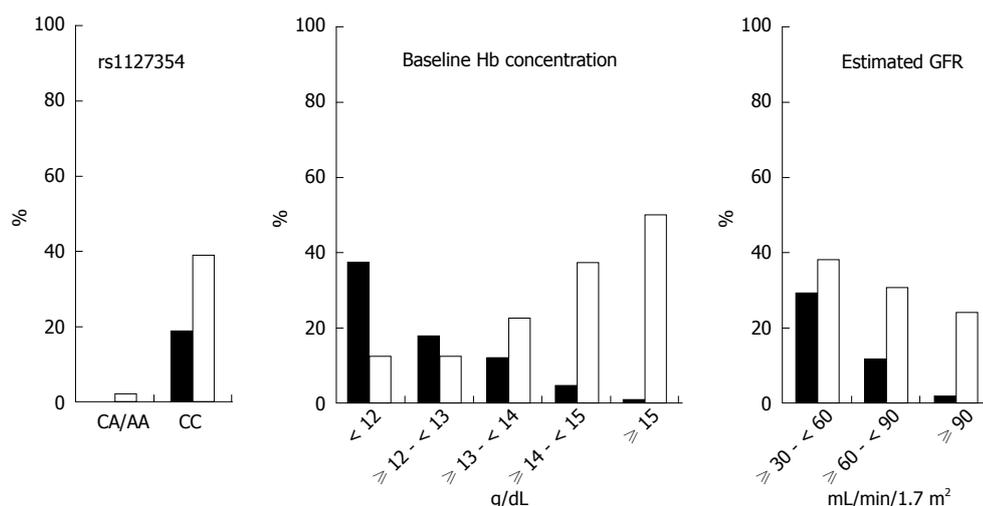


Figure 1 Anemic event rates in subset groups of each significantly independent baseline factor. The inosine triphosphatase single nucleotide polymorphism rs1127354 genotype, baseline hemoglobin (Hb) concentration and estimated glomerular filtration rate (GFR) were significantly associated with anemic events. Black and white vertical rectangles indicate significant anemia (< 10.0 g/dL at week 4 of treatment) and significant Hb decline (> 3.0 g/dL at week 4 of treatment), respectively.

genotype CC ($P = 8.04 \times 10^{-10}$).

Multiple logistic regression analysis identified three independent variables that were significantly associated with significant Hb decline (Table 3): baseline Hb [$P = 1.29 \times 10^{-9}$, odds ratio (OR) = 1.89 (g/dL), 95% confidence interval (CI): 1.54-2.32], SNP rs1127354 ($P = 1.60 \times 10^{-7}$, OR = 28.26, 95%CI: 8.10-98.62), and GFR [$P = 6.46 \times 10^{-4}$, OR = 0.959 (mL/min/1.73 m²), 95%CI: 0.942-0.977]. The model was expressed as: $S = -9.369 + 0.635 \times \text{baseline Hb} + 3.342 \times \text{SNP rs1127354}$ (where genotype CC was 1 and CA/AA was 0) $-0.041 \times \text{GFR}$. P values were 0.401 and 9.79×10^{-24} in the Hosmer-Lemeshow test and likelihood-ratio χ^2 test, respectively. Positive and negative predictive values and predictive accuracy were 67.5%, 79.9% and 77.0%, respectively. To validate the prediction model, it was used for the confirmatory group. Positive and negative predictive values and predictive accuracy were 76.7%, 79.7% and 79.0%, respectively. For the overall cohort, these values were 70.8%, 79.9% and 77.7%, respectively. Significant Hb decline was not associated with treatment outcome in the overall cohort [SVR, 40% (69/171); VR, 32% (55/171); and NVR 27% (47/171)] or split groups.

Baseline factors associated with significant anemia

Female ($P = 0.00896$) and older ($P = 0.0443$) patients, and those with lower albumin ($P = 0.0197$), lower white blood cell count ($P = 0.0226$), lower baseline Hb ($P = 5.34 \times 10^{-13}$), lower Ccr ($P = 1.06 \times 10^{-4}$), lower GFR ($P = 2.69 \times 10^{-4}$), lower CL/F ($P = 6.59 \times 10^{-5}$), lower BW ($P = 0.00309$), smaller BSA ($P = 0.0254$), and rs1127354 major genotype CC ($P = 2.76 \times 10^{-5}$) were more likely to have significant anemia than those who did not. In multiple logistic regression analysis, the model could not be constructed by these variables, because no patients with rs1127354 minor genotype CA/AA suffered from

significant anemia in this study population (Figure 1). All patients with significant anemia had rs1127354 major genotype CC. When SNP rs1127354 was excluded from the multivariate analysis, baseline Hb [$P = 1.67 \times 10^{-9}$, OR = 0.376 (g/dL), 95%CI: 0.274-0.517] and GFR [$P = 0.00233$, OR = 0.962 (mL/min/1.73 m²), 95%CI: 0.938-0.986] were significantly independent variables. Significant anemia was not associated with treatment outcome in the overall cohort [SVR, 31% (25/81); VR, 36% (29/81); and NVR 33% (27/81)] or split groups.

Figure 1 shows the incidence rates of significant anemia and significant Hb decline in the overall cohort according to the three significantly independent factors. Specifically, SNP rs1127354 had an overwhelming impact on the anemic events. In 431 patients with major genotype CC, significant anemia and significant Hb decline developed in 81 (19%) and 168 (39%) patients, respectively. In contrast, none (0%) and three (2%) of 130 patients with minor genotype CA/AA showed each anemic event, respectively, as described above. Positive predictive values of SNP rs1127354 alone for the likelihood of significant anemia and significant Hb decline were 14.3% and 39.1%, respectively. Negative predictive values were 100% and 97.7%, respectively. Values of predictive accuracy were 35.7% and 53.5%, respectively. Figure 2 depicts time-course changes in qualitative Hb decline from baseline according to SNP rs1127354 genotypes. The SNP genotype significantly influenced Hb decline at week 2 as well as week 4 ($P = 5.437 \times 10^{-3}$).

Contribution of Hb decline at week 2 of treatment

Hb decline from baseline at week 2 of treatment, an on-treatment factor, significantly influenced significant Hb decline ($P = 1.96 \times 10^{-33}$). An ROC curve was depicted to identify an optimal cut-off point for prediction of significant Hb decline by using Hb decline at week 2

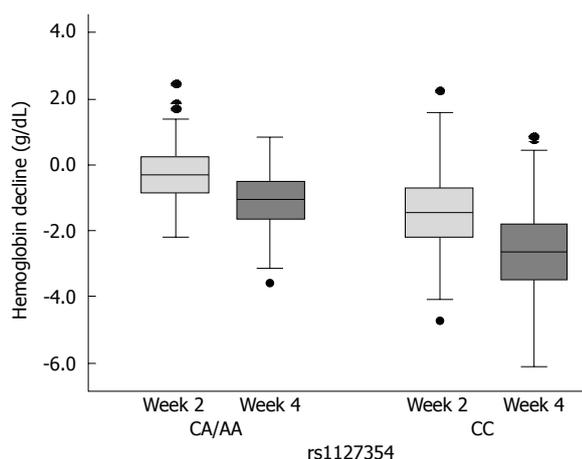


Figure 2 Hemoglobin decline from baseline at week 2 and 4 of treatment according to the inosine triphosphatase single nucleotide polymorphism rs1127354 genotypes. Bars within boxes denote the median value of hemoglobin (Hb) decline from baseline. The boxes and the lower and upper bars represent the 25th to 75th percentiles, and the 10th and 90th percentiles, respectively. The single nucleotide polymorphism genotype significantly influenced Hb decline at week 2 and 4 ($P = 5.437 \times 10^{-9}$ in repeated measures analysis of variance).

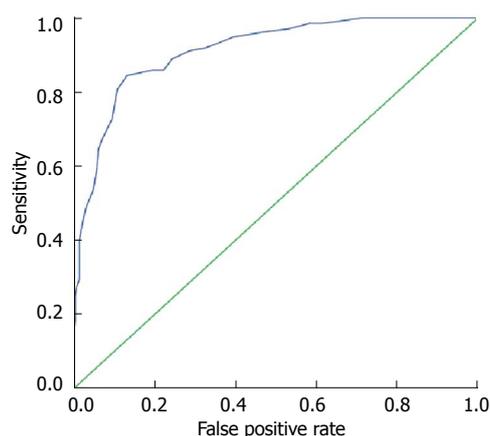


Figure 3 Receiver operating characteristic curves generated with every cut-off point of predicted probability of significant hemoglobin decline (> 3.0 g/dL at week 4 of treatment) corresponding to each hemoglobin decline from baseline at week 2 of treatment. The area under the curve was 0.913 (95% confidence interval: 0.885-0.941, $P = 4.08 \times 10^{-43}$). When the sensitivity and false-positive rate were 0.844 and 0.131, the Youden's index value of 0.713 was maximal. The optimal cut-off point of hemoglobin decline at 2 wk was 1.45 g/dL.

(Figure 3). The AUC was 0.913 (95%CI: 0.885-0.941, $P = 4.08 \times 10^{-43}$). The maximal value of Youden's index was 0.713. The sensitivity and false-positive rate were 0.844 and 0.131, respectively. The optimal cut-off point of Hb decline at week 2 was 1.45 g/dL.

When this variable, together with baseline variables, was incorporated into multiple logistic regression analysis to generate a statistic model for predicting significant Hb decline, the re-performed analysis using the derivation group data identified four significantly independent variables: Hb decline at week 2 [$P = 3.29 \times 10^{-17}$, OR = 7.54 (g/dL), 95%CI: 4.71-12.05], GFR [$P = 2.16 \times 10^{-4}$,

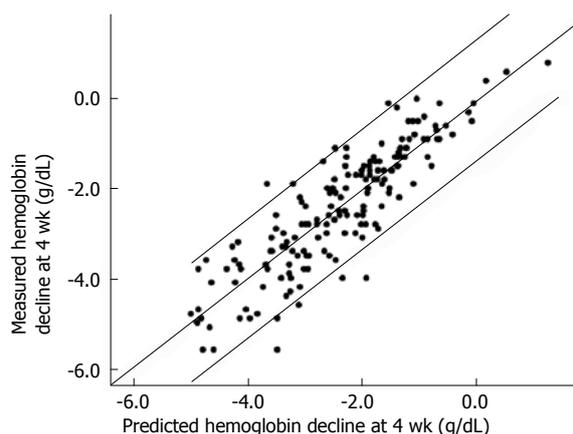


Figure 4 Correlation between predicted and measured values of hemoglobin decline at week 4 of treatment. Predicted values were yielded by the multiple linear regression model that was constructed in the derivation group. Measured values were derived from the confirmatory group. There was a significant correlation between predicted and measured values (Spearman's $\rho = 0.880$, $P = 1.16 \times 10^{-56}$). The area between lower and upper parallel lines of the diagonal line includes 95% of patients analyzed.

OR = 0.962 (mL/min/1.73 m², 95%CI: 0.942-0.982], rs1127354 ($P = 5.75 \times 10^{-4}$, OR = 10.94, 95%CI: 2.80-42.71), baseline Hb [$P = 7.86 \times 10^{-4}$, OR = 1.50 (g/dL), 95%CI: 1.18-1.90]. The model was expressed as: $S = -8.285 - 2.020 \times \text{Hb decline at week 2} - 0.039 \times \text{GFR} + 2.393 \times \text{SNP rs1127354}$ (where genotype CC was 1 and CA/AA was 0) + 0.405 \times baseline Hb. P values were 0.587 and 1.59×10^{-58} in the Hosmer-Lemeshow test and likelihood-ratio χ^2 test, respectively. Positive and negative predictive values and predictive accuracy were 79.8%, 88.8% and 86.2%, respectively. These values were 83.3%, 91.0% and 88.3% in the confirmatory group, and 81.3%, 89.0% and 86.7% in the overall cohort.

Prediction of Hb decline value

To predict qualitative Hb decline value at week 4 of treatment, the multiple linear regression model was constructed using data from the derivation group. The statistic model was expressed as: $\hat{y} = 0.784 - 0.748 \times \text{Hb decline at week 2} - 0.878 \times \text{SNP rs1127354}$ (where genotype CC was 1 and CA/AA was 0) - 0.178 \times baseline Hb + 0.012 \times GFR ($R = 0.842$, $R^2 = 0.709$, adjusted $R^2 = 0.706$, Durbin-Watson test = 1.984, $P = 2.42 \times 10^{-7}$). There was a significant correlation between predicted values in the model and measured values in the confirmatory group (Spearman's $\rho = 0.880$, $P = 1.16 \times 10^{-56}$; Figure 4).

Next, qualitative Hb decline value at week 2 was estimated by significantly independent variables in the derivation group. The model was expressed as: $\hat{y} = 2.922 - 1.067 \times \text{SNP rs1127354}$ (where genotype CC was 1 and CA/AA was 0) - 0.276 \times baseline Hb + 0.008 \times GFR ($R = 0.528$, $R^2 = 0.279$, adjusted $R^2 = 0.274$, Durbin-Watson test = 0.537, $P = 4.49 \times 10^{-31}$). The correlation between predicted values in the model and measured values in the confirmatory group was statistically significant but rela-

tively weak (Spearman's $\rho = 0.566$, $P = 2.41 \times 10^{-17}$).

DISCUSSION

As mentioned in the introduction section, peg-IFN α plus RBV combination will be in demand for the foreseeable future. Patients at a high risk of developing RBV-induced hemolysis will expose themselves to a more increased risk for treatment-induced anemia in triple combination treatment. Identifying such high-risk patients and predicting the severity of anemia in individuals may provide an early decision to commence treatment with normal or reduced dosage and to keep the dose reduction to a minimum to lessen the disadvantages of anemia with adequate exposure to RBV continuing. To date, many studies have proposed factors that could influence the probability of clinically significant anemia in RBV-based treatment: age, sex^[11,12], race, pre-existing cirrhosis^[14], baseline Hb concentration^[11,20], Ccr^[14,20], CL/F^[8,9], drug exposure^[12-14], plasma RBV concentration^[10], Hb decline at week 2 of treatment^[12,14,20], and SNPs at the *ITPA*^[15-18], *C20orf194*^[15] and nucleoside transporter genes^[19]. However, the definition of anemia or end point of analysis varied a little among previous studies, possibly leading to alteration of significant predictors. Despite these useful predictors, there is no convenient prediction model or formula for estimating the likelihood of clinically significant anemia that has been defined previously and used generally^[15]. This study provided relevant numerical expressions constructed by independent variables for predicting the differentially defined anemia: Hb concentration < 10.0 g/dL (significant anemia) and a decline in Hb concentration > 3.0 g/dL (significant Hb decline) at week 4 of treatment and qualitative Hb decline at week 2 and 4. This is believed to be the first report to construct the prediction models by using reliable factors: the *ITPA* SNP rs1127354, baseline Hb concentration, estimated GFR, and quantitative Hb decline at week 2 of treatment, irrespective of the different definitions of anemia. The significant baseline factors that were shown in this study appear to influence treatment-induced anemia in triple combination treatment (under investigation, data not shown).

Two functional *ITPA* variants conferring *ITPA* deficiency or reduced activity are known to contribute most to protection against RBV-induced hemolytic anemia^[15-18]. Inosine triphosphate (ITP) is hydrolyzed by *ITPA* to inosine monophosphate. Therefore, *ITPA* deficiency or low activity causes the accumulation of ITP in red blood cells (RBCs)^[24-26]. The accumulated ITP may compete with the accumulated triphosphate form of RBV that could mediate oxidative damage to the RBC membrane and extravascular destruction^[25-27], thereby protecting RBCs against RBV-induced hemolysis. As also shown in this study, one functional SNP rs1127354 is prominently associated with differentially defined anemia. Of note, however, the SNP was not always a factor

of the top significance. The combined *ITPA* activity variable with another functional SNP rs7270101 is a stronger determinant of anemia than either *ITPA* SNP alone in European-Americans^[16], whereas rs7270101 is not polymorphous in the Japanese population as registered in the HapMap database and reported by others^[17,18,23]. One SNP, rs6051702 at the *C20orf194* located near the *ITPA*, linked to the *ITPA* SNPs, also confers protection against anemia in European-Americans^[15], while the association was statistically significant but weak in one Japanese cohort^[18]. This Japanese study population showed no significant association (Table 3), supporting that rs1127354 is a single causal variant responsible for protection against anemia in the Japanese genetic cohort^[17].

Certainly, the *ITPA* SNP rs1127354 minor variant A is a strong protective allele for anemia. In this overall cohort, none (0%) and three (3%; who had genotype CA) of patients with minor variant A had significant anemia and significant Hb decline, respectively (Figure 1). Therefore, negative predictive value of minor variant A was 100% and 97.7%, respectively. The noticeable distinction was in excellent agreement with other studies^[15,18]. With respect to the likelihood of these anemic events, patients with minor variant A may be monitored less intensively and recommended to receive normal RBV doses, even in patients with relatively low baseline Hb, or more aggressive dose escalation strategies irrespective of baseline Hb. It is noteworthy that genotype AA patients with predicted *ITPA* deficiency, including seven patients with baseline Hb < 13.0 g/dL (range, 11.7-12.9 g/dL), showed no or little change in the Hb concentration (data not shown), although the number was small.

As shown in this study and another^[18], however, only 25% of the Japanese population has minor variant A. The remaining 75% have major genotype CC. Positive predictive values of major genotype CC alone for significant anemia and significant Hb decline were low (14.3% and 39.1%, respectively), and values of predictive accuracy were low (35.7% and 53.5%, respectively). The range of Hb decline varied widely among individuals with genotype CC, indicating that some of them showed little or no change in Hb decline. Even in minor genotype CA carriers, it also varied widely and was similar to that of genotype CC patients (Figure 2). These findings strongly suggest that any factors other than the strong predictor *ITPA* SNP could affect hemolysis positively or negatively. Therefore, it is highly unlikely that the *ITPA* SNP (except genotype AA) is used alone to determine clinical decision making for treatment modification. In fact, several factors independently and strongly influenced treatment-induced anemia as well as the *ITPA* SNP in this study.

The clearance rate of RBV from the body is of critical importance for influencing treatment outcome and RBV-induced anemia, because the clearance parameters, such as CL/F and Ccr, reflect plasma/serum RBV con-

centrations at week 4 of treatment, which means the steady phase of treatment^[8-10,14,20,28]. Higher or lower values of the parameters are correlated closely with lower or higher plasma/serum concentrations, respectively. Higher plasma/serum concentrations lead to an increased risk for progression of anemia as well as the higher probability of achieving SVR. Indeed, this study confirmed that the clearance rate is associated significantly and independently with RBV-induced anemia irrespective of the different definitions. This study also analyzed which of three parameters estimated by the formulae were the most stable for predicting clinically significant anemia. These formulae are composed by age, sex, BW and serum creatinine. Age and sex have been reported to affect treatment-induced anemia and dose reduction, and could reflect reactivity to treatment, tolerance and pharmacological metabolism^[11,12,29]. Japan is one of the countries with the longest living people and the world's fastest aging society, therefore, the clearance rate should especially be taken into account in RBV-based treatment of Japanese patients. The reason that estimated GFR remained an independent factor in the final model may be that the formula has been built up based on data from the Japanese population.

Higher baseline Hb concentration was significantly associated with the likelihood of significant Hb decline. Conversely, lower baseline Hb concentration was linked to significant anemia. These findings may be a matter of course. However, most of this study population received treatment without RBV dose reduction as scheduled, suggesting that kinetics of Hb decline within the first 4 wk of treatment might be delayed in patients with lower baseline Hb concentration. A certain threshold of Hb concentration might limit the progression of anemia independent of baseline Hb concentration. At least in Japanese patients, the two different definitions of anemia, significant Hb decline and significant anemia, should be separately analyzed and discussed.

In this multivariate analysis, qualitative Hb decline at week 2 of treatment was most highly predictive of significant Hb decline, compared to the strong predictor *ITPA* SNP rs1127354 and other baseline factors. Previous studies have shown that Hb decline of 2.0 g/dL at week 2 of treatment was predictive of Hb concentration < 10 g/dL or < 8.5 g/dL during the treatment^[12,20]. In another study, Hb decline of 1.5 g/dL at week 2 was predictive of Hb decline \geq 2.5 g/dL at week 4^[14]. In this ROC analysis, the best cutoff value for Hb decline at week 2 was 1.45 g/dL. Taken together, Hb decline at week 2 is an excellent early predictor of subsequent Hb decline and could identify candidates for early intervention to maintain RBV dosing and adequate exposure. Indeed, the formula including this on-treatment variable improved positive and negative predictive values and predictive accuracy for significant anemia and significant Hb decline. When considered along with other independent baseline factors predictive of qualitative Hb decline at week 4, the final model yielded high significant values

that represented goodness of fit. Using such a timely on-treatment variable and formula, more exact identification of patients prone to clinically significant anemia, early intervention with RBV dose reduction, and more careful monitoring may be indicated to reduce anemia-related adverse effects and avoid premature discontinuation of RBV.

ITPA SNP rs1127354, baseline Hb concentration and estimated GFR influenced Hb decline at week 2 significantly and independently, as well as that at week 4. However, it appears to be difficult to predict qualitative Hb decline at week 2 by using the multiple linear regression model. The point for attention is that the models and formulae did not perfectly predict the likelihood of the anemia, strongly suggesting the possibility that other unidentified factors associated with early occurring anemia might be lost, such as rare SNPs, brittleness of the RBC membrane against intracellular triphosphate form of RBV, or intracellular concentration of ITP.

In conclusion, convenient formulae for qualitatively or quantitatively predicting the likelihood of differentially defined anemia could be generated by significant independent factors in RBV-based treatment for chronic HCV infection. Such trial modeling may be useful in guiding clinical decision making on treatment modification: identifying the predisposition to develop RBV-induced anemia before treatment initiation or at the early treatment phase, and developing the individual tailoring and optimization of RBV dosage to maximize the treatment efficacy and minimize RBV-related adverse effects.

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COMMENTS

Background

In ribavirin (RBV)-based treatment for chronic hepatitis C, hemolytic anemia is a major adverse effect and makes it difficult to continue treatment as scheduled. Many factors have been reported to influence clinically significant anemia that could modify or discontinue treatment. However, the definition of anemia or end point of analysis varied somewhat among studies, leading to alteration of significant predictors. Despite these useful predictors, there is no convenient prediction model for estimating the probability of clinically significant anemia.

Research frontiers

Host genetic variation at the inosine triphosphatase (*ITPA*) gene that leads to *ITPA* deficiency or low activity are known to contribute greatly to protection against RBV-induced hemolytic anemia. However, it is highly unlikely that the *ITPA* single nucleotide polymorphism (SNP) alone is used to determine clinical decision making for treatment modification. Any factors other than the strong predictor *ITPA* SNP could affect hemolytic anemia positively or negatively.

Innovations and breakthroughs

This study provided relevant numerical expressions constructed by using significantly independent factors for predicting the differentially defined anemia. The reliable factors were the *ITPA* SNP rs1127354, baseline hemoglobin (Hb) concentration, estimated glomerular filtration rate and quantitative Hb decline

at week 2 of treatment, irrespective of the different definitions of anemia. These factors independently and strongly influenced RBV-induced anemia, as well as the ITPA SNP. The ITPA SNP was not always a factor of major significance.

Applications

Such modeling may be useful in guiding clinical decision making on treatment modification: more exactly identifying candidates at a high risk for clinically significant anemia or predicting the severity of anemia in individuals before treatment initiation or at the early treatment phase, and developing the individual tailoring and optimization of RBV dosage to maximize the treatment efficacy and minimize RBV-related adverse effects with adequate exposure to RBV continuing.

Peer review

Pegylated interferon plus RBV still assumes an important role in the treatment of chronic hepatitis C. The manuscript is well written and the study has investigated a crucial point of anti-HCV treatment. Interestingly, the authors observed patients who showed significant Hb decline and significant anemia, respectively, and showed that factors associated with anemia differed according to the definitions.

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Characteristics of deslanoside-induced modulation on jejunal contractility

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Abstract

AIM: To characterize the dual effects of deslanoside on the contractility of jejunal smooth muscle.

METHODS: Eight pairs of different low and high contractile states of isolated jejunal smooth muscle fragment (JSMF) were established. Contractile amplitude of JSMF in different low and high contractile states was selected to determine the effects of deslanoside, and Western blotting analysis was performed to measure the effects of deslanoside on myosin phosphorylation of jejunal smooth muscle.

RESULTS: Stimulatory effects on the contractility of JSMF were induced ($45.3\% \pm 4.0\%$ vs $87.0\% \pm 7.8\%$, $P < 0.01$) by deslanoside in 8 low contractile states, and inhibitory effects were induced ($180.6\% \pm 17.8\%$ vs $109.9\% \pm 10.8\%$, $P < 0.01$) on the contractility of JSMF in 8 high contractile states. The effect of deslanoside on the phosphorylation of myosin light chain of JSMF in low ($78.1\% \pm 4.1\%$ vs $96.0\% \pm 8.1\%$, $P < 0.01$) and high contractile state ($139.2\% \pm 8.5\%$ vs 105.5 ± 7.34 , $P < 0.01$) was also bidirectional. Bidirec-

tional regulation (BR) was abolished in the presence of tetrodotoxin. Deslanoside did not affect jejunal contractility pretreated with the Ca^{2+} channel blocker verapamil or in a Ca^{2+} -free assay condition. The stimulatory effect of deslanoside on JSMF in a low contractile state (low Ca^{2+} induced) was abolished by atropine. The inhibitory effect of deslanoside on jejunal contractility in a high contractile state (high Ca^{2+} induced) was blocked by phentolamine, propranolol and L-NG-nitroarginine, respectively.

CONCLUSION: Deslanoside-induced BR is Ca^{2+} dependent and is related to cholinergic and adrenergic systems when JSMF is in low or high contractile states.

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Key words: Deslanoside; Bidirectional regulation; Contractile state; Jejunal smooth muscle

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INTRODUCTION

More than 200 naturally occurring cardiotonic glycosides (CGs, cardiac glycosides) have been identified to date^[1].

CGs have long been and will continue to be used in the treatment of congestive heart failure and have entered the clinical trial phase for treating cancer^[2-5]. CGs enhance the myocardial contraction by increasing intracellular Ca^{2+} *via* inhibiting the activities of Na^+/K^+ -ATPase^[6-8]. CGs ouabain has been found to induce excitation on colonic smooth muscle^[9]. Toxic effects of CGs are observed in clinics, e.g., atrioventricular block, bradycardia, and gastrointestinal irritation^[1]. Probably due to the fact that no therapeutic applications are yet known, the characteristics of CGs on the intestinal motility have rarely been investigated.

Intestinal motility is mainly modulated by neurotransmitters and hormones; the neuronal regulation of intestinal motility involves intrinsic, e.g., enteric nervous system (ENS), as well as extrinsic nerves, e.g., the sympathetic and parasympathetic nervous system (SPNS)^[10]. The central nervous system is able to modulate, but not entirely control, the motor activity by sending instructions *via* SPNS, and ENS modulates the motility of intestinal smooth muscle even when isolated from the body to fulfill pivotal functions^[10,11]. In this study, we proposed a hypothesis that inducible bidirectional regulation (BR) is the major autonomous control of intestinal motility in the absence of CNS control, and that both low and high contractile states of intestinal smooth muscle can be regulated back toward normal contractile state by a single CGs deslanoside-induced BR. To test the hypothesis, different low and high contractile states of intestinal smooth muscle were established. Considering both colon and small intestine are sites of “abnormal” motility in intestinal smooth muscle disorders, e.g., irritable bowel syndrome (IBS)^[12-14], and that the jejunum is a “typical” region of the small intestine, we chose to investigate the contractility of isolated jejunal smooth muscle fragment (JSMF) and its underlying mechanisms involved in deslanoside-induced BR.

MATERIALS AND METHODS

Experimental models of diarrhea and constipation

The animal protocol was approved by Dalian Medical University Animal Care and Ethics Committee, and all experimental procedures described were carried out in accordance with the Declaration of Helsinki. Sprague-Dawley rats (200-250 g) were used in the assay. Constipation-predominant (CP) rats were established by daily gavage with cool water (0 °C-4 °C) for 14 d, and the control rats were prepared by daily gavage with water at room temperature^[15,16]. Diarrhea-predominant (DP) rats were established by intracolonic instillation of acetic acid and restraint stress, and control rats received intracolonic instillation with saline^[17-19]. The granule number and the moisture content of the feces from the control group and the model group were measured daily, and the body mass was recorded once every 3 d.

Tissue preparation

Tissue fragments from the intact tubular jejunum were

prepared according to the methods described previously^[20,21]. Jejunum was isolated from normal, CP and DP rats. Jejunal fragments were cut into approximately 2 cm in length (tubes). One end of the jejunal fragment in longitudinal direction was fixed to the wall of a tissue bath chamber (20 mL volume), and the other end was connected to a force-displacement transducer. This montage measured the contractile response of JSMF.

Contractility determination

The organ bath was maintained at 37 °C, and the resting tension was set optimally at 1.0 g. Preliminary experiments showed that this load stretched tissues to their optimal length for force development during contraction. JSMF was allowed to equilibrate in aerated Krebs buffer for 50 min and the bath solution was replaced every 10 min. Contractile amplitude of JSMF was measured from the baseline to the peak and was expressed as a percentage of normal contractile amplitude. Contractile amplitude was recorded and identical time-interval of each assay with the same start and stop time was chosen to compare the amplitude before and after drug treatment in different assay conditions. The mean amplitude was calculated from six independent assays.

Ex vivo assay condition

The contractility of JSMF was measured in Krebs buffer (118 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L KH_2PO_4 , 1.2 mmol/L MgSO_4 , 4.2 mmol/L NaHCO_3 , 2.5 mmol/L CaCl_2 , 10 mmol/L glucose; pH 7.4) and selected as the normal contractile state (NCS). The jejunal contractility measured in modified low Ca^{2+} (1.25 mmol/L) and high Ca^{2+} (5.0 mmol/L) Krebs buffer was selected as the representative low contractile state (RLCS) and representative high contractile state (RHCS), respectively, since spontaneous contractions of intestinal smooth muscle were paralleled to intracellular Ca^{2+} concentration^[22,23]. One pair of low-high contractile states was established from jejunal smooth muscle isolated from CP and DP rats. The other six pairs of low-high contractile states were generated by incubating JSMF in modified low K^+ (2.5 mmol/L)-high K^+ (10.0 mmol/L) Krebs buffer, low Na^+ (100 mmol/L)-high Na^+ (150 mmol/L) Krebs buffer, high Mg^{2+} (3.0 mmol/L)-low Mg^{2+} (1.0 mmol/L) Krebs buffer, adrenaline (5.0 $\mu\text{mol/L}$)-ACh (5.0 $\mu\text{mol/L}$) Krebs buffer, quercetin (10.0 $\mu\text{mol/L}$)-capsaicin (10.0 $\mu\text{mol/L}$) and nitric oxide (NO) donor sodium nitroprusside (SNP) (5 $\mu\text{mol/L}$)-erythromycin (10 $\mu\text{mol/L}$) Krebs buffer^[24,25]. After the stable contractile state of jejunal contraction was obtained, deslanoside was added to the bath to make a final concentration of 20 $\mu\text{mol/L}$, unless otherwise indicated.

Western blotting analysis

The phosphorylation of myosin light chain (PMLC) in jejunum was examined by Western blotting as described previously^[26,27]. JSMF was immediately treated with low Ca^{2+} or high Ca^{2+} Krebs buffer for 1 min in the absence or presence of 20 $\mu\text{mol/L}$ deslanoside, and then were

Table 1 Effects of deslanoside on the contractility of jejunal smooth muscle pretreated with receptor antagonist

Agents	Normal contractile state		Low contractile state		High contractile state	
	Pre-deslanoside	Post-deslanoside	Pre-deslanoside	Post-deslanoside	Pre-deslanoside	Post-deslanoside
Krebs buffer	100.0 ± 12.1	149.0 ± 13.0 ^b	39.1 ± 2.8	89.1 ± 5.1 ^b	177.7 ± 16.0	109.3 ± 11.9 ^b
Atropin	93.3 ± 6.3	155.0 ± 15.1 ^b	29.1 ± 1.1	32.4 ± 3.3	149.7 ± 11.0	100.3 ± 11.2 ^b
Diphenhydramine	109.0 ± 14.3	145.2 ± 13.1 ^b	46.5 ± 3.8	94.0 ± 4.9 ^b	169.6 ± 15.2	108.5 ± 11.6 ^b
Cimetidine	100.0 ± 11.8	153.4 ± 13.0 ^b	42.9 ± 3.9	93.4 ± 8.1 ^b	180.1 ± 17.4	113.3 ± 12.1 ^b
Phentolamine	92.5 ± 6.5	160.6 ± 16.2 ^b	55.4 ± 5.5	90.6 ± 5.2 ^b	189.3 ± 19.2	184.5 ± 17.2
Propranolol	89.4 ± 9.8	145.5 ± 14.2 ^b	33.2 ± 2.6	95.6 ± 7.8 ^b	163.1 ± 16.1	161.7 ± 17.3
L-NNA	103.0 ± 11.3	150.0 ± 13.2 ^b	51.3 ± 4.6	119.6 ± 12.5 ^b	190.5 ± 18.2	195.1 ± 19.1

Contractile amplitude of isolated jejunal smooth muscle fragment in normal contractile state is defined as 100% (control). Other data are the relative value (%) compared with control, and are expressed as the mean ± SE; ^b*P* < 0.01 vs the data prior to deslanoside treatment (*n* = 6). L-NNA: L-NG-nitroarginine.

frozen and stored in liquid nitrogen. Ground product was incubated for 30 min in ice-cold homogenization buffer. The blots on nitrocellulose filter membrane were probed with phosphor-myosin light chain 2 (Ser 19) antibody (1:1000) [No. 3671, Cell Signaling Technology, Inc (CST), United States] and myosin light chain 2 (total myosin light chain) antibody (1:1000) (No. 3672, CST, United States) at 4 °C with gentle shaking overnight. Anti-rabbit IgG secondary antibodies were used at 1:2500 for 60 min at room temperature and bands were detected and quantified using Multispectral imaging system (UVP, United States).

Drugs

Injectable deslanoside was obtained from Sine Pharmaceutical (Shanghai, China). Capsaicin and quercetin was purchased from Chengdu Biopurify Phytochemicals Co. Ltd, China. Tetrodotoxin (TTX) was a product of Aladdin Chemistry Co. Ltd (Shanghai, China). Unless otherwise indicated, chemicals were obtained from Sigma (United States).

Statistical analysis

Student's *t* test was used to compare statistical differences between two groups, and *P* < 0.05 was considered statistically significant.

RESULTS

Deslanoside-induced BR on the contractility of JSMF

Deslanoside exerted stimulatory effects on JSMF in NCS in a dose range of 5-160 μmol/L (Figure 1A).

Eight low and 8 high contractile states of jejunal smooth muscle were established as described in Materials and Methods. The contractility of JSMF in both low and high contractile states was statistically different from that of normal control (Figure 1). Deslanoside (20 μmol/L) was used in all the assays based on the fact that deslanoside-induced BR on jejunal contractility was observed in a dose range of 10-40 μmol/L. Deslanoside produced significant stimulatory effects (45.3% ± 4.0% vs 87.0% ± 7.8%, *P* < 0.01) on the contractility of JSMF in all 8 low contractile states (Figure 1B), and produced significant inhibitory effects (180.6% ± 17.8% vs 109.9% ± 10.8%, *P* < 0.01) on the contractility of JSMF in all 8

high contractile states (Figure 1C).

Western blotting analysis

The PMLC in jejunum was significantly decreased in RLCS in comparison with that in NCS (100.0% ± 9.4% vs 78.1% ± 4.1%, *P* < 0.01), and was significantly increased at RHCS in comparison with that in NCS (100.0% ± 6.7% vs 139.2% ± 8.5%, *P* < 0.01) (Figure 2). Deslanoside significantly increased the PMLC in RLCS (78.1% ± 4.1% vs 96.0% ± 8.1%, *P* < 0.01), and significantly decreased the PMLC in RHCS (139.2% ± 8.5% vs 105.5 ± 7.34, *P* < 0.01).

Effects of deslanoside on the contractility of JSMF in the presence of TTX

In the presence of TTX, BR was not observed when deslanoside was tested on the contractility of JSMF in RLCS and RHCS (Figure 3).

Underlying mechanisms involved in deslanoside-induced BR

Deslanoside, at bath concentrations of 5 μmol/L, 20 μmol/L and 80 μmol/L, did not affect jejunal contractility in a Ca²⁺-free assay condition, and 20 μmol/L deslanoside did not stimulate the contractility of JSMF pre-incubated with the Ca²⁺ channel blocker verapamil at normal, low and high contractile states (Figure 4).

The underlying mechanisms involved in deslanoside-induced BR were investigated. Muscarinic receptor antagonist atropine abolished the stimulatory effect of deslanoside on the contractility of JSMF in RLCS (Table 1; Figure 5A). Neither histamine H1-receptor antagonist diphenhydramine nor histamine H2-receptor antagonist cimetidine blocked deslanoside-induced stimulatory effects on the contractility of JSMF in RLCS (Table 1; Figure 5A). α-adrenergic receptor antagonist phentolamine, β-adrenergic receptor antagonist propranolol and NO synthase inhibitor L-NNA abolished deslanoside-induced inhibitory effect on the contractility of JSMF in RHCS (Table 1; Figure 5B).

DISCUSSION

Eight pairs of low-high contractile states were established to imitate intestinal hyper- and hypomotility and

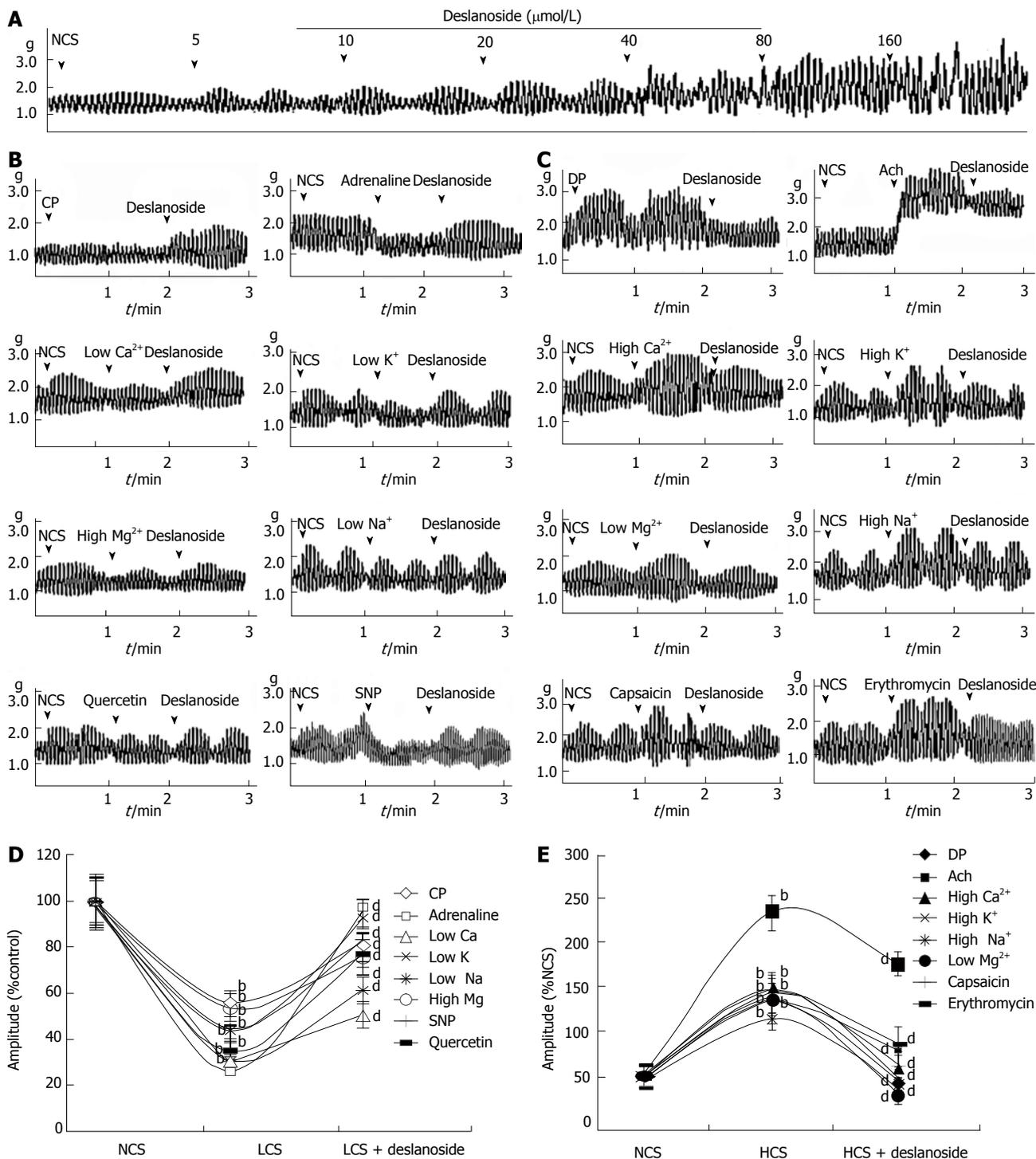


Figure 1 Deslanoside-induced bidirectional regulation on the contractility of jejunal smooth muscle fragment. A: Dose response relationship of deslanoside; B, D: Representative traces and statistical analysis of total traces from six independent experiments of deslanoside-induced bidirectional regulation on the contractility of jejunal smooth muscle fragment (JSMF) in eight low contractile states (LCS); C, E: Representative traces and statistical analysis of total traces from six independent experiments of deslanoside-induced bidirectional regulation on the contractility of JSMF in eight high contractile states (HCS). The median value of contractile amplitude of JSMF in normal contractile state is set to 100%, normal contractile state (NCS, control). Low and high contractile states of JSMF are the relative values compared with NCS. Data are expressed as the mean ± SE (%NCS, n = 6); ^bP < 0.01 vs the control; ^dP < 0.01 vs contractile amplitude of JSMF in LCS or HCS before deslanoside administration. CP: Constipation-prominent rats; DP: Diarrhea-prominent rats; SNP: Sodium nitroprusside; Ach: Acetylcholine.

to evaluate the characteristics of deslanoside-induced BR and potential clinical implication. IBS is known as one of the major functional gastrointestinal disorders, affecting approximately 10% of all adults worldwide^[28]. IBS is usually categorized into three subclasses: IBS with

constipation (hypo-motility), IBS with diarrhea (hyper-motility), and IBS with alternating symptoms of both constipation and diarrhea (IBS-A)^[13,14,29]. None of the currently available drugs are globally effective in treating all IBS symptoms^[30], and developing treatment strategies

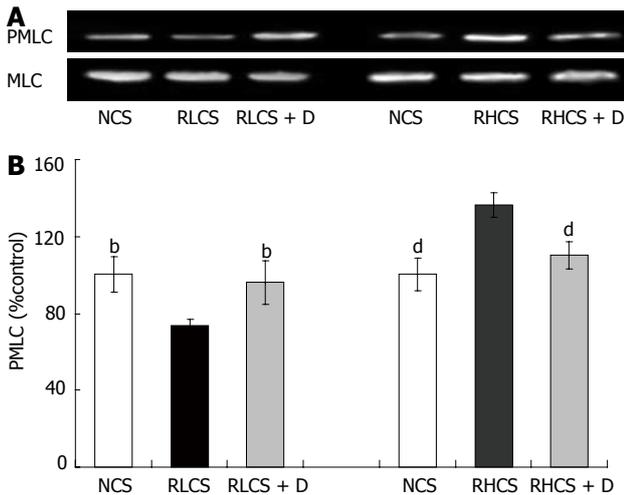


Figure 2 Western blotting analysis of the phosphorylation of myosin light chain. A: Representative images of Western blotting of the phosphorylation of myosin light chain (PMLC) of jejunum; B: Statistical analysis of band intensities of the PMLC in 4 independent experiments in normal contractile state (NCS, control), representative low contractile state (RLCS), and representative high contractile state (RHCS). To correct for loading variations, the result is expressed as a ratio of phosphor-myosin light chain to myosin light chain and NCS control is defined as 100%. Data represent mean \pm SE from 4 independent experiments; ^b*P* < 0.01 vs RLCS; ^d*P* < 0.01 vs RHCS. D: Deslanoside.

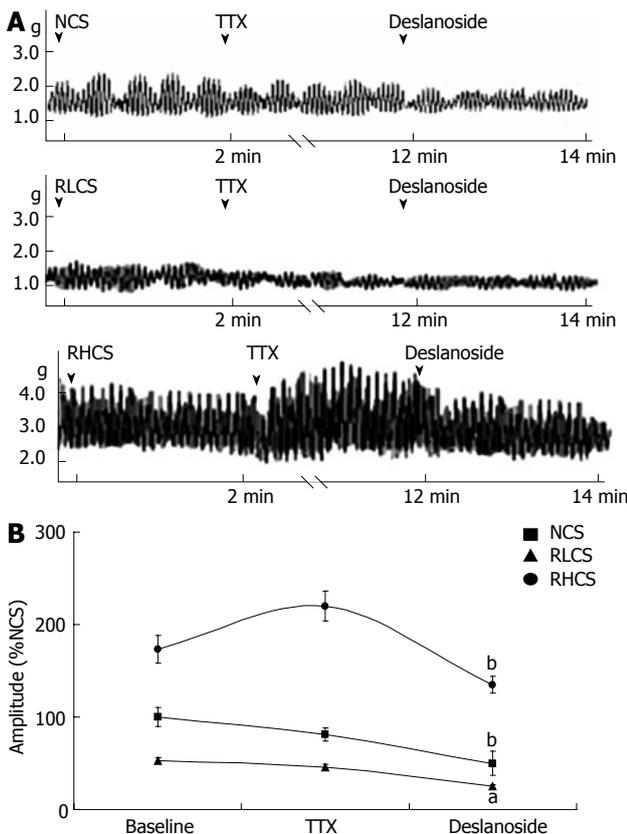


Figure 3 Effects of deslanoside on the contractility of jejunal smooth muscle fragment pretreated with tetrodotoxin. A: Representative traces of deslanoside on the contractility of jejunal smooth muscle fragment (JSMF) pre-treated with tetrodotoxin in normal contractile state (NCS), representative low contractile state (RLCS) and representative high contractile state (RHCS); B: Statistical analysis obtained from independent assays. Other data are the relative values compared with NCS. Data are expressed as the mean \pm SE (%NCS, *n* = 6); ^a*P* < 0.05, ^b*P* < 0.01 vs contractile amplitude of JSMF after treatment with tetrodotoxin (TTX), (0.1 μ mol/L).

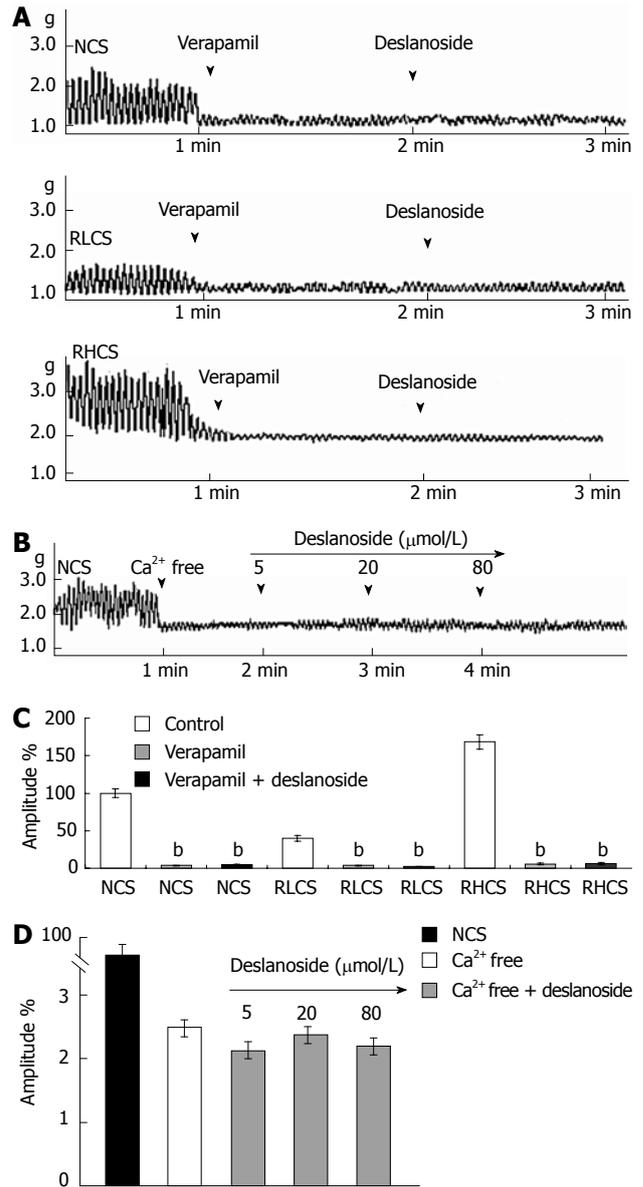


Figure 4 Effects of deslanoside on the contractility of jejunal smooth muscle fragment in Ca^{2+} -free conditions. A: Representative traces of deslanoside (20 μ mol/L) on the contractility of jejunal smooth muscle fragment (JSMF) pre-treated with verapamil (0.1 μ mol/L) in normal contractile state (NCS), representative low contractile state (RLCS) and representative high contractile state (RHCS); B: Representative traces of deslanoside (5-80 μ mol/L) on the contractility of JSMF pre-treated with Ca^{2+} -free Krebs buffer; C, D: Statistical analysis obtained from 6 independent assays in identical assay conditions as (A) and as (B). The mean contractile amplitude of JSMF without drug treatment in NCS is set to a relative value of 100%, other data are expressed as mean \pm SE (% NCS, *n* = 6); ^b*P* < 0.01 vs the control group.

for patients with IBS has been difficult because of the lack of pharmacological targets and the wide range of symptomatology^[31]. Considering that the precise cause of IBS is unknown and it is unlikely that one single factor could explain all instances of IBS^[32], we established various assay conditions to mimic the possible intestinal hyper- and hypo-motility. These low and high contractile states of isolated intestinal smooth muscle were established (1) by changing ionic concentration in assay buffers; (2) using inhibitory and stimulatory neurotransmitters, or using exog-

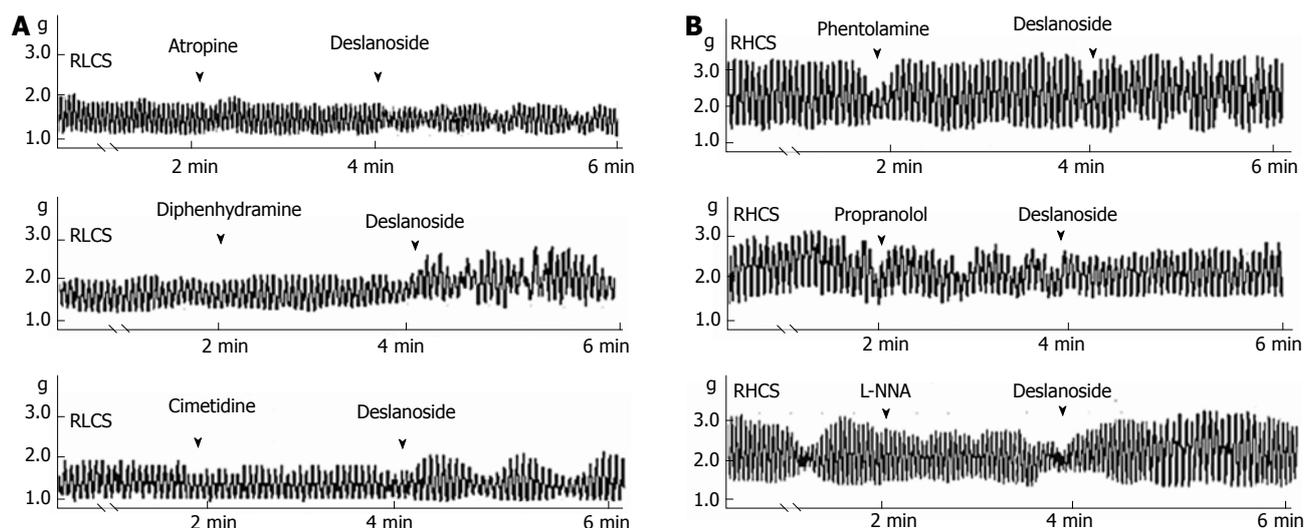


Figure 5 Effects of deslanoside on the contractility of jejunal smooth muscle fragment pretreated with receptor antagonist. A: Effects of deslanoside on the contractility of jejunal smooth muscle fragment (JSMF) pretreated with 10 $\mu\text{mol/L}$ atropine, 10 $\mu\text{mol/L}$ diphenhydramine and 10 $\mu\text{mol/L}$ cimetidine in the representative low contractile state (RLCS), respectively; B: Effects of deslanoside on the contractility of JSMF pretreated with 10 $\mu\text{mol/L}$ phentolamine, 5 $\mu\text{mol/L}$ propranolol and 10 $\mu\text{mol/L}$ L-NG-nitroarginine (L-NNA) in the representative high contractile state (RHCS), respectively.

enous inhibitors and stimulators in the assay buffers; and (3) using isolated intestinal smooth muscle obtained from constipation-prominent rat model and diarrhea-prominent rat model.

In this study, we tried to evaluate the possibility that whether deslanoside-induced adverse gastrointestinal irritation could be beneficialized as a potential therapeutic effect on the intestinal smooth muscle dysfunction, and characterized deslanoside-induced BR on the contractility of JSMF. Deslanoside was found to induce stimulatory effects on JSMF in all eight low contractile states and induced inhibitory effects on JSMF in all eight high contractile states. In accordance with deslanoside-induced BR on the contractility of JSMF, the effects deslanoside on myosin phosphorylation of JSMF were also bidirectional.

Activation of muscarinic receptor increases the intestinal motility and stimulation of α and β -adrenoceptors inhibits intestinal motility. Inhibition of intestinal motility is also mediated by NO, a nonadrenergic, noncholinergic neurotransmitter, producing its effect by directly acting on smooth muscle and by indirectly inhibiting acetylcholine and substance P releasing^[33,34]. Based on the aforementioned mechanisms involved in the modulation of intestinal contractility, our results have the following implications. The evidence that atropine blocked the stimulatory effects of deslanoside on JSMF in RLCS implies that stimulatory effects of deslanoside on JSMF in low contractile state are correlated with M receptor linked stimulation; and the evidence that phentolamine, propranolol and L-NNA abolished the inhibitory effects of deslanoside on JSMF in RHCS suggests that the inhibitory effects of deslanoside are correlated with adrenergic α , β receptor, as well as NO synthase linked relaxing mechanisms. Deslanoside-induced BR is Ca^{2+} -dependent, since it neither affected jejunal contractility in a Ca^{2+} -free assay condition, nor stimulated jejunal contractility pre-incubated with the Ca^{2+} channel blocker

verapamil in normal, low and high contractile states (Figure 4). The evidence that deslanoside-induced BR is not observed in the presence of TTX implies that deslanoside-induced BR is based on the presence of ENS.

Compared with controls (85.3 ± 37.3 min), the transit times (a measurement of bowel movement) obtained in constipation-predominant (67.4 ± 19.6 min) and diarrhea-predominant patients with IBS (108.4 ± 34.3 min) were decreased and increased, respectively ($P < 0.05$)^[35]. The results implicate that deslanoside-induced BR on jejunum is informative for preclinical investigation of a drug with potential value for the modulation of both abnormally low and high contractility of intestinal smooth muscle. To relieve the symptoms of functional bowel disorders, such as alternating-type IBS, BR-inducer deslanoside could be considered for the potential future clinical application.

It is known that ENS is highly interconnected and responsible for secreting at least 50 different modulators, regulating intestinal motility and other functions^[36]. We are still not clear about the diverse mechanisms for BR induction, including how dozens of neurotransmitters in intestinal smooth muscle are interrelated in normal contractile state, and how they correlate with BR in both the low and high contractile states. Although we have partially revealed the characteristics of deslanoside-induced BR, further study is still required to identify the detailed mechanisms.

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The authors wish to thank Zhi Lin and Fan Yuan for their comments.

COMMENTS

Background

Irritable bowel syndrome (IBS) is known as one of major functional gastro-

intestinal disorders, contracting approximately 10% of all adults world wide. Cardiotonic glycosides (CGs) have long been and continue to be used in the treatment of congestive heart failure and have entered clinical trials for treating cancer. Gastrointestinal irritation of CGs has been reported, however, the characteristics of CGs on intestinal motility remain unknown.

Research frontiers

Developing treatment strategies for patients with IBS has been difficult because of the lack of pharmacological targets and the wide range of symptomatology, especially in the alternating-type IBS (IBS-A) which is a functional gastrointestinal disorder with alternating symptoms of both constipation and diarrhea.

Innovations and breakthroughs

The present study established 8 pairs of low-high contractile states to mimic the possible intestinal smooth muscle disorders. These different low and high contractile states of isolated intestinal smooth muscle were established by changing ionic concentration in assay buffers; using inhibitory and stimulatory neurotransmitters; exogenous inhibitors and stimulators, respectively in the assays; and isolated intestinal smooth muscle obtained from both constipation-prominent rat model and diarrhea-prominent rat model. The results indicate that the contractile state determines deslanoside-induced effects to be stimulatory or inhibitory, namely, stimulatory effects on the contractility of intestinal fragment were induced by deslanoside in all low contractile states, and inhibitory effects were induced on the contractility of jejunal smooth muscle fragment (JSMF) in all high contractile states. The present study indicates that deslanoside-induced Bidirectional regulation (BR) requires the presence of enteric nervous system and is Ca²⁺ dependent. The possible mechanism of deslanoside-induced BR is related to cholinergic system when jejunal smooth muscle is in a low contractile state, and related to adrenergic system and nitric oxide relaxing mechanism when in a high contractile state.

Applications

The results implicate that deslanoside-induced BR on jejunum is informative for preclinical investigation of a drug with potential value for the modulation of both abnormally low and high contractility of intestinal smooth muscles. To relieve the symptoms of functional bowel disorders, such as IBS-A, BR-inducer deslanoside could be considered for the potential future clinical application.

Terminology

IBS is usually classified into three subclasses: IBS with constipation (hypomotility), IBS with diarrhea (hyper-motility), and IBS with alternating symptoms of both constipation and diarrhea.

Peer review

This is a well done study that provides interesting insight into the action of deslanoside. The study is complete, well-written and suitable for publication.

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Expression characteristics and diagnostic value of annexin A2 in hepatocellular carcinoma

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Abstract

AIM: To investigate the characteristics and diagnostic value of annexin A2 (ANXA2) expression in cancerous tissues and sera of patients with hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC).

METHODS: Levels of liver *ANXA2* gene transcription or protein expression were analyzed in HCC-, their self-controlled precancerous-, and distant cancerous- tissues from 30 HCC. Serum levels of ANXA2 expression in 115 patients with HCC, 25 with metastatic liver can-

cer, 35 with chronic hepatitis, 28 with acute hepatitis, 38 with cirrhosis, and 30 healthy controls were determined. Clinicopathological characteristics of circulating ANXA2 expression were analyzed, and its diagnostic efficiency and clinical values in HCC were evaluated.

RESULTS: ANXA2 expression was localized in both cell membrane and cytoplasm in HCC tissue, mainly in the cytoplasm of matched adjacent cancerous tissue, and there was almost no positive staining in matched distant cancerous tissue. Abnormal expression of liver ANXA2 was present in HCC tissues compared with self-controlled adjacent- and distant-cancerous tissues at protein or mRNA level. Circulating ANXA2 in HCC patients was significantly higher than that of other liver diseases ($P < 0.01$) except metastatic liver cancer. If the diagnostic cutoff value of ANXA2 level was more than 18 ng/mL, the incidence of serum ANXA2 was 86.96% in the HCC group, 80% in the metastatic liver cancer group, 31.58% in the liver cirrhosis group, none in the chronic hepatitis or acute hepatitis or normal control group, respectively. Serum ANXA2 expression in HCC patients was correlated with HBV infection (27.38 ± 5.67 ng/mL vs 18.58 ± 7.83 ng/mL, $P < 0.01$), extrahepatic metastasis (26.11 ± 5.43 ng/mL vs 22.79 ± 5.64 ng/mL, $P < 0.01$), and portal vein thrombus (26.03 ± 5.99 ng/mL vs 23.06 ± 5.03 ng/mL, $P < 0.01$), and was significantly higher ($P < 0.01$) in the moderately- (26.19 ± 5.34 ng/mL) or the poorly- differentiated group (27.05 ± 5.13 ng/mL) than in the well differentiated group (20.43 ± 4.97 ng/mL), and in the tumor node metastasis stages III-IV ($P < 0.01$) than in stages I - II. ANXA2 was not correlated with patient sex, age, size or α -fetoprotein (AFP) level. Area under the receiver operating characteristic curve for the whole range of sensitivities and specificities was 0.796 for ANXA2 and 0.782 for AFP. Combining detection of serum ANXA2 and AFP substantially improved the diagnostic efficiency (96.52%) and the negative predictive value (96.61%) for HCC.

CONCLUSION: The characteristics and distribution

of ANXA2 expression has good diagnostic potential for HCC diagnosis.

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Key words: Hepatocellular carcinoma; Annexin A2; Liver; Upregulation; Clinicopathological characteristics; Diagnosis; Expression; Biomarker

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Zhang HJ, Yao DF, Yao M, Huang H, Wu W, Yan MJ, Yan XD, Chen J. Expression characteristics and diagnostic value of annexin A2 in hepatocellular carcinoma. *World J Gastroenterol* 2012; 18(41): 5897-5904 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v18/i41/5897.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i41.5897>

INTRODUCTION

Hepatocellular carcinoma (HCC), as one of the most malignant tumors, is the third leading cause of cancer-related death, especially in the inshore area of the Yangtze River^[1,2]. Surgical resection is not suitable for a considerable number of HCC cases because of metastasis, and the long-term survival of postoperative HCC patients is not satisfactory^[3,4]. Its early detection and treatment is an effective way to improve patient survival. Detection of circulating markers is the most effective method because it is simple, accurate and low cost, but no ideal biomarker has been found so far^[5,6]. Recent studies showed that annexin A2 (ANXA2) plays an important role in hepatocyte malignant transformation and HCC development^[7-9]. ANXA2, as the best characterized of the Annexin family, is a calcium-dependent phospholipid-binding protein that plays a key role in the regulation of cellular growth and signal transduction pathways^[10]. It is reported that ANXA2 expression is upregulated in HCC compared with benign liver disease. Furthermore, its phosphorylation at residues of Tyr23 by c-Src is also increased^[11,12], and overexpression and tyrosine phosphorylation of ANXA2 may be of functional relevance.

Evaluation of the diagnostic value of ANXA2 in highly differentiated liver tumors suggests that adding sinusoidal ANXA2 expression to the marker panel [glypican 3, hepatoma-specific gamma-glutamyl transferase (HS-GGT), and heat shock protein 70] increases the reliability and objectivity of HCC diagnosis^[13]. In addition, serum ANXA2 levels in HCC patients are elevated by a quantitative sandwich enzyme linked immunosorbent assay (ELISA) method^[14]. It may be a serological marker for HCC to enable early diagnosis, as well as monitoring of aggressiveness, treatment responsiveness, recurrence and survival. However, the clinicopathologic characteristics of hepatic ANXA2 expression and the evaluation of its

diagnostic value for hepatitis B virus (HBV)-related HCC have not been reported up to now. In this study, the expression of hepatic and circulating ANXA2 was investigated in HCC patients and compared with expression in benign liver diseases to evaluate the pathologic characteristics and efficiency in HCC diagnosis.

MATERIALS AND METHODS

Collection of serum samples

We evaluated 115 HCC patients (88 men and 27 women) who were treated at the Affiliated Hospital of Nantong University, Nantong, China. Patient age ranged from 25 to 81 years (median, 48.3 years). Other cases studied included 35 with chronic hepatitis, 28 with acute hepatitis, 38 with cirrhosis, and 25 with metastatic liver cancer (liver metastasis of lung cancer, 6; gastric cancer, 6; acute myeloid leukemia, 3; breast cancer, 3; colorectal cancer, 3; cervical cancer, 2; and pancreatic cancer, 2) and samples from 30 healthy people with hepatitis viral markers [HBV-DNA, HBV surface antigen, and anti-hepatitis C virus (HCV)] and a normal alanine aminotransferase level obtained from the Nantong Central Blood Bank as controls. All cases were diagnosed by biochemical tests, viral histology, and B-ultrasonic examination. Blood samples (5 mL) were collected with heparin in the morning and sera separated immediately. α -fetoprotein (AFP) level was detected by a radiological method^[15].

Collection of liver specimens

The cancerous-, the self-matched adjacent cancerous- (more than 3 cm to cancer focus), and the distant cancerous- (more than 5 cm) specimens after surgical operation were respectively taken from 30 HCC patients who were treated at the Affiliated Hospital of Nantong University, Nantong, China. One portion of each specimen was immediately frozen in liquid nitrogen for total RNA extraction [ANXA2 mRNA by real time quantitative polymerase chain reaction (qPCR)], an extract was used to determine liver ANXA2 by Western blotting, and the remaining sample was fixed with 10% (vol/vol) formalin for ANXA2 immunohistochemistry. The diagnosis of HCC and viral hepatitis was based on the criteria proposed by the Chinese National Collaborative Cancer Research Group^[16] and at the Chinese National Viral Hepatitis Meeting^[17], respectively. Prior written informed consent was obtained from all patients according to the World Medical Association Declaration of Helsinki, and the study received ethics board approval from the Affiliated Hospital of Nantong University, Jiangsu Province, China.

ELISA

The level of serum ANXA2 was detected by using a human ANXA2 ELISA kit (Usn Life Science Inc., Wuhan, China) according to the manufacturer's instructions. To each well was added 100 μ L of serum sample or standard separately, and then 100 μ L of detection reagent A was

added and incubated for 1 h at 37 °C. Subsequently, 100 μ L of detection reagent B was added and incubated for 0.5 h at 37 °C. Then, 90 μ L of substrate solution was added and incubated for 25 min at 37 °C. Finally, 50 μ L of stop solution was added to each well, and absorbance was read at 450 nm. During the procedure, washing the plate was according to the ELISA routine method.

Total RNA isolation and synthesis of cDNA

Total RNA was isolated from 50 mg of liver tissue, using Trizol reagent (Invitrogen, United States) according to the manufacturer's instructions. The integrity of the total RNA was examined by 1% agarose gel electrophoresis, the quantity was determined based on absorbance at 260 nm (A_{260}), and the purity was analyzed based on the absorbance ratio at 260 nm and 280 nm ($A_{260/280}$) (Bio-RAD smartspec™ plus, United States). The ANXA2 cDNA was synthesized from 1 μ g of total RNA using First Strand cDNA Synthesis Kit (Fermentas, Canada) according to the manufacturer's instructions.

qPCR

The qPCR was run on an Applied Biosystems StepOne™ real-time PCR system according to the manufacturer's recommendations. The reaction solution contained 25 μ L 2 \times SYBR Premix Ex Taq (TaKaRa, Japan), 2 μ L primer mix, 1 μ L 50 \times ROX Reference Dye I, 4 μ L cDNA, and 18 μ L deionized water to make a total volume of 50 μ L. ANXA2 primers were as follows: forward, 5'-TGAGC-GGGATGCTTTGAAC-3', and; reverse, 5'-ATCCT-GTCTC TGTGCATTGCTG-3'; β -actin primers were as follows: forward, 5'-ATTGCC GACAGGATGCAGA-3', and reverse, 5'-GAGTACTTGCGCTCAGGAGGA-3' used as an internal control^[18], while no template control (H₂O) was included in each reaction run. The optimized PCR conditions were as follows: 1 cycle at 95 °C for 2 min; 40 cycles at 95 °C for 10 s, 62 °C for 1 min and final extension at 60 °C for 15 s. The relative quantitative analysis was performed by comparison of the 2^{- $\Delta\Delta$ Ct} values.

Western blotting

Liver tissues were homogenized in an ice-cold homogenization buffer containing 50 mmol/L 3-(N-Morpholino) propanesulfonic acid buffer (pH 7.4), 100 mmol/L KCl, 320 mmol/L sucrose, 50 mmol/L NaF, 0.5 mmol/L MgCl₂, 0.2 mmol/L dithiothreitol, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L Na₃VO₄, 20 mmol/L sodium pyrophosphate, 20 mmol/L β -phosphoglycerol, 1 mmol/L p-nitrophenyl phosphate, 1 mmol/L benzamide, 1 mmol/L phenylmethylsulfonyl fluoride, and 5 μ g/mL each of leupeptin, aprotinin, and pepstatin A. The homogenates were centrifuged at 800 g for 10 min at 4 °C. The supernatants were collected, and total protein concentrations were determined by an enhanced bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, China). A total of 20 mg of protein of each sample was run on a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were

then transferred onto polyvinylidene fluoride membranes and blocked with 5% bovine serum albumin in tris-buffered saline, pH 7.5 (100 mmol/L NaCl, 50 mmol/L Tris, and 0.1% Tween-20). Membranes were immunoblotted overnight at 4 °C with the anti-ANXA2 and anti- β -actin antibodies (Santa Cruz Biotechnology, United States), followed by respective horseradish peroxidase-conjugated secondary antibodies. The bands were subsequently visualized by a chemiluminescence detection system (Millipore, United States), and density analysis was performed by an image analyzer. The ANXA2 level was expressed with the relative ratio (RR), which was calculated by the following formula using signal intensity (SI) of ANXA2 and β -actin. $RR = SI_{ANXA2}/SI_{\beta-actin}$.

Immunohistochemistry for ANXA2

The 3 μ m thick sections were prepared from formalin-fixed, paraffin-embedded tissue blocks. Sections were deparaffinized in xylene twice for 10 min, then dehydrated through graded ethanol to distilled water for 5 min. Deparaffinized 5 μ m thick liver sections were washed three times with phosphate buffered solution (PBS) (pH 7.4), incubated in endogenous peroxidase blocking solution (Immunostain EliVision Kit, Maxim Biotech, United States), and then treated with 0.01 mol/L citrate buffer pH 6.0 for 10 min in a microwave oven at 650 W. Non-specific-antibody binding was blocked by pretreatment with PBS containing 0.5% bovine serum albumin (fraction V powder, Sigma, United States). Sections were then rinsed in PBS and incubated overnight at 4 °C with diluted anti-human ANXA2 antibody (1:500, Santa Cruz Biotechnology, United States) followed by three washes in PBS containing 0.05% Tween-20. The steps were performed using Immunostain EliVision kit according to the manufacturer's instructions. Sections were stained with 3,3'-diamino-benzidine tetrahydrochloride as a chromogen. The slide was rinsed with distilled water, counterstained with hematoxylin, dehydrated, air dried, and mounted. The negative control slides were treated with nonspecific mouse IgG. The sections were examined under light microscopy. ANXA2 staining was assessed using the immunoreactive score. In detail, the percentage of positive cells was assessed semiquantitatively and classified as follows: diffuse positive staining (+++) of more than 50% of total cells; moderate staining (++) , 16%-50%; weak staining (+), 5%-15%; and negative staining (-), < 5%^[19]. The results of staining were evaluated by two independent pathologists without knowledge of the clinicopathologic features, and any difference in interpretation was resolved by consensus. Duplicate tissue cores for each tumor showed high levels of homogeneity for staining intensity and percentage of positive cells. The higher score was taken as the final score in cases of a difference between duplicate tissue cores.

Statistical analysis

The data are expressed as mean \pm SD. Differences between different groups were evaluated by using a Student

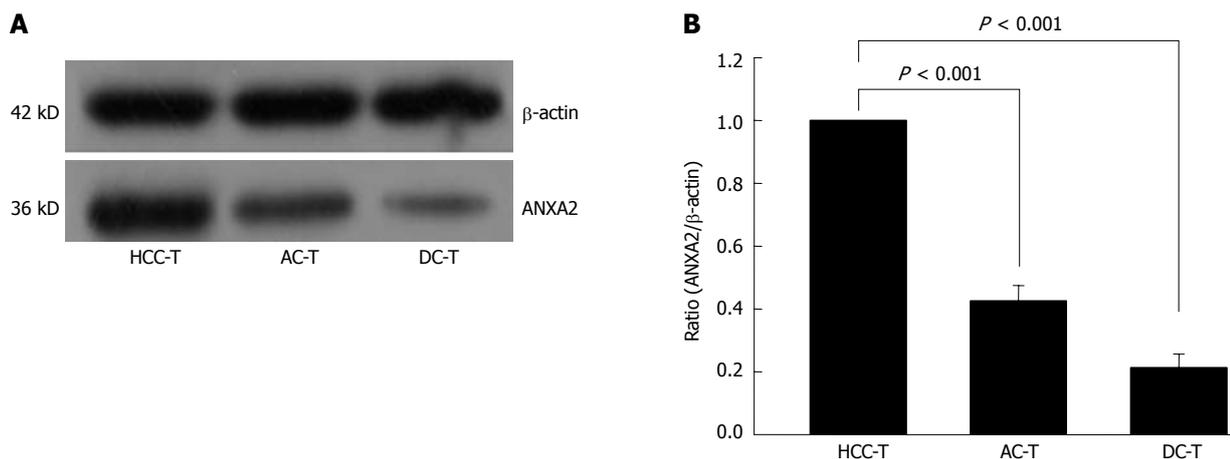


Figure 1 Annexin A2 protein level in liver tissue of hepatocellular carcinoma. A: Representative images of Western blotting. The protein levels of annexin A2 (ANXA2) and β -actin were detected and the latter served as the control; B: The statistical results indicated that the level of ANXA2 expression was obviously increased in hepatocellular carcinoma (HCC) tissues compared with the matched adjacent- or the distant-cancerous tissues ($P < 0.001$). Three independent experiments were repeated, and the results are given as mean \pm SD. HCC-T: HCC tissues; AC-T: Adjacent cancerous tissues; DC-T: Distant cancerous tissues.

Table 1 Relative quantity of hepatic annexin A2 mRNA and annexin A2 expression in hepatocellular carcinoma-, adjacent-, and distant-cancerous tissues ($n = 30$) (mean \pm SD)

Group	C_{tANXA2}	$C_{t\beta-actin}$	ΔCt	$2^{-\Delta\Delta Ct}$	Annexin A2 intensity				Z
					-	+	++	+++	
HCC tissues	21.63 \pm 0.09	21.19 \pm 0.07	0.44 \pm 0.06	1	0	1	7	22	
Adjacent cancerous tissues	24.29 \pm 0.09	22.60 \pm 0.08	1.70 \pm 0.12	0.43 \pm 0.10 ^b	3	16	11	0	6.113 ^b
Distant cancerous tissues	24.71 \pm 0.06	22.13 \pm 0.06	2.57 \pm 0.07	0.23 \pm 0.07 ^b	30	0	0	0	7.328 ^b

^b $P < 0.01$ vs the hepatocellular carcinoma (HCC) tissue group. -: Negative staining; +: Weak staining; ++: Moderate staining; +++: Diffuse positive staining.

t test, a χ^2 test or a rank-sum test. $P < 0.05$ was considered significant. Receiver operating characteristic (ROC) curves were constructed by calculating the sensitivities and specificities at several cutoff points^[15]. Efficiency evaluation was calculated according to sensitivity, specificity, accuracy, positive predictive value, and negative predictive value.

RESULTS

ANXA2 expression level in HCC tissues

ANXA2 protein and mRNA levels were detected in 30 self-controlled HCC tissues, and their matched adjacent- and distant-cancerous specimens by Western blotting and real-time PCR, respectively. As shown in Figure 1, the ANXA2 protein level was obviously higher in HCC tissues than in the self-controlled adjacent- and distant-cancerous specimens ($F = 498.221$, $P < 0.001$). The relative qPCR analysis (Table 1) indicated that the level of ANXA2 mRNA expression in the HCC tissues ($2^{-\Delta\Delta Ct} = 1.00$) was significantly higher ($F = 7908.11$, $P < 0.001$) than in the matched adjacent cancerous tissues ($2^{-\Delta\Delta Ct} = 0.43 \pm 0.10$) or the distant cancerous tissues ($2^{-\Delta\Delta Ct} = 0.23 \pm 0.07$). In short, ANXA2 was overexpressed in HCC tissues compared with the self-controlled adjacent- and distant-cancerous tissues, whether protein level or mRNA level.

Immunohistochemistry for ANXA2 expression and cell distribution

The expression and distribution of hepatic ANXA2 in 30 self-controlled HCC tissues, their matched adjacent- and distant-cancerous specimens are shown in Figure 2. The positive ANXA2 protein was localized in both cell membrane and cytoplasm (Figure 2A) in HCC tissue (30 of 30, 100%), mainly in the cytoplasm (Figure 2B) in matched adjacent cancerous tissue (27 of 30, 90%), and there was almost no positive staining (Figure 2C) in their matched distant cancerous tissue (0 of 30, 0%). The intensity and comparative analysis of ANXA2 expression in different liver tissues are shown in Table 1. Although no significant difference in the positive rate of ANXA2 expression ($\chi^2 = 3.518$, $P = 0.070$) was found between the HCC group and the adjacent cancerous group, the intensity of ANXA2 expression in the HCC group was significantly higher than that in the adjacent cancerous group ($Z = 6.113$, $P < 0.001$) or the distant cancerous group ($Z = 7.328$, $P < 0.001$).

Circulating ANXA2 and AFP level in patients with liver diseases

The levels of circulating ANXA2 and AFP expression in 241 patients with liver diseases are shown in Table 2. The mean level of serum ANXA2 expression in HCC patients was significantly higher than in the cases with

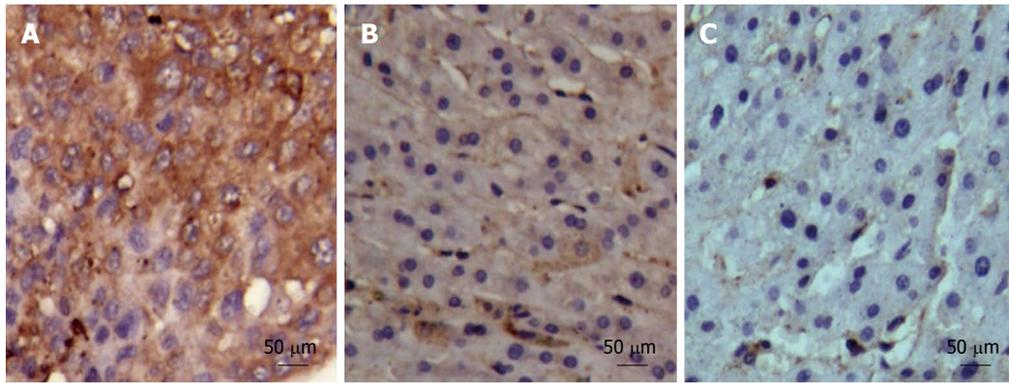


Figure 2 Immunohistochemistry of hepatic annexin A2 expression and distribution. Representative images of immunohistochemistry in the hepatocellular carcinoma (HCC), self-controlled adjacent- and distant- cancerous tissues (400 × magnification). A: Hepatocyte annexin A2 (ANXA2) expression in HCC tissue; B: Hepatocyte ANXA2 expression in matched adjacent cancerous tissue; C: Hepatocyte ANXA2 expression in matched distant cancerous tissue. The level of ANXA2 expression was obviously increased in HCC tissues compared with that in the matched adjacent- or the distant-cancerous tissues.

Table 2 Serum annexin A2 and α -fetoprotein levels in 241 patients with liver diseases (mean \pm SD)

Group	n	(ng/mL)	q	P value	Pos. (%)	χ^2	P value
ANXA2							
HCC	115	24.60 \pm 7.60			100 (86.96) ¹		
MLC	25	24.22 \pm 9.15 ³	0.482	0.803	20 (80.00) ³	0.812	0.368
LC	38	16.35 \pm 8.86 ³	11.621	< 0.001	12 (31.58) ³	44.652	< 0.001
CH	35	6.85 \pm 1.56 ³	22.566	< 0.001	0 (0.00) ³	91.304	< 0.001
AH	28	6.92 \pm 1.41 ³	20.948	< 0.001	0 (0.00) ³	80.971	< 0.001
NC	30	6.16 \pm 1.27 ³	22.757	< 0.001	0 (0.00) ³	84.058	< 0.001
AFP							
HCC	115	1446.76 \pm 1573.46			81 (70.44) ²		
MLC	25	1241.76 \pm 1349.14 ³	1.087	0.442	12 (48.00) ³	4.635	0.031
LC	38	73.73 \pm 168.03 ³	7.969	< 0.001	6 (15.79) ³	34.771	< 0.001
CH	35	69.05 \pm 106.73 ³	6.761	< 0.001	5 (14.29) ³	34.583	< 0.001
AH	28	70.54 \pm 107.11 ³	7.306	< 0.001	4 (14.29) ³	29.446	< 0.001
NC	30	6.06 \pm 1.63 ³	7.506	< 0.001	0 (0.00) ³	47.874	< 0.001

¹Serum annexin A2 (ANXA2) level > 18 ng/mL or ²Serum α -fetoprotein (AFP) level > 50 ng/mL was abnormal; ³Compared with the hepatocellular carcinoma (HCC) group. MLC: Metastatic liver cancer; LC: Liver cirrhosis; CH: Chronic hepatitis; AH: Acute hepatitis; NC: Normal controls; Pos. (%): Positive case number (%).

liver cirrhosis, chronic hepatitis, or acute hepatitis, or in control subjects ($P < 0.001$), but not compared with the metastatic liver cancer group. If the diagnostic cutoff value was more than 18 ng/mL, the incidence of circulating ANXA2 was 86.96% in the HCC group, 80% in the metastatic liver cancer group, 31.58% in the liver cirrhosis, and zero in the chronic hepatitis group, the acute hepatitis group or the normal control group. The incidence of serum AFP in the HCC patients (81 of 115, 70.44%) was significantly higher than in the metastatic liver cancer group (12 of 25, 48%, $P < 0.05$), the liver cirrhosis group (6 of 38, 15.79%), the chronic hepatitis group (5 of 35, 14.29%), the acute hepatitis group (4 of 28, 14.29%), and was zero in the normal control group.

Clinicopathologic features of circulating ANXA2 expression

The clinicopathologic features of circulating ANXA2 expression in 115 HCC patients are shown in Table 3. The

higher level of serum ANXA2 expression was correlated with HCC patients with HBV infection (Figure 3A, $t = 6.820$, $P < 0.001$), extrahepatic metastasis ($t = 3.191$, $P = 0.002$), or portal vein thrombus ($t = 2.859$, $P = 0.005$). Serum ANXA2 expression was graded, with 20.43 ± 4.97 ng/mL in the well differentiated group and significantly lower ($P < 0.001$) in the moderately- (26.19 ± 5.34 ng/mL) or the poorly- differentiated (27.05 ± 5.13 ng/mL) group, and levels of ANXA2 expression were obviously higher ($P < 0.001$) in tumor node metastasis (TNM) stage III or IV than in TNM stage I or II. ANXA2 expression was not correlated with patient sex, age, tumor size or serum AFP level (Figure 3B). Both circulating ANXA2 and AFP are useful biomarkers for HCC diagnosis.

Evaluation of serum ANXA2 level for HCC diagnosis

The evaluation of serum ANXA2 and AFP levels for HCC diagnosis is shown in Figure 4. The comparative analysis of two markers for the whole range of sensitivities and specificities was 0.796 in ANXA2 and 0.782 in AFP according to under the area under the ROC curve. The clinical evaluation of serum ANXA2 or/and AFP levels for HCC diagnosis is shown in Table 4. The sensitivity of serum ANXA2 only was 86.96%, while a combination of ANXA2 with AFP could increase the rate of HCC diagnosis (96.52%), and the negative predictive value was improved to 96.61%.

DISCUSSION

HCC prognosis is poor, and early detection is of the utmost importance. Although serum AFP is a useful biomarker for the detection and monitoring of HCC, the false-negative rate using the AFP level alone may be as high as 40% for HCC patients with small size tumors. A previous report implied that ANXA2 expression was upregulated in HCC and it could be a useful molecular marker for HCC^[10-12]. In this study, the expression of hepatic and circulating ANXA2 was investigated in HCC patients and compared with that in cases of benign liver diseases to explore the clinicopathological characteristics

Table 3 Pathologic characteristics of annexin A2 expression in sera of 115 hepatocellular carcinoma patients (mean \pm SD)

Group		<i>n</i>	(ng/mL)	<i>t</i>	<i>P</i> value	Pos. (%) > 18 ng/mL	χ^2	<i>P</i> value
Sex	Male	88	24.56 \pm 5.84	0.150	0.881	78 (88.64)	0.933	0.334
	Female	27	24.79 \pm 5.33			22 (81.48)		
Age (yr)	\geq 50	87	24.84 \pm 6.07	0.798	0.427	76 (87.36)	0.050	0.822
	< 50	28	23.83 \pm 4.56			24 (85.71)		
Tumor size (cm)	\geq 5.0	75	24.38 \pm 5.68	0.850	0.397	66 (88.00)	0.207	0.649
	< 5.0	40	25.35 \pm 5.95			34 (85.00)		
α -fetoprotein (ng/mL)	\geq 400	53	24.91 \pm 5.52	0.531	0.596	45 (84.91)	0.365	0.546
	< 400	62	24.34 \pm 5.96			55 (88.71)		
HBsAg	Positive	79	27.38 \pm 5.67	6.820	< 0.001	73 (92.41)	6.605	0.010
	Negative	36	18.58 \pm 7.83			27 (75.00)		
Differentiated grading	Well	37	20.43 \pm 4.97	4.966 ¹	< 0.001 ¹	25 (67.57)	10.631 ¹	0.001 ¹
	Moderate	43	26.19 \pm 5.34			41 (95.35)		
	Poor	35	27.05 \pm 5.13			34 (97.14)		
TNM staging	Stages I - II	52	21.16 \pm 5.97	5.594 ²	< 0.001 ²	38 (71.43)	16.122 ²	< 0.001 ²
	Stages III-IV	63	27.44 \pm 6.01			62 (97.06)		
Extrahepatic metastasis	With	62	26.11 \pm 5.43	3.191	0.002	58 (93.55)	5.514	0.023
	Without	53	22.79 \pm 5.64			42 (79.25)		
Portal vein thrombus	With	60	26.03 \pm 5.99	2.859	0.005	56 (93.33)	4.498	0.034
	Without	55	23.06 \pm 5.03			44 (80.00)		

¹Compared with the well differentiated group; ²Compared with the tumor node metastasis (TNM) stage I - II group. HBsAg: Surface antigen of the hepatitis B virus; Pos. (%): Positive case number (%).

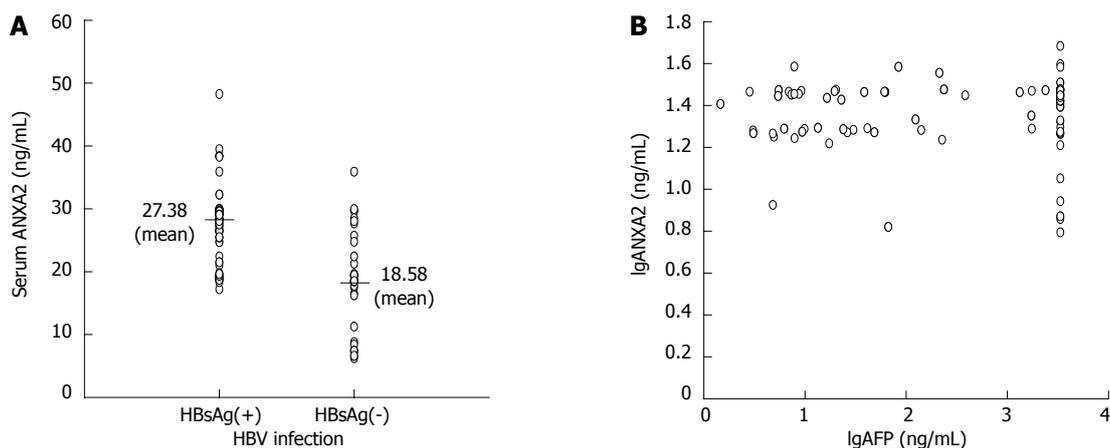


Figure 3 Relationship between serum annexin A2 level and hepatitis B virus infection or serum α -fetoprotein level in hepatocellular carcinoma patients. A: The scatter figure indicated that serum annexin A2 (ANXA2) level was obviously correlated with hepatitis B virus (HBV) infection. The serum ANXA2 level in hepatocellular carcinoma (HCC) patients was higher ($t = 6.820$, $P < 0.001$) in the positive-surface antigen of the HBV (HBsAg) group than that in the negative-HBsAg group; B: The scatter figure of denary logarithm indicated no significant correlation between serum ANXA2 and α -fetoprotein (AFP) level in HCC patients.

and diagnostic value in HCC.

The expression of hepatic ANXA2 was associated with hepatocyte malignant transformation. Hepatic ANXA2 was overexpressed in HCC tissues compared with their matched adjacent- and distant tissue, not only at protein level (Figure 1) but also at mRNA level (Table 1). Although no significant difference in the positive rate of ANXA2 expression was found between the HCC group and the adjacent cancerous group, the intensity of ANXA2 expression in the HCC group was significantly higher than in the adjacent cancerous group or the distant cancerous group (Table 1). There was a consistent overexpression of ANXA2 protein level and ANXA2 mRNA level. It is reported that adding ANXA2 to the established marker panel for the detection of early and well-differentiated

HCC should increase the diagnostic reliability and objectivity, which may particularly improve the accuracy of HCC diagnosis in minute tissue samples^[13]. It is worth mentioning that ANXA2 levels in HCC adjacent tissue present a very good intermediate state at protein or mRNA level, which might result from the change in the tumor microenvironment and the transfer of tumor cells, because a previous report suggested that ANXA2 binds with plasminogen and tissue plasminogen activator on the cell surface and promotes tumor metastasis by inducing the conversion of plasminogen to plasmin, which leads to activation of matrix metalloproteinases and degradation of extra-cellular matrix components^[7-9].

Although the mechanisms of hepatocarcinogenesis have not been elucidated, a long-lasting inflammation

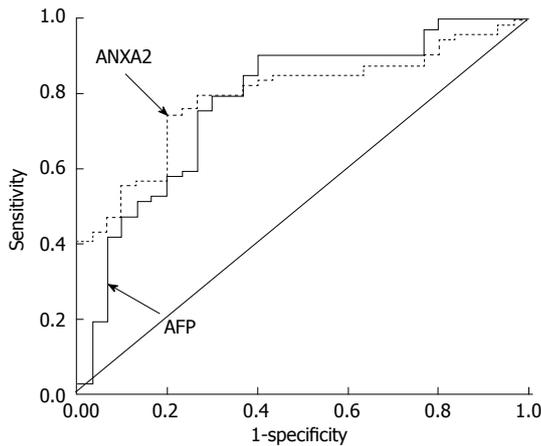


Figure 4 Receiver operating characteristic curves for circulating annexin A2 level in hepatocellular carcinoma diagnosis. The area under the receiver operating characteristic curves was 0.782 for α -fetoprotein (AFP) and 0.796 for annexin A2 (ANXA2).

induced by hepatitis virus infection is a definite risk for neoplastic degeneration and the accumulation of genetic alterations. The diagnosis and monitoring of small size tumors have been always difficult due to the lack of effective biomarkers that can characterize the formation and progression of HCC development^[20,21]. The ability of several biomarkers, such as lens culinaris agglutinin-reactive AFP-L3^[22], HS-GGT, and glypican-3^[23], to detect early HCC has been examined, but the sensitivity and specificity are still not satisfactory. In this study, the circulating ANXA2 level was investigated in HCC patients and cases with benign liver diseases (Table 1). If the cutoff value of ANXA2 abnormality is more than 18 ng/mL, its incidence in patients with HCC (86.96%) or metastatic liver cancer (80%) was significantly higher than in cases with liver cirrhosis (31.58%), chronic hepatitis (0%), acute hepatitis (0%), or controls (0%). The value of ANXA2 in patients with cirrhosis is intermediate, and it may be considered as an early marker during malignant transformation of liver cells. Serum AFP is a useful serological marker for HCC diagnosis; however, a high false negative rate has been found in patients with benign liver diseases, and our study suggests that serum ANXA2 is superior to AFP and a relatively distinct marker for HCC diagnosis.

The *ANXA2* gene is upregulated in HBV- and/or HCV-associated HCC^[24]. ANXA2 induces cell migration and neoangiogenesis *via* tissue plasminogen activator-dependent plasmin generation^[25], represents metastatic potential^[18], and promotes invasion and migration of HCC *in vitro* *via* its interaction with HAb18G/CD147^[7]. Moreover, Tyr23 phosphorylation-dependent cell-surface localization of ANXA2 is required for invasion and metastases^[12]. The clinicopathologic features of circulating ANXA2 expression in HCC patients (Table 3) demonstrated that there is a very close relationship between ANXA2 level and invasion and metastasis, as well as HBV infection. The higher level of ANXA2 expression

Table 4 Efficiency evaluation of serum annexin A2 or/ α -fetoprotein levels for hepatocellular carcinoma diagnosis¹

Project of evaluation	ANXA2 (%)	AFP (%)	Both (%)
Sensitivity	86.96	70.43	96.52
Specificity	66.67	73.08	68.67
Accuracy	75.28	71.96	80.07
Positive predictive value	65.79	65.85	68.10
Negative predictive value	87.39	77.03	96.61

¹Serum annexin A2 (ANXA2) level >18 ng/mL or α -fetoprotein (AFP) level > 50 ng/mL was abnormal.

in HCC patients was correlated with HBV infection (Figure 3A), extra-hepatic metastasis, portal vein thrombus, differentiated grading, and TNM staging. However, no significant correlation was found between serum ANXA2 level and tumor size, or AFP level (Figure 3B). It deserves to be mentioned that there was no significant difference between the moderately differentiated group and the poorly differentiated group, or TNM staging III-IV. The efficiency evaluation of serum ANXA2 or/ α -fetoprotein level for HCC diagnosis (Table 4, Figure 4) indicated that serum ANXA2 detection has higher sensitivity, accuracy, negative predictive value and complementary diagnostic value in combination with AFP for HCC diagnosis.

In conclusion, hepatic ANXA2 expression is associated with hepatocyte malignant transformation and plays an important role in hepatic active metabolism, development, microenvironment, and prognosis of HCC. The higher intensity of ANXA2 expression in HCC tissues and circulating ANXA2 was correlated with HBV infection, extrahepatic metastasis and portal vein thrombus. Therefore, it could be developed as an effective diagnostic marker for HCC by a series of further independent and prospective studies, and become a research hotspot to reveal the mechanism of metastasis resulting from ANXA2 in the near future.

COMMENTS

Background

Hepatocellular carcinoma (HCC), as one of the most malignant tumors, is the third leading cause of cancer-related death. The development of biomarkers for early diagnosis and accurate prognosis of HCC is important for improving patients' survival. Annexin A2 (ANXA2) could be used as a new marker for HCC in the future.

Research frontiers

It is reported that ANXA2 expression and its phosphorylation is upregulated in HCC compared with benign liver disease. Moreover, the dose-response relationship between ANXA2 and optical density was linear in the range of 0-10 μ g/mL. However, little research on the about expression characteristics and diagnostic value of ANXA2 in HCC have been reported to date. In this study, the authors analyzed the expression characteristics and specific distribution of ANXA2 as well as its diagnostic value in HCC.

Innovations and breakthroughs

This is the first report on the expression characteristics and specific distribution of ANXA2 as well as its diagnostic value in HCC. ANXA2 overexpression in HCC patients was correlated with hepatitis B virus infection, extrahepatic metastasis, portal vein thrombus, differentiated grading and tumor node metastasis staging, but not with patient sex, age, size or α -fetoprotein (AFP) level. Joint diagnosis using serum ANXA2 and AFP substantially improved the diagnostic efficiency.

Applications

The expression characteristics and specific distribution of ANXA2 have good diagnostic potential for HCC, and could be developed into an effective diagnostic marker for HCC by a series of further independent and prospective studies.

Terminology

ANXA2 as a member of the Annexin family is a calcium-dependent phospholipid-binding protein and is involved in the regulation of cellular growth and signal transduction pathways. Its expression is upregulated in HCC with increased molecular phosphorylation at residues of Tyr23 by c-Src.

Peer review

The evaluated manuscript reports ANXA2 as biomarker of HCC. The study has been well conducted and provide further validation.

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Comparative effectiveness of *i*-SCAN™ and high-definition white light characterizing small colonic polyps

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Abstract

AIM: To evaluate accuracy of *in vivo* diagnosis of adenomatous vs non-adenomatous polyps using *i*-SCAN digital chromoendoscopy compared with high-definition white light.

METHODS: This is a single-center comparative effectiveness pilot study. Polyps ($n = 103$) from 75 average-risk adult outpatients undergoing screening or surveillance colonoscopy between December 1, 2010 and April 1, 2011 were evaluated by two participating endoscopists in an academic outpatient endoscopy center. Polyps were evaluated both with high-definition white light and with *i*-SCAN to make an *in vivo* prediction of

adenomatous vs non-adenomatous pathology. We determined diagnostic characteristics of *i*-SCAN and high-definition white light, including sensitivity, specificity, and accuracy, with regards to identifying adenomatous vs non-adenomatous polyps. Histopathologic diagnosis was the gold standard comparison.

RESULTS: One hundred and three small polyps, detected from forty-three patients, were included in the analysis. The average size of the polyps evaluated in the analysis was 3.7 mm (SD 1.3 mm, range 2 mm to 8 mm). Formal histopathology revealed that 54/103 (52.4%) were adenomas, 26/103 (25.2%) were hyperplastic, and 23/103 (22.3%) were other diagnoses include "lymphoid aggregates", "non-specific colitis," and "no pathologic diagnosis." Overall, the combined accuracy of endoscopists for predicting adenomas was identical between *i*-SCAN (71.8%, 95%CI: 62.1%-80.3%) and high-definition white light (71.8%, 95%CI: 62.1%-80.3%). However, the accuracy of each endoscopist differed substantially, where endoscopist A demonstrated 63.0% overall accuracy (95%CI: 50.9%-74.0%) as compared with endoscopist B demonstrating 93.3% overall accuracy (95%CI: 77.9%-99.2%), irrespective of imaging modality. Neither endoscopist demonstrated a significant learning effect with *i*-SCAN during the study. Though endoscopist A increased accuracy using *i*-SCAN from 59% (95%CI: 42.1%-74.4%) in the first half to 67.6% (95%CI: 49.5%-82.6%) in the second half, and endoscopist B decreased accuracy using *i*-SCAN from 100% (95%CI: 80.5%-100.0%) in the first half to 84.6% (95%CI: 54.6%-98.1%) in the second half, neither of these differences were statistically significant.

CONCLUSION: *i*-SCAN and high-definition white light had similar efficacy predicting polyp histology. Endoscopist training likely plays a critical role in diagnostic test characteristics and deserves further study.

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Key words: Colonoscopy; Adenoma; Virtual chromoen-

doscopy; Colonic polyps; Comparative effectiveness

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INTRODUCTION

Fourteen million colonoscopies are performed annually in the United States, with the majority performed for screening with the goal of detecting and resecting pre-cancerous polyps^[1]. Small polyps (≤ 10 mm in size) make up the majority of polyps removed during screening colonoscopy, yet the rate of advanced neoplasia and invasive carcinoma in these polyps is low. The current standard of practice is removal of all detected polyps, regardless of size, for the purpose of histopathologic diagnosis and prevention of colorectal cancer^[2]. However, this practice may not be the most cost-effective way to utilize limited endoscopic resources^[3]. Accurate methods to predict histology of small polyps *in vivo* could prevent unnecessary polypectomies and/or promote a “resect and discard” practice^[4], thus minimizing risk to patients as well as improving efficiency and cost of endoscopy as a screening tool. However, clinical impression of lesion histology using standard white light colonoscopy has been shown to correlate poorly with neoplasia regardless of endoscopist experience^[5]. This climate creates an opportunity to expand the role for advanced colonoscopic techniques to predict histology *in vivo* and perform polypectomy in a targeted fashion. In fact, chromoendoscopy with optical magnification and pit pattern analysis can be highly accurate in optical diagnosis^[6]. Ideally, the benefit of chromoendoscopy in “optical diagnosis” could be harnessed with more time-efficient digital chromoendoscopic techniques that visually enhance mucosal surface aspects or vessel patterns without significantly prolonging endoscopic procedure time^[7].

PENTAX *i*-SCAN™, a PENTAX Medical Company digital chromoendoscopy technology, uses post-processing computer algorithms integrated into the standard high-definition processor to modulate light reflected from mucosa and highlight surface contrast. Several small studies have evaluated the accuracy of *i*-SCAN to assist physicians in predicting polyp histology *in vivo* and have shown promising results^[8-10]. However, the generalizability of these studies is limited by their reliance on experts with non-validated diagnostic criteria.

The goal of this pilot study is to build on this early

work by evaluating the accuracy of *i*-SCAN in predicting histology of small polyps (less than 10 mm) throughout the colon.

MATERIALS AND METHODS

Study design

This was a single center, prospective, comparative effectiveness pilot study conducted at a single academic medical center. The study protocol and equipment were approved by the local Institutional Review Board. The sponsor had no role in the conduct, analysis or reporting of study results.

Study population

Consecutive outpatient adults at least 45 years of age referred for screening or surveillance endoscopy at Duke University Medical Center between December 1, 2010 and April 1, 2011 were eligible for study enrollment. Based on readily available data from chart review at the time of the referral, we excluded patients with any of the following conditions: history of colorectal cancer or polyposis syndrome, acute gastrointestinal bleed, history of inflammatory bowel disease, use of anti-platelet or anticoagulant agents that prevent biopsy or polypectomy during colonoscopy, ASA Class III or greater, or inability to provide informed consent. All enrolled subjects provided written informed consent. Patient demographics including age, sex, race, and personal history of prior adenomatous polyps were recorded.

Endoscopy equipment

Endoscopies were performed using PENTAX high-definition adult and pediatric colonoscopies equipped with *i*-SCAN. A button-operated control head on the endoscope permits instant switch between high-definition white light (HDWL) and *i*-SCAN modes.

Endoscopic procedures

All colonoscopies were performed by two experienced endoscopists who have completed at least 2000 colonoscopies. Previous work with *i*-SCAN capitalized on its ability to enhance surface patterns for polyp histology prediction^[8,10], and thus Kudo pit pattern analysis was chosen for systematic histology prediction. The endoscopists had not previously used *i*-SCAN for the purpose of polyp prediction and thus underwent review of Kudo pit pattern characterization prior to enrolling patients. Posters showing pit pattern characteristics were also available for reference during all procedures.

After colonic preparation, patients underwent moderate sedation per endoscopy unit protocol. The endoscope was inserted to the cecum in standard fashion without attempt to detect polyps on insertion. Procedure time and quality of bowel preparation were graded by the endoscopist and recorded.

Upon reaching the cecum, the colonoscope was withdrawn in standard fashion using HDWL to visualize the

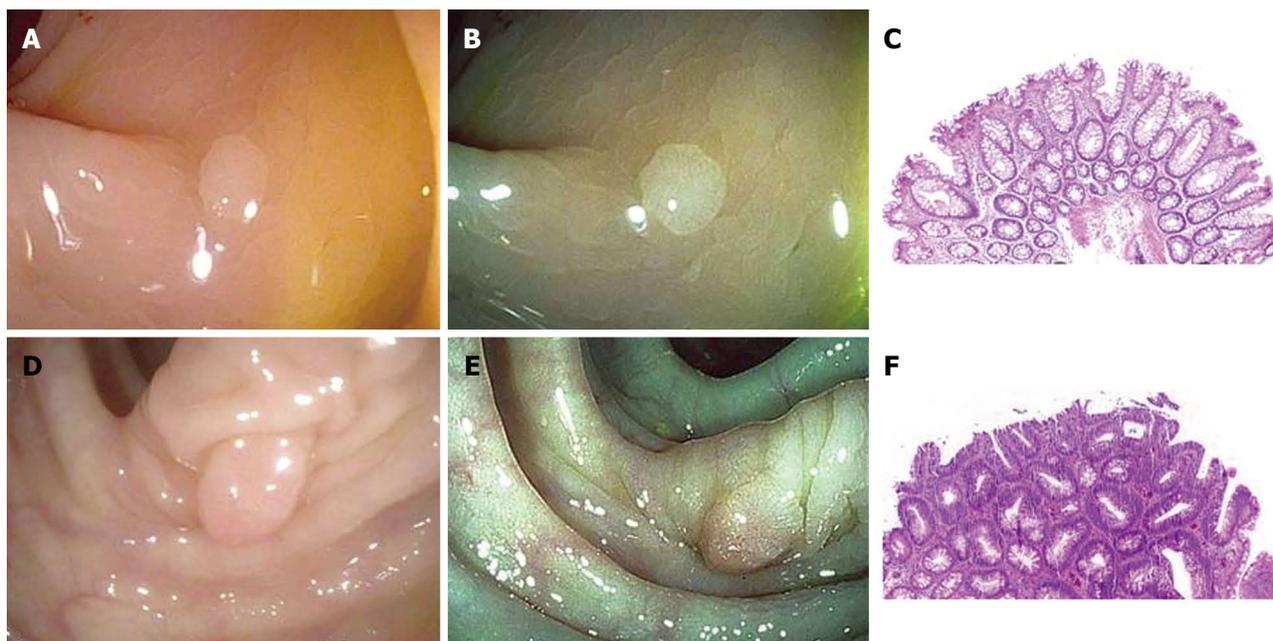


Figure 1 Correct prediction of small colonic polyps. A: Correctly predicted as hyperplastic, visualized under high-definition white light; B: Correctly predicted as hyperplastic, visualized under *i*-SCAN; C: Pathology demonstrating colonic mucosa with serrated architecture extending midway down the glands and non-dysplastic cytology, consistent with a hyperplastic polyp [hematoxylin and eosin (HE); 4 ×]; D: Correctly predicted as adenoma, visualized under high-definition white light; E: Correctly predicted as adenoma, visualized under *i*-SCAN; F: Pathology demonstrating colonic mucosa with hyperchromatic, elongated, and pseudostratified nuclei, consistent with a tubular adenoma (HE; 4 ×).

colonic mucosa. All polyps detected during the procedure were documented for size, location, and morphology. Size was estimated using open biopsy forceps for reference. Small polyps, those defined as less than 10 mm in size, were further evaluated as below.

When polyps less than 10 mm in size were identified by white light endoscopy, surface characteristics were first assessed using HDWL followed by *i*-SCAN. Endoscopists were not limited to any one *i*-SCAN mode. Endoscopists were asked to predict histology of the polyp in real time using Kudo pit pattern classification for each diagnostic modality. Images of the polyp in HDWL and *i*-SCAN were captured by the endoscopist (Figure 1). Polyp morphology was described using Paris classification system^[1]. Subsequently, the polyp was resected, collected in an individual specimen jar with fresh neutral buffered formalin, and sent to pathology. The samples were processed, and two hematoxylin and eosin sections were created and reviewed by a single surgical pathologist blinded to the colonoscopy findings. A maximum of five consecutive polyps per patient were examined using the *i*-SCAN modality. This restriction was implemented so as to avoid skewing results by the rare patient who might have multiple hyperplastic polyps in the rectum.

At the midpoint of the study as part of a pre-determined intervention, the study team reviewed incorrect predictions with each endoscopist. This intervention included a review of incorrectly predicted polyp images in both HDWL and *i*-SCAN.

Role of funding source

The protocol was an investigator-initiated study funded

by PENTAX Medical Company. PENTAX provided funding for the study coordinator and pathology costs. The funding source had no role in the study conduct, data collection, statistical analysis, interpretation, manuscript preparation, or decision to submit the manuscript for publication.

Statistical analysis

The primary outcome for the analysis is sensitivity, specificity, accuracy, and test characteristics of *i*-SCAN and HDWL in predicting the histology of small polyps in real time. Diagnostic test characteristics were evaluated by comparing with histopathologic diagnosis as the gold standard.

The test characteristics were calculated as binomial proportions from one-way frequency tables, and 95% confidence intervals were constructed using the exact confidence limits. Patient and polyp characteristics were compared between providers using Wilcoxon rank-sum test for continuous variables and χ^2 or Fisher's exact test for categorical variables. To assess the impact of patient and provider characteristics on the likelihood of accurate prediction, a generalized linear mixed model was used, with random effect of intercept and slope of polyp size to take within-patient dependency into account, and to allow intercept and slope differ across patients. We explored the learning curve with *i*-SCAN by comparing the endoscopists' prediction accuracy on polyps from patients from the first half of the study *vs* those from the second half. All data were analyzed by using the SAS version 9.2 (SAS Institute Inc., Cary, NC).

Table 1 Patient baseline characteristics *n* (%)

	All patients	Patients with polyps
Patients (<i>n</i>)	75	43
Age (yr), mean ± SD	60.6 ± 9.58	61.9 ± 8.98
Male sex	43 (57.3)	31 (72.1)
Race ¹		
White	56 (75.7)	33 (78.6)
Black	15 (20.3)	7 (16.67)
Other	3 (4.00)	2 (4.76)
Proportion of patients with +family history of colorectal cancer	14 (19.2)	10 (23.8)
Proportion of patients with personal history of adenomatous polyps	24 (32.9)	32 (74.4)
Prep quality		
Excellent	14 (19.2)	7 (17.5)
Good	48 (65.8)	27 (67.5)
Fair	10 (13.7)	5 (12.5)
Poor	1 (1.4)	1 (2.5)

¹Missing data includes family history (*n* = 2) and prep quality (*n* = 2).

Table 2 Endoscopist quality measures

	Endoscopist A	Endoscopist B
Patients (<i>n</i>)	48 (64)	27 (36)
Polyps/patient (<i>n/n</i>), mean ± SD	1.5 ± 1.7	1.2 ± 1.9
Polyp size (mm), mean ± SD	3.67 ± 1.39	3.77 ± 1.04
Cases in which polyp identified <i>n</i> (%)	29 (60.4)	14 (51.9)
Cases in which adenomatous polyp identified <i>n</i> (%)	22 (45.8)	10 (37.0)
Procedure time (min), mean ± SD	21 ± 6.8 ^a	26 ± 9.6 ^a

^a*P* < 0.05 vs Endoscopist A.

RESULTS

Study population characteristics

Eighty-two patients met enrollment criteria; seven patients declined to participate. Thus, 75 patients were enrolled during the study period between December 1, 2010 and April 1, 2011. The cecum was successfully intubated in 100% of cases. Baseline patient demographic data (Table 1) are shown. There were no significant differences between the two endoscopists in terms of patient age, sex, race, family history of colorectal cancer, or personal history of adenomatous polyps.

Quality metrics for the two endoscopists involved in the study are shown in Table 2. Due to scheduling variability, the majority of procedures in the study were performed by Endoscopist A. Endoscopist B took longer, on average, to complete procedures. There was no difference in polyp detection (*P* = 0.47) or polyp size (*P* = 0.34) between endoscopists.

Polyp characteristics

One hundred and three small polyps were included in the analysis among the 43 patients with polyps. The average

Table 3 Test characteristics for adenoma prediction, % (95%CI)

	Combined	Endoscopist A	Endoscopist B
White light			
Accuracy	71.8 (62.1-80.3)	63.0 (50.9-74.0)	93.3 (77.9-99.2)
Sensitivity	74.1 (60.4-85.0)	66.7 (49.0-81.4)	88.9 (65.3-98.6)
Specificity	69.4 (54.6-81.8)	59.5 (42.1-75.3)	100 (73.5-100)
Positive predictive value	72.7 (61.0-84.5)	61.5 (44.8-77.5)	100 (79.4-100)
Negative predictive value	70.8 (58.0-83.7)	64.7(46.5-80.3)	85.7(57.2-98.2)
<i>i</i> -SCAN			
Accuracy			
Total	71.8 (62.1-80.3)	63.0 (50.9-74.0)	93.3 (77.9-99.2)
First Half	71.4 (57.8-82.7)	59.0 (42.1-74.4)	100 (80.5-100)
Second half	72.3 (57.4-84.4)	67.6 (49.5-82.6)	84.6 (54.6-98.1)
Sensitivity	72.2 (58.4-83.5)	63.9 (46.2-79.2)	88.9 (65.3-98.6)
Specificity	71.4 (56.7-83.4)	62.2 (44.7-77.5)	100 (73.5-100)
Positive predictive value	73.6 (61.7-85.5)	62.2 (44.6-76.6)	100 (79.4-100)
Negative predictive value	70.0 (57.3-82.7)	63.9 (46.2-79.2)	85.7 (57.2-98.2)

size of the polyps was 3.7 mm (SD 1.3 mm, range: 2-8 mm). Six of the 103 polyps (5.8%) were located in the rectum, 30 (29.1%) in the sigmoid, 7 (6.8%) were located in the descending colon, 35 (34.0%) in the transverse colon, and 25 (24.3%) in the ascending colon/cecum. By morphology, 101 of the 103 (98.1%) were described as by Paris Is, with only 1 polyp described as Paris Ip, and 1 described as Paris Iia.

Pathology revealed 54/103 (52.4%) adenomas, 26/103 (25.2%) hyperplastic, and 23/103 (22.3%) other diagnoses including “lymphoid aggregates”, “non-specific colitis”, and “no pathologic diagnosis”.

Test characteristics

The sensitivity, specificity, and accuracy of HDWL for the *in vivo* prediction of polyp histology are shown in Table 3. Overall sensitivity with all patients combined was 74.1%, specificity was 69.4%, and accuracy was 71.8%. Table 3 shows the sensitivity, specificity, and accuracy of *i*-SCAN for the *in vivo* prediction of polyp histology. Test characteristics with all patients combined showed sensitivity of 72.2%, specificity of 71.4%, and accuracy of 71.8%.

In addition to evaluating differences in the accuracy of prediction with *i*-SCAN between endoscopists, we also assessed for the presence of a learning effect. Namely, we compared the accuracy of *i*-SCAN in the first and second half of polyps examined by each endoscopist. Endoscopist A increased his accuracy from 59.0% to 67.6% whereas Endoscopist B decreased his accuracy from 100% to 84.6%. These differences were not statistically significant.

DISCUSSION

In the current study, we did not detect a difference in the

diagnostic efficacy of *i*-SCAN and HDWL in determining small colorectal polyp histology during screening and surveillance colonoscopy. The observed accuracy of HDWL in this study (74.1%) was similar to other studies in the literature^[12-14]. This suggests that poor physician performance or effort was less likely to explain suboptimal results. Furthermore, both endoscopists showed a baseline high sensitivity rate using HDWL, thus decreasing room for additional improvement when *i*-SCAN was then applied. Endoscopist B in particular showed such high baseline sensitivity and specificity for adenoma prediction (88.9% and 100% respectively) using HDWL alone that any additional improvement of *i*-SCAN as a diagnostic modality was virtually impossible.

In general, digital chromoendoscopic techniques including Fujinon intelligent chromoendoscopy (FICE), narrow band imaging, and *i*-SCAN have been shown to be practical for *in vivo* differentiation between adenomatous and hyperplastic polyps, but the accuracy has ranged across the literature from 70 to over 90 percent^[8-10,15-21]. The accuracy of *i*-SCAN in our study (71.8%) was lower than we would have expected based on published results and below the accuracy needed for clinical application^[22]. Promising studies using *i*-SCAN thus far have reported up to 90% accuracy^[10]. In addition, Hoffman *et al*^[8] showed sensitivity of 82% (9/11 adenomas) and specificity of 96% (52/54 hyperplastic polyps) in the distal 30 cm of the colon.

Our finding may be explained by a number of factors. First, in both of the above studies, endoscopies were performed by a single operator experienced in real-time polyp diagnosis. Our endoscopists, both of whom are experienced faculty members at an academic institution, did not have prior experience with digital chromoendoscopic techniques nor with pit pattern analysis prior to this study and thus underwent training with *i*-SCAN and pit pattern recognition. A specific, validated method for training practitioners in *i*-SCAN use and pit pattern recognition has yet to be described. It is promising that training methods have been validated in other chromoendoscopic techniques and have shown to improve diagnostic accuracy and interobserver agreement^[23,24]. Our findings highlight the importance of training *i*-SCAN in a standardized fashion, not only for replication of published results but also for potential future application in a general practice setting.

Another possible explanation for our results rests in the fact that magnification was not used in the study. We felt that the undue increase in procedure time and sedation for our patients, as well as poor quality of stored high magnification images, did not merit using high magnification. However, there may be an important role for high magnification in terms of improving diagnostic efficacy in combination with digital chromoendoscopic technique. For example, Kim *et al*^[17] reported in 2011 that the most significant improvements in diagnostic efficacy were found with FICE in conjunction with high magnifi-

cation, with a difference in 80.4% accuracy without high magnification to 87.0% with high magnification. In fact, high magnification was particularly helpful when evaluating polyps less than 5 mm, which was the size of the majority of polyps in our analysis.

Finally, it should also be noted that studies have employed a number of endoscopic classification schemes in studying the usefulness of digital chromoendoscopy. These include the Kudo pit pattern classification, the Japanese Society for Cancer of Colon and Rectum criteria, and specific classification schemes developed by the investigators^[10,15,25]. It remains unclear how generalizable these classification schemes are, especially when using different virtual chromoendoscopic techniques. Even with other well-studied chromoendoscopic techniques, the importance of standardizing nomenclature for surface pattern characteristics and defining interobserver variability within individual techniques has been recognized^[26].

This study does have a number of limitations. First, the training offered to the endoscopists involved in the study was not standardized, and it is unclear to what extent results may have changed with more formal training. We did not detect a significant learning effect during the course of the study though our sample size was small. Secondly, the Kudo polyp classification system used in this study has not been specifically validated for histology prediction using *i*-SCAN, though several groups have utilized surface characterization patterns to aid polyp histology prediction^[8,10]. Further studies validating a specific polyp classification system using *i*-SCAN may be helpful in this regard. Thirdly, patients were not randomized to the two imaging modalities nor was there a cross-over design. As such, it is unlikely that the accuracy of *i*-SCAN would be worse than HDWL because the polyp was first evaluated in HDWL.

There has been continued interest in real-time prediction of polyp histology for a number of practical reasons including the avoidance of unnecessary polypectomy, reducing complication risks, and improving cost efficiency from a histopathologic standpoint. While there have been many promising studies using multiple digital chromoendoscopic techniques, including *i*-SCAN, our study did not identify a benefit to using *i*-SCAN to predict polyp histology. The markedly different accuracy rate between endoscopists strongly suggests that there are endoscopist factors that predict success with *in vivo* diagnosis, similar to how endoscopist factors may predict adenoma detection rates^[27]. Further understanding these factors will be important to help guide training before the widespread application of virtual chromoendoscopic techniques in clinical practice.

COMMENTS

Background

The majority of polyps detected and removed during screening colonoscopy are small polyps (less than 10 mm in size) that are unlikely to represent advanced

neoplasm or invasive carcinoma. Accurate methods to predict histopathology of small polyps *in vivo* could potentially prevent unnecessary polypectomies or encourage a cost-effective “resect and discard” strategy during screening colonoscopy. PENTAX *i*-SCAN, a digital chromoendoscopy technology, might aid *in vivo* prediction of polyp histology. In this study, they assessed the accuracy of *in vivo* histology prediction of small colonic polyps using *i*-SCAN as compared to high-definition white light, using formal histopathology as the gold standard comparison.

Research frontiers

Several small studies using *i*-SCAN have shown promising results in improving accuracy of *in vivo* polyp histology prediction. However, the reliance on experienced experts with non-validated diagnostic criteria limits generalizability of published results.

Innovations and breakthroughs

The authors found no significant difference between the accuracy of *i*-SCAN and high-definition white light, with high baseline accuracy using high-definition white light. Interestingly, they did find a significant difference in accuracy between endoscopists, regardless of imaging modality. This suggests an important role for individual endoscopist factors and experience.

Applications

Understanding endoscopist factors and standardizing training using *i*-SCAN may improve not only the ability to reproduce published results, but also the future possibility to apply these technologies in a general practice setting.

Terminology

PENTAX *i*-SCAN is a digital chromoendoscopy technology that uses post-processing computer algorithms integrated into the standard high-definition processor to modulate light reflected from mucosa. This highlights surface contrast by “virtual chromoendoscopy” technique, analogous to the way conventional chromoendoscopy highlights surface contrast using indigo carmine dye.

Peer review

This study provides potentially useful information for improving the clinical applications of *i*-SCAN. It explains that histologic assessment of polyps is high using white light, that interobserver variability is high, and that the new refinements probably facilitate the general use of tissue recognition but may not be essential for experienced endoscopists. Endoscopist training likely plays a critical role and deserves further study and standardization.

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Human thrombin for the treatment of gastric and ectopic varices

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Abstract

AIM: To evaluate the efficacy of human thrombin in the treatment of bleeding gastric and ectopic varices.

METHODS: Retrospective observational study in a Tertiary Referral Centre. Between January 1999-October 2005, we identified 37 patients who were endoscopically treated with human thrombin injection therapy for bleeding gastric and ectopic varices. Patient details including age, gender and aetiology of liver disease/segmental portal hypertension were documented. The thrombin was obtained from the Scottish National Blood Transfusion Service and prepared to give a solution of 250 IU/mL which was injected *via* a standard injection needle. All patient case notes were reviewed and the total dose of thrombin given along with the number of endoscopy sessions was recorded. Initial haemostasis rates, rebleeding rates and mortality were catalogued along with the incidence of any immediate complications which could be attributable to the thrombin therapy. The duration of follow up was also listed. The study was conducted according to the United Kingdom research ethics guidelines.

RESULTS: Thirty-seven patients were included. 33

patients (89%) had thrombin (250 U/mL) for gastric varices, 2 (5.4%) for duodenal varices, 1 for rectal varices and 1 for gastric and rectal varices. (1) Gastric varices, an average of 15.2 mL of thrombin was used per patient. Re-bleeding occurred in 4 patients (10.8%), managed in 2 by a transjugular intrahepatic portosystemic shunt (TIPSS) (one unsuccessfully who died) and in other 2 by a distal splenorenal shunt; (2) Duodenal varices (or type 2 isolated gastric varices), an average of 12.5 mL was used per patient over 2-3 endoscopy sessions. Re-bleeding occurred in one patient, which was treated by TIPSS; and (3) Rectal varices, an average of 18.3 mL was used per patient over 3 endoscopy sessions. No re-bleeding occurred in this group.

CONCLUSION: Human thrombin is a safe, easy to use and effective therapeutic option to control haemorrhage from gastric and ectopic varices.

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Key words: Variceal haemorrhage; Ectopic Varices; Gastric varices; Portal hypertension; Thrombin

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INTRODUCTION

Haemorrhage from gastric or ectopic varices is associated with high morbidity and mortality and can account for up to one third of all cases of variceal haemorrhage^[1]. In

the presence of oesophageal varices, the prevalence of gastric varices ranges from 15% to 100%^[2,3] with the risk of bleeding generally regarded to range from 4%-65% over the first 2 years after diagnosis^[2,4]. More importantly it has been reported that although gastric varices are less likely to bleed than oesophageal varices, once they bleed, they tend to do so more severely and haemostasis can be difficult to achieve^[2].

Unlike oesophageal variceal haemorrhage, bleeding from gastric varices has not been extensively studied. The natural history of bleeding gastric varices differs from that of oesophageal varices and thus the precipitating event for gastric variceal haemorrhage remains uncertain. Predictive factors for oesophageal variceal haemorrhage such as a hepatic venous pressure gradient (HVPG) of > 20 mmHg^[5] is not felt to be as relevant to gastric variceal bleeding and this is partly attributed to the development of gastrosplenic shunts. Indeed we have previously demonstrated that a portal pressure gradient (PPG) of < 12 mmHg does not necessarily protect against gastric variceal bleeding and that a PPG < 7 mmHg is a better safeguard against rebleeding^[6]. The variceal size, tortuosity and stigmata of recent bleeding such as red signs however remain alarming features^[4].

Thrombin was first used for the management of gastric varices in 1947^[7] and affects haemostasis by converting fibrinogen to a fibrin clot. It also has other influences on the coagulation system with one effect being the enhancement of local platelet aggregation. Endoscopic treatment with thrombin has been reported in the treatment of bleeding oesophageal, gastric and duodenal varices^[8-10] with a low rate of rebleeding. The most recent study from Ramesh *et al*^[11] reported that haemostasis was achieved in the acute setting in 92% patients presenting with bleeding gastric varices, with only one patient requiring a transjugular intrahepatic portosystemic shunt (TIPSS) to control bleeding. The majority of these studies are however retrospective and include small patient numbers.

The aim of this study is therefore to evaluate the use of human thrombin in the treatment of gastric and ectopic varices.

MATERIALS AND METHODS

We identified 37 consecutive patients who were treated with human thrombin (Scottish National Blood Transfusion Service) from January 1999-October 2005 for isolated bleeding from gastric and ectopic varices. Thrombin was injected rather than cyanoacrylate as this was our Units protocol. The case notes were reviewed and total volume of thrombin used and the incidence of complications recorded, as was the incidence of re-bleeding or death. Those patients with bleeding oesophageal varices who underwent banding of varices or any patient in which there was diagnostic doubt as to the aetiology of bleeding were excluded. The study was conducted ac-

ording to the United Kingdom research ethics guidelines. Following consideration by the local ethics committee, further specific ethical review and approval was not required, as the study was considered a retrospective audit using anonymised data obtained as part of usual patient care.

Patient characteristics

Twenty-eight of the patients were male (male:female ratio = 28:9) with a mean age at presentation of 53.2 years (range: 18-83 years). The underlying aetiology was alcoholic liver disease in 15 patients, splenic vein thrombosis in 6, cryptogenic cirrhosis in 6, primary biliary cirrhosis in 2, chronic active hepatitis in 2, portal vein thrombosis in 2, primary sclerosing cholangitis in 2, α 1-antitrypsin deficiency in 1, congenital hepatic fibrosis in 1 and hepatitis C in 1 patient. The Childs-Pugh grade: grade A = 5 patients, grade B = 16 patients and grade C = 10 patients. Segmental portal hypertension was defined as extrahepatic portal hypertension in the absence of liver cirrhosis and was seen in 6 patients whose underlying aetiology was splenic vein thrombosis.

Endoscopic therapy

All patients had an upper gastrointestinal endoscopy/flexible sigmoidoscopy performed by an experienced operator within 12 h of presentation. Gastric variceal haemorrhage was defined as visible spurting or oozing of blood from the lesser curve or fundal vessels at the time of endoscopy with varices subdivided into fundal and non fundal. Sarin's classification for gastric varices was used but it was noted that it is often difficult to differentiate the types of fundal varices in patients who are actively bleeding. The gastric and duodenal varices were also considered to have bled if there were stigmata of recent bleeding such as red spots or adherent clot. Rectal variceal haemorrhage was defined by the presence of rectal varices with either adherent clot or visible active bleeding combined with a history of profuse fresh blood loss per rectum.

In those patients with splenic vein thrombosis, an alternative therapy such as splenectomy may be considered by some but it is important that bleeding is controlled and therefore all these patients underwent endoscopy and stabilization of bleeding prior to consideration for splenectomy.

Protocol for thrombin therapy

All patients were adequately resuscitated at the time of endoscopy. Human thrombin concentrate obtained from the Scottish National Blood Transfusion Service and each vial was reconstituted with 5 mL of water to give a concentration of 250 U/mL. As thrombin was being used outwith its licensed use, informed written consent was obtained from each patient prior to endoscopy. The thrombin was injected directly into the varices using a standard injection sclerotherapy needle to a maximum

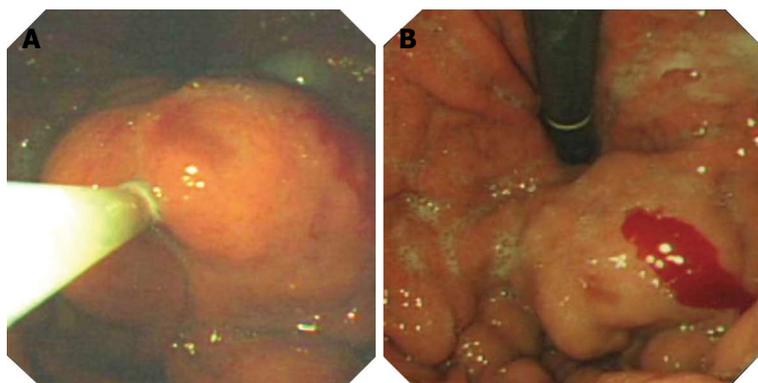


Figure 1 Injection of gastric varices with thrombin. A: During thrombin injection the gastric varix swells; B: Post thrombin injection.

volume of 10 mL at any one session by multiple injections. Repeat endoscopy was arranged initially for one week then at two weekly intervals until further injection was deemed unnecessary by the endoscopist as the overlying mucosa had returned to normal in which the varix appeared well covered with no stigmata of recent haemorrhage. The number of endoscopy sessions, the total volume of thrombin used and the incidence of complications were documented.

RESULTS

Thirty-three patients (89%) had thrombin for gastric varices, two (5.4%) for duodenal varices, one for rectal varices and one for gastric and rectal varices. A small number of patients in this cohort were in our original pilot study^[12]. Twenty seven patients (82%) also had oesophageal varices with 19 patients (58%) already in a banding programme. Only 3 patients were on beta-blocker therapy prior to admission. The average total volume of thrombin used per patient was 15.2 mL (range: 5-47.5 mL, combined rectal and gastric varices) over 1-7 endoscopy sessions (median 3.6 sessions).

For those patients with gastric varices, 82% were classified as gastro-oesophageal varices (GOV) type 2 with the remainder being classified as isolated gastric varices (IGV) type 1 (12%) and type 2 (6%). In two cases where it was not absolutely clear whether they were GOV type 1 or type 2 they were included as type 2. An average of 15.2 mL of thrombin was used per patient (Figure 1). Re-bleeding occurred in four patients (10.8%), three of which bled before the 2nd endoscopy session (i.e., within 7 d of index bleed) and two of whom were managed by TIPSS. One of the TIPSS procedures was unsuccessful and the patient subsequently died after a rebleed. The two other patients were successfully treated by distal splenorenal shunt.

For those two patients with duodenal varices, an average of 12.5 mL was used per patient over 2-3 endoscopy sessions. Re-bleeding occurred at day 3 in one patient which was treated successfully by TIPSS insertion.

For the patient with rectal varices, an absolute volume

of 18.3 mL was used over three endoscopy sessions. No re-bleeding occurred in this group.

Only a small proportion of patients (15%) had additional therapy with vasopressor agents, the use of which did not change outcome. All patients with liver disease received five days of intravenous antibiotics as per our units protocol. In addition, true eradication of varices was rare with varices deemed visually eradicated in only two patients. No HVPG measurements were obtained in any patient as this is not our Units protocol. Overall mortality was 2.7% after median follow up of 22 mo.

Complications

No clinically significant adverse events occurred following thrombin therapy with median follow up of 22 mo.

DISCUSSION

Gastric varices are generally classified by their location in the stomach and their relationship with oesophageal varices, as proposed by Sarin *et al*^[21]. GOV is the term used to describe gastric varices that are associated with oesophageal varices and are classified as either type 1 or type 2. IGV is used to refer to gastric varices that occur independently of oesophageal varices. It is observed that type 1 GOV (which are primarily supplied by the left gastric vein) represent 75% of all gastric varices observed. However it is generally regarded that the most serious haemorrhage occurs when type 1 IGV gastric varices (primarily supplied by the short gastric vein) bleed.

Various treatment options exist for the management of bleeding gastric varices and include endoscopic methods, TIPSS and other radiological procedures. Surgical procedures such as under running of gastric varices or devascularization procedures has previously been used but in the small studies performed have shown no great benefit and thus are rarely performed today.

Although there is debate regarding optimum treatment of gastric variceal haemorrhage, endoscopic therapy is an established treatment and it is currently recommended in the United Kingdom to be the first line treatment in the management of acute gastric variceal

haemorrhage^[13]. Endoscopic treatment options include standard sclerotherapy, band and snare ligation or endoscopic injection with cyanoacrylate or thrombin.

Standard sclerotherapy using ethanolamine as the sclerosing agent has been used with moderate success in the treatment of bleeding oesophageal varices but has limited success in the treatment of bleeding gastric varices. It is widely accepted that sclerotherapy for gastric varices requires significantly greater volumes of sclerosant^[2] which is thought to account for the increased number of side effects that are observed^[14]. The initial haemostasis rates obtained when using sclerotherapy have been reported to vary widely from 26%-100%^[11-16] which may reflect different operator ability and injection techniques. However a rebleeding rate of 60%-90% has been reported in most studies which is generally observed in patients with bleeding fundal varices^[1,17]. The majority of the rebleeding episodes have been reported to be related to ulceration at the injection site.

Endoscopic injection therapy with tissue adhesives such as cyanoacrylate has also been shown to be of benefit in the management of bleeding gastric varices and is becoming more popular due to the high rebleeding rates observed with sclerotherapy. A 90% success rate in achieving initial haemostasis has been reported in a number of non randomised studies^[18-20] but Raymond *et al*^[21] also reported a rebleeding rate of 50%. Several studies have also demonstrated that the use of tissue adhesives is superior to standard sclerotherapy. Oho *et al*^[3] have published results of a controlled but non-randomised study which demonstrated that initial control of gastric variceal bleeding and survival was significantly greater in patients treated with cyanoacrylate than standard sclerotherapy. Sarin *et al*^[22] have published results for a small randomised control trial which again suggested that the use of cyanoacrylate was superior to standard sclerotherapy. Complications rates between the two procedures have been reported to be similar but of course this depends primarily on the expertise available. In the hands of inexperienced operators, tissue adhesives can cause irreparable damage to the endoscope. In addition, a few centres have reported the occurrence of systemic emboli after the use of tissue adhesives to control variceal haemorrhage^[23,24].

Gastric variceal band ligation using 'O' rings and detachable snares have also been used in the management of gastric variceal haemorrhage but with high rebleeding rates being observed^[25]. Yoshida *et al*^[26,27] have however published data on the combined use of the detachable snare and simultaneous injection sclerotherapy and O-ring ligator. In a study of 43 patients^[26], 97% had eradication of gastric varices with an 8% rebleeding rate observed within a 2 year follow up period.

The optimal treatment for the management of gastric varices therefore remains uncertain but as our results suggest, a promising therapeutic option is the use of intravariceal injection of thrombin. The use of thrombin

in bleeding gastric or ectopic varices has only previously been reported in a handful of studies in which a variety of thrombin preparations have been used. To our knowledge, this study is currently the largest published study using human thrombin.

For the management of bleeding gastric varices, our rates of achieving initial haemostasis were in keeping with 93% published by Datta *et al*^[10] and 100% by Williams *et al*^[9]. Our rebleed rate of 14% was again in keeping with data published by Williams *et al*^[9] but it should however be recorded that the median follow up time in these studies varied greatly from six weeks to nine mo. It should also be documented that this rebleed rate was observed without us actively attempting to visually eradicate the varices and may suggest that continued thrombin injection to achieve visual eradication appears unnecessary. This point was emphasised when we examined the number of endoscopic sessions in which thrombin was given as initially the procedure was performed out to 7 sessions. It was only after analysis of these pilot data that rebleeding was deemed extremely rare after 3 endoscopic sessions and that eradication of gastric varices was unnecessary. It is therefore now our Units adopted policy that patients should be treated with thrombin for 3 endoscopic sessions. Overall, our mortality rate of 2.7% highlights how effective thrombin therapy can be, but it should be recorded that these results were obtained after 5 patients who rebleed underwent further interventions: namely TIPSS or splenorenal shunt surgery.

The thrombin used in this study was obtained free of charge from Blood Transfusion Service but we have now changed to using a recombinant thrombin preparation at cost of £250 for concentration of 250 IU/mL. This is comparable to the cost of cyanoacrylate and the cost of TIPSS (at £5000).

Currently, no randomised controlled trials comparing thrombin with tissue adhesives or sclerotherapy have been performed. Interestingly however, Kojima *et al*^[28] have published results for 30 patients with bleeding fundal varices that underwent sclerotherapy with ethanolamine under fluoroscopic guidance with the injection site being sealed with topical thrombin glue. All participants also received intravenous vasopressin and transdermal nitroglycerin. They reported an initial haemostasis rate of 93% with a rebleed rate of 19% after 5 years of follow up. The efficacy of the topical thrombin is however difficult to determine and the specialized technique and equipment required for this procedure may limit its future application.

With regards to the safety of human thrombin, we did not record any complications of thrombin use and this is again in keeping with all of the previously published studies that have used human thrombin. Complications such as anaphylaxis or altered thrombogenesis that have previously been reported with the use of bovine thrombin did not occur^[29].

Although in this study we have not monitored the

effect of thrombin by any means, we have evidence that results can be further improved by assessing clot formation with other means such as endoscopic ultrasound^[30].

In conclusion, We have shown that human thrombin is a safe, easy to use and effective therapeutic option in the management of bleeding gastric and ectopic varices. Our study also suggests that continued thrombin injection to achieve visual eradication appears unnecessary. Larger randomised control trials are necessary to compare the use of human thrombin with the current available therapeutic modalities.

COMMENTS

Background

Haemorrhage from gastric or ectopic varices is associated with high morbidity and mortality and can account for up to one third of all cases of variceal haemorrhage.

Research frontiers

Unlike oesophageal variceal haemorrhage, bleeding from gastric varices has not been extensively studied. The natural history of bleeding gastric varices differs from that of oesophageal varices and thus the precipitating event for gastric variceal haemorrhage remains uncertain.

Innovations and breakthroughs

Initial haemostasis rates, rebleeding rates and mortality were catalogued along with the incidence of any immediate complications which could be attributable to the thrombin therapy. The duration of follow up was also listed. The study was conducted according to the United Kingdom research ethics guidelines.

Applications

Human thrombin is a safe, easy to use and effective therapeutic option in the management of bleeding gastric and ectopic varices.

Peer review

The results of a retrospective study concerning 37 patients, who were treated with thrombin injection for bleeding gastric or ectopic varices. Their major finding is that in patients with gastric varices this treatment is effective. The authors conclude that thrombin injection may be used to treat patients with gastric or ectopic variceal bleeding.

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High-fibre diet and *Lactobacillus paracasei* B21060 in symptomatic uncomplicated diverticular disease

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Abstract

AIM: To investigate in symptomatic uncomplicated diverticular disease the efficacy of symbiotics associated with a high-fibre diet on abdominal symptoms.

METHODS: This study was a multicentre, 6-mo randomized, controlled, parallel-group intervention with a preceding 4-wk washout period. Consecutive outpatients with symptomatic uncomplicated diverticular disease, aged 40-80 years, evaluated in 4 Gastroenter-

ology Units, were enrolled. Symptomatic uncomplicated diverticular disease patients were randomized to two treatment arms A or B. Treatment A ($n = 24$ patients) received 1 symbiotic sachet Flortec[®] (*Lactobacillus paracasei* B21060) once daily plus high-fibre diet for 6 mo. Treatment B ($n = 21$ patients) received high-fibre diet alone for 6 mo. The primary endpoint was regression of abdominal symptoms and change of symptom severity after 3 and 6 mo of treatment.

RESULTS: In group A, the proportion of patients with abdominal pain < 24 h decreased from 100% at baseline to 35% and 25% after 3 and 6 mo, respectively ($P < 0.001$). In group B the proportion of patients with this symptom decreased from 90.5% at baseline to 61.9% and 38.1% after 3 and 6 mo, respectively ($P = 0.001$). Symptom improvement became statistically significant at 3 and 6 mo in group A and B, respectively.

The proportion of patients with abdominal pain > 24 h decreased from 60% to 20% then 5% after 3 and 6 mo, respectively in group A ($P < 0.001$) and from 33.3% to 9.5% at both 3 and 6 mo in group B ($P = 0.03$). In group A the proportion of patients with abdominal bloating significantly decreased from 95% to 60% after 3 mo, and remained stable (65%) at 6-mo follow-up ($P = 0.005$) while in group B, no significant changes in abdominal bloating was observed ($P = 0.11$). After 6 mo of treatment, the mean visual analogic scale (VAS) values of both short-lasting abdominal pain (VAS, mean \pm SD, group A: 4.6 ± 2.1 vs 2.2 ± 0.8 , $P = 0.02$; group B: 4.6 ± 2.9 vs 2.0 ± 1.9 , $P = 0.03$) and abdominal bloating (VAS, mean \pm SD, group A: 5.3 ± 2.2 vs 3.0 ± 1.7 , $P = 0.005$; group B: 5.3 ± 3.2 vs 2.3 ± 1.9 , $P = 0.006$) decreased in both groups, whilst the VAS values of prolonged abdominal pain decreased in the Flortec[®] group, but remained unchanged in the high-fibre diet group (VAS, mean \pm SD, group A: 6.5 ± 1.5 vs 4.5 ± 2.1 , $P = 0.052$; group B: 4.5 ± 3.8 vs 5.5 ± 3.5).

CONCLUSION: A high-fibre diet is effective in relieving

abdominal symptoms in symptomatic uncomplicated diverticular disease. This treatment may be implemented by combining the high-fibre diet with Flortec[®].

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Key words: Symptomatic uncomplicated diverticular disease; *Lactobacillus paracasei* B21060; Probiotics; Symbiotics; Diverticular disease; High-fibre diet

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INTRODUCTION

Diverticular disease (DD) of the colon is a very common disorder which remains asymptomatic in nearly 80% of patients. The remaining patients develop recurrent abdominal symptoms and some complications, such as diverticulitis and bleeding, requiring hospital admission and surgery^[1-3]. The main goals of symptomatic DD management are both relief of abdominal symptoms and prevention of acute diverticulitis^[4].

The standard therapeutic approach for symptomatic uncomplicated DD still remains to be defined. Guidelines of the American College of Gastroenterology, the European Association for Endoscopy Surgery, and the World Gastroenterology Organization recommend a high-fibre diet in patients with symptomatic uncomplicated DD^[5,6]. Some data would suggest that cyclic treatment with nonabsorbable antibiotics plus high-fibre diet is more effective in obtaining symptom relief as compared to diet alone^[7,8], and it reduces the incidence of first episode of acute diverticulitis at 1 year^[9]. However, the level of evidence of superiority of nonabsorbable antibiotics over dietary fibre or fibre supplementation is poor^[10], and both the cost and efficacy of a long-life cyclic treatment with nonabsorbable antibiotics to prevent diverticulitis in all symptomatic DD patients has been questioned^[11,12].

A recent systematic review suggest the potential usefulness of fibre, rifaximin, mesalazine, and probiotics, and their possible combination in symptomatic uncomplicated DD treatment, but reliable controlled therapeutic trials are still lacking^[12].

Probiotics, prebiotics, and symbiotics may modify

the gut microbial balance leading to health benefits^[13-16]. Changes in peri-diverticular bacterial flora have been suggested as a potential key step in the pathogenesis of diverticular microscopic inflammation. This, in turn, may play a role in generating abdominal symptoms in uncomplicated DD, thus making probiotics an appealing therapy for DD. Some data suggest that probiotic therapy is safe and potentially useful in the management of DD patients^[17]. Flortec[®] is a totally natural symbiotic agent, consisting of the synergistic combination of *Lactobacillus paracasei* (*L. paracasei*) B21060 (probiotic component) and arabinogalactan/xylooligosaccharides (prebiotic component). Flortec[®] treatment has been shown to be effective in relieving symptoms associated with irritable bowel syndrome^[18], and in the treatment of acute diarrhea in adults treated at a primary care setting^[19]. The therapeutic benefit of this symbiotic formulation in addition to a high dietary fibre intake in symptomatic uncomplicated DD remains to be defined. The primary aim of this cluster randomized study was to investigate the efficacy of a patented symbiotic preparation containing *L. paracasei* B21060 in association with high-fibre diet compared to high-fibre diet alone on relief of abdominal symptoms in patients with symptomatic uncomplicated DD.

MATERIALS AND METHODS

Study population

Consecutive outpatients were evaluated in 4 Gastroenterology Units (1 academic and 3 nonacademic) for enrolment in the study. Inclusion criteria were a well-established diagnosis of symptomatic uncomplicated DD and age ranging from 40 to 80 years. The study was performed over a 10 mo period from March, 2010 to January, 2011.

Symptomatic uncomplicated DD was defined as the presence of colonic diverticula associated with abdominal pain and/or bloating for at least 6 mo before recruitment, without signs of acute diverticulitis^[20]. Signs of acute inflammation were excluded by physical examination (to ascertain the absence of abdominal rigidity, rebound tenderness, and/or guarding in one or more abdominal quadrants), as well as routine biochemistry (complete blood count, erythrocyte sedimentation rate, C-reactive protein, protein electrophoresis). To quantify and localize the colonic diverticula, double contrast enema and/or colonoscopy was performed. Exclusion criteria were: presence of less than 5 diverticula, recent history (< 3 mo) or actual clinical evidence of acute diverticulitis, previous colonic surgery, antibiotics, mesalazine, nonsteroidal anti-inflammatory drugs or laxative use during the four weeks before enrolment, coexisting inflammatory bowel disease, diseases with possible small intestine bacterial overgrowth. Patients were also excluded if dyspeptic symptoms were predominant over abdominal symptoms and when low compliance or motivation could be expected for any reason. All patients provided written informed consent.

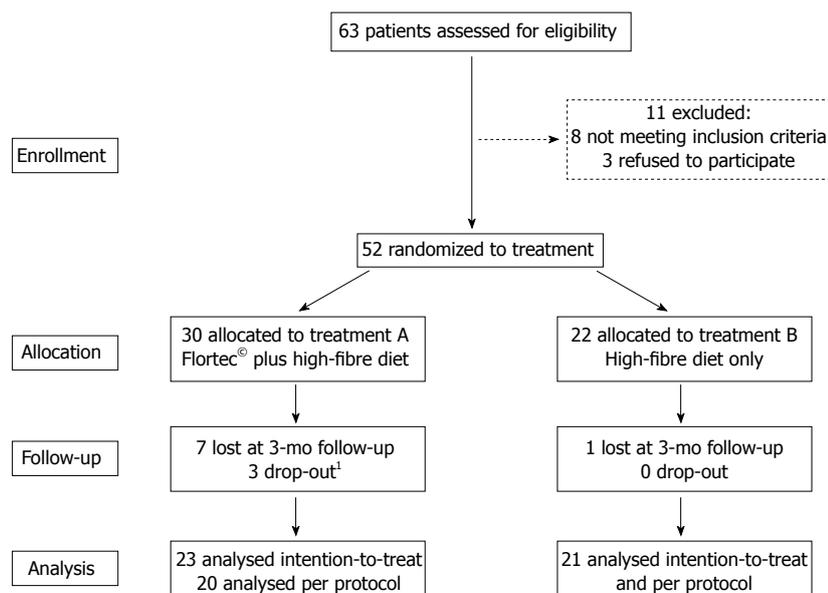


Figure 1 Flowchart of the patients throughout the study. ¹3 patients dropped out after 3 mo of treatment, 1 patient for new onset of constipation and 2 patients for worsening of abdominal symptoms.

Study design

This study was a multicentre, 6-mo randomized, controlled, parallel-group intervention with a preceding 4-wk washout period. All patients were instructed to follow a high-fibre diet containing at least 30 g daily intake of dietary fibre as well as a daily water intake of at least 1.5 L. For this purpose, all patients were given an information sheet regarding the content of dietary fibre in commonly consumed fruits, vegetables and cereals, and dietary counselling was performed. According to cluster randomization^[21], each participating centre was randomly assigned to recruit patients for either treatment A or B. For 6 mo, treatment arm A received a once daily dose of the symbiotic preparation Flortec[®] administered orally, plus high-fibre diet, while treatment arm B was treated with high-fibre diet only (Figure 1). Rescue medication was not allowed during the study period.

All patients underwent 3 clinical interviews: at study entry and after 3 and 6 mo of intervention. Patients were evaluated for abdominal symptoms, compliance to therapy assessed by a structured questionnaire, and routine biochemistry (complete blood count, erythrocyte sedimentation rate, C-reactive protein, protein electrophoresis) was done to exclude signs of acute inflammation. In order to assess compliance to the high-fibre diet and to verify eventual changes in dietary fibre intake, at study entry and 3- and 6-mo follow-up clinical interviews, the daily fibre intake during the 7 d before the interview was recorded (a semiquantitative score ranging from 0–28 was used: for each day of the week max 4 points were assigned: 1 point for intake of fruit and another point for intake of vegetables or whole grain cereals at lunch and/or dinner). The primary endpoint considered was the regression of abdominal symptoms and change in symptom severity after 3 and 6 mo of treatment. As a secondary endpoint the tolerability of treatment - i.e., oc-

currence of adverse effects was considered.

Symptom assessment

Symptoms of patients were evaluated at study entry and after 3 and 6 mo of treatment by assessing the presence/absence and intensity of abdominal pain lasting more or less than 24 h and the presence/absence and intensity of abdominal bloating^[19,21]. Patients were asked to grade the intensity of abdominal symptoms on a visual analogic scale (VAS) consisting of a 10 cm long line with 0 cm indicating “no sensation” and 10 cm indicating “the strongest sensation ever felt”.

Symbiotic preparation, Flortec[®]

Flortec[®] (Bracco Co, Milan, Italy) is a composite symbiotic formulation and each 7 g sachet contains 5×10^9 colony-forming units viable lyophilized *L. paracasei* B12060. The dry powder bacteria were mixed with the following excipients: xylo-oligosaccharides (700 mg), glutamine (500 mg), and arabinogalactone (1243 mg). As glutamine and oligosaccharides have some prebiotic activities on human fecal flora, the Flortec formulation combines the synergistic effect of a prebiotic with a probiotic (a symbiotic formulation). The study preparation was in powder form. Patients were instructed to store the preparation at room temperature (< 20 °C) in a dry place and to dissolve the powder preparation in 100 mL of water once daily and to ingest it immediately 2 h after lunch.

Statistical analysis

The sample size was calculated considering data reported in literature: we expected that dietary fibre supplementation would be effective in 30% of cases, accepting a range from 15% to 45% (5, 12) s Because the combined efficacy of high-fibre diet and symbiotic supplementation is not known in literature, for this pilot study a superiority

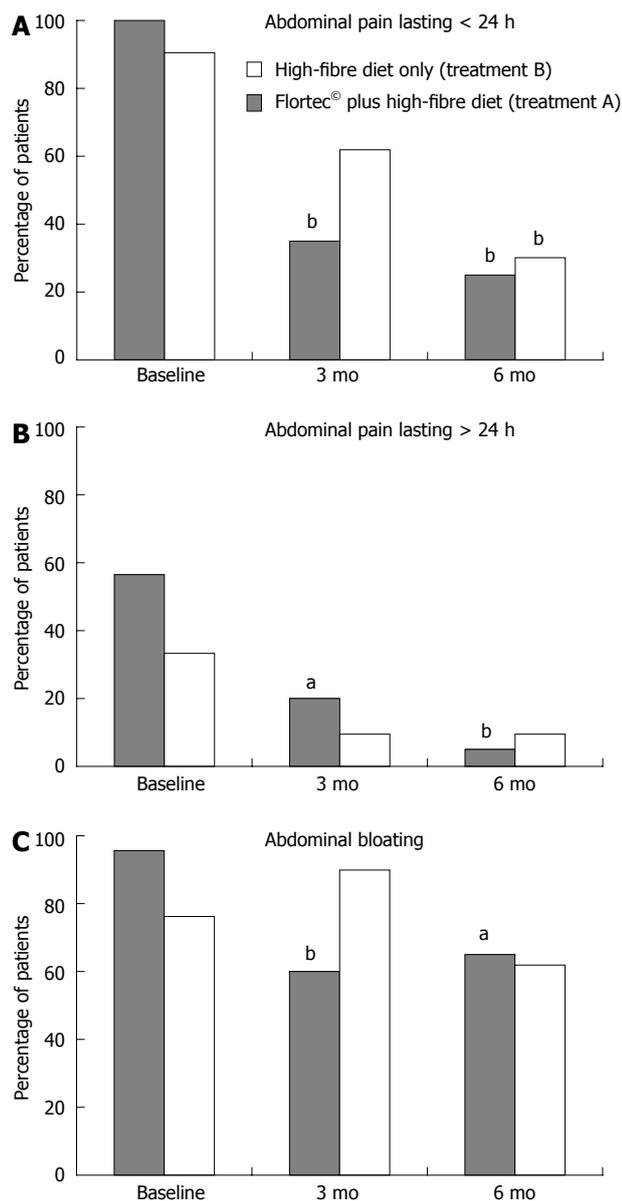


Figure 2 Change of abdominal symptoms after treatment according to intention-to-treat analysis. A: Abdominal pain lasting < 24 h; B: Abdominal pain lasting > 24 h; C: Abdominal bloating. ^a $P < 0.05$, ^b $P < 0.01$ vs baseline.

of about 30% for the second treatment arm over the first one was supposed, and a total of 50 cases (25 for each arm) were needed, with an α error of 10% and a study power of 80%.

The analysis was carried out on the intention-to-treat (ITT) population, defined as all randomized patients who performed at least one follow-up assessment after baseline, and on the per-protocol population, defined as all patients who completed the prescribed treatment in the 6-mo-treatment period. The presence of abdominal symptoms was expressed as number (%) of total patients and in terms of severity as mean \pm SD of VAS. Data were analysed by Fisher's exact and/or Student's *t*-test. To test for differences between the baseline, 3- and 6-mo sets of proportion of patients presenting abdominal pain or bloating, Cochran's *Q* test was performed. The results

Table 1 Demographic characteristics and baseline symptoms

	High-fibre diet plus probiotics (<i>n</i> = 30)	High-fibre diet alone (<i>n</i> = 22)	<i>P</i> value
Demographics			
Age, yr	68.1 \pm 8.6	63.8 \pm 10.3	0.10
Gender, female	22 (73.3)	13 (59.1)	0.43
Body mass index	26.4 \pm 2.9	24.9 \pm 2.9	0.07
Smoking habit	12 (40.0)	11 (50.0)	0.66
Alcoholic drinks	9 (30.0)	11 (50.0)	0.24
Coffee	28 (93.3)	20 (90.9)	0.84
Localization of colon diverticula			
Left colon	27 (90.0)	20 (90.9)	0.49
Left and right	3 (10.0)	2 (9.1)	0.28
colon			
Symptoms			
Dyspeptic symptoms	3 (10.0)	3 (13.6)	0.97
Abdominal pain	28 (93.3)	20 (90.9)	0.48
lasting < 24 h			
VAS	4.6 \pm 2.2	4.6 \pm 2.8	0.97
Abdominal pain	14 (46.7)	8 (36.4)	0.49
lasting > 24 h			
VAS	6.7 \pm 1.7	5.2 \pm 2.8	0.12
Abdominal bloating	25 (83.3)	17 (77.2)	0.49
VAS	5.4 \pm 2.2	5.2 \pm 3.1	0.80

VAS: Visual analog scale. Data are presented by mean \pm SD or *n* (%).

were coded 0 for absence and 1 for presence of abdominal symptoms. The compliance to high-dietary fibre intake was assessed by analysis of variance. The *P* values were considered significant if they were less than 0.05. The statistical analyses were carried out using a dedicated software package (MedCalc Software, Mariakerke, Belgium, version 10.1.2).

RESULTS

Baseline characteristics

Of the 52 randomized patients with symptomatic uncomplicated DD (35 females, mean age 66.3 ± 9.5 years), at baseline 48 (92.3%) had abdominal pain lasting less than 24 h, 22 (42.3%) had abdominal pain lasting more than 24 h, and 42 (80.8%) had abdominal bloating, whereas dyspeptic symptoms were present in only 6 (11.5%) patients. Demographic and clinical characteristics of patients are given in Table 1. No differences between the treatment groups were observed with respect to baseline characteristics and gastrointestinal symptoms. The dietary fibre intake score was not statistically different between groups (13.3 ± 7.3 vs 16.0 ± 9.1 , $P = 0.30$).

The flowchart in Figure 1 shows the progress of patients from recruitment until the end of the study. Of the 52 randomized patients, 30 (57.7%) were allocated to the Flortec[®] plus high-fibre diet group (group A) and 22 (42.3%) to the high-fibre diet group (group B). Eight patients were lost at 3-mo follow-up, and, therefore, 44 patients were included in the ITT population. In group A, 3 patients dropped out after 3 mo of treatment, 1 patient for new onset of constipation and 2 patients for worsening of abdominal symptoms, while in group B all 21

patients completed the 6-mo treatment period. Thus, the PP population consisted of 41 patients.

Compliance to high-fibre diet

At baseline, 3- and 6-mo evaluation, the dietary fibre intake scores were not different between the Flortec[®] group and the high-fibre diet group. In particular, in group A patients, the dietary fibre intake score was 16 ± 9.1 at baseline (17.9 ± 7.3 vs 16 ± 9.1 , $P < 0.01$ at 3 mo; 18.3 ± 7 vs 16 ± 9.1 , $P < 0.01$ at 6 mo). In group B patients, this score increased from 13.3 ± 7.3 at baseline to 18.4 ± 6.1 at 3 mo ($P < 0.0001$) and to 21.4 ± 4.5 at 6 mo ($P < 0.0001$). Dietary fibre intake similarly increased in both groups over the study period ($P = 0.702$).

Efficacy of treatment

As shown in Figure 2, in group A the proportion of patients with abdominal pain lasting less than 24 h significantly decreased from 100% at baseline to 35% after 3 mo and to 25% after 6 mo of treatment ($P < 0.001$ by Cochran's Q test). In group B the proportion of patients with this symptom decreased from 90.5% at baseline to 61.9% after 3 mo and to 38.1% after 6 mo of treatment ($P = 0.001$ by Cochran's Q test). The symptom improvement became statistically significant at 3 and 6 mo in groups A and B, respectively.

In group A, the proportion of patients with abdominal pain lasting more than 24 h (Figure 2) significantly decreased from 60% at baseline to 20% after 3 mo and further decreased to 5% after 6 mo of treatment ($P < 0.001$ by Cochran's Q test). In group B, the proportion of patients with prolonged abdominal pain significantly decreased from 33.3% at baseline to 9.5% after 3 mo, and remained stable (9.5%) at 6-mo follow-up ($P = 0.03$ by Cochran's Q test).

As shown in Figure 2, in group A the proportion of patients with abdominal bloating significantly decreased from 95% to 60% after 3 mo, and remained stable (65%) at 6-mo follow-up ($P = 0.005$ by Cochran's Q test). In group B, no significant changes in abdominal bloating was observed, the proportion of patients complaining such a symptom being 76.2%, 80.9%, and 61.9% at entry, 3-, and 6-mo follow-up, respectively ($P = 0.11$ by Cochran's Q test).

In the high-fibre diet group, 3 patients described the new onset of abdominal symptoms during the study period; 1 patient experienced prolonged abdominal pain and 2 patients abdominal bloating, whilst no onset of new symptoms occurred in the Flortec[®] group.

After 6 mo of treatment, the mean VAS values of both short-lasting abdominal pain (VAS , mean \pm SD, group A: 4.6 ± 2.1 vs 2.2 ± 0.8 , $P = 0.02$; group B: 4.6 ± 2.9 vs 2.0 ± 1.9 , $P = 0.03$) and abdominal bloating (VAS , mean \pm SD, group A: 5.3 ± 2.2 vs 3.0 ± 1.7 , $P = 0.005$; group B: 5.3 ± 3.2 vs 2.3 ± 1.9 , $P = 0.006$) decreased in both groups, whilst the VAS values of prolonged abdominal pain decreased in the Flortec[®] group, but remained unchanged in the high-fibre diet group (VAS ,

mean \pm SD, group A: 6.5 ± 1.5 vs 4.5 ± 2.1 , $P = 0.052$; group B: 4.5 ± 3.8 vs 5.5 ± 3.5).

None of the patients developed altered biochemical inflammatory parameters, acute diverticulitis or other diverticular disease complications throughout the 6-mo study period. In both groups no adverse event was registered over the 6-mo treatment period.

DISCUSSION

High-fibre diet is largely suggested for symptomatic uncomplicated DD patients^[5,6]. Probiotic therapy may be of benefit in DD patients, but its efficacy when combined with high-fibre diet remains to be established. This pilot study investigated the efficacy of a continuous 6-mo treatment with a symbiotic preparation containing *L. paracasei* B21060 associated with a high-fibre diet compared to a high-fibre diet alone in patients with symptomatic uncomplicated DD. The main findings of this study were that (1) a high-fibre diet alone is effective on some abdominal symptoms of symptomatic uncomplicated DD patients, but the combination of this approach with a symbiotic preparation containing *L. paracasei* B21060 allows an increase in the therapeutic response; and (2) the prescription of a high-fibre diet increases the intake of dietary fibre over time, regardless of whether a single diet or combined approach with symbiotic supplementation is used.

In detail, the high-fibre diet alone was effective in reducing short-lasting abdominal pain following 6 mo of treatment, but using the combined approach with Flortec[®] a regression of this symptom was already observed after 3 mo. With the dietary approach alone regression of prolonged abdominal pain was observed ($P = 0.03$), but this therapeutic response was more accentuated with the combined treatment strategy. Finally, abdominal bloating significantly regressed only with the symbiotic treatment, while high-fibre diet alone had no beneficial effect on this symptom. Taken together, these findings show that the combined approach offers an advantage over the dietary approach alone in improving the therapeutic response of patients with symptomatic uncomplicated DD with regard to abdominal symptoms.

Our study also showed that both groups significantly increased dietary fibre intake over the study period. This result may be explained by the fact that according to our study design the prescription of a high-fibre diet was supported by a dietary information sheet and followed over time by registering an intake score. It is likely that this systematic approach may have increased the intake of dietary fibre over time, perhaps due to an increased awareness that the prescription of diet needs to be taken seriously, like a real treatment option and not as a simple suggestion.

To date, the underlying mechanisms of the therapeutic benefit of dietary fibre in diverticular disease are not fully understood, albeit a relationship with stool volume and transit time has been hypothesized^[5,22]. More recently,

it has been shown that vegetarians are less likely than non-vegetarians to have radiologically confirmed diverticulosis (12% *vs* 33%), and that the insoluble component of fibre is associated with a decreased risk (relative risk 0.63, 95%CI: 0.36 to 0.75) of DD^[23], thus giving an indirect rationale for the high-fibre diet in symptomatic uncomplicated DD.

However, abdominal bloating was not effectively treated with a high-fibre diet, but a good therapeutic response was obtained in the Flortec[®] group only. This result is not surprising because it is well known that a high-fibre diet may increase the presence of intestinal gas due to an increase in the gas-producing intestinal microflora^[24]. Indeed, among our study population, it was only in the high-fibre diet group that patients with a new onset of abdominal bloating during the study period were registered.

The rationale for the use of probiotics in symptomatic uncomplicated DD is given by their anti-inflammatory effects and capability to enhance anti-infective defences by (1) maintaining an adequate bacterial colonization in the gastrointestinal tract; (2) inhibiting colonic bacterial overgrowth and metabolism of pathogens; and (3) reducing proinflammatory cytokines^[13,14]. In DD, local alterations of the peridiverticular colonic flora have been included as one of the causes leading to periods of symptomatic disease^[1-3]. Thus, the therapeutic benefit of the supplementation of *L. paracasei* B21060 observed in our study may be explained by the ability of probiotics to ensure an optimal colonic microenvironment, which is probably able to prevent local diverticular inflammation and to reduce abdominal symptoms. This idea is supported by experimental data showing that *L. paracasei* is able to survive the passage through the gastrointestinal tract, to persist in stools after administration is discontinued, and to temporarily associate throughout different sites of the entire human colon, suggesting a positive ecological role played by this probiotic strain^[25,26].

Literature data on the role of probiotics in the management of DD are still scant. The benefit of a cyclic 6-mo supplementation with a *L. paracasei sub. paracasei* F19 in association with a high-fibre diet on prolonged abdominal pain and bloating in symptomatic uncomplicated DD has been described, while the high-fibre diet alone appeared to be ineffective^[19]. Compared to the current study, in this study the prescription of a high-fibre diet was not accompanied by detailed dietary information and compliance to the high-fibre diet was not assessed, thus making it difficult to evaluate the therapeutic response in this treatment arm. Other previous studies investigated the efficacy of probiotics, as a non-pathogenic *Escherichia coli* strain or *Lactobacillus casei* and VSL#3 together with other therapeutic agents such as antibiotics or mesalazine in patients with DD^[27-31], making the results of these studies not comparable with our findings.

We are aware that the relative low sample size of this pilot study may have limited the statistical power of results. However, we preferred to analyse data with

respect to single abdominal symptoms rather than to a global symptom score, thus further reducing the sample number. But in this way, the efficacy of treatment on each single symptom could be evaluated more accurately. Furthermore, in this study two treatment arms were compared without a true control and cluster randomization was performed, thus limiting the interpretation of the results with particular regard to placebo effect. Considering that symptoms in symptomatic uncomplicated DD are likely to be influenced by the placebo effect, a placebo-controlled study is necessary to confirm our results.

To conclude, this study provides evidence that a high-fibre diet alone is effective in relieving abdominal pain in patients with symptomatic uncomplicated DD. This therapeutic response may be implemented by combining the dietary approach with Flortec[®] treatment which is effective in abdominal bloating, too. Data from this pilot study need to be confirmed in other larger trials.

COMMENTS

Background

The standard therapeutic approach for symptomatic uncomplicated diverticular disease (DD) still remains to be defined. Guidelines of American and European Gastroenterology Associations recommend a high-fibre diet in patients with symptomatic uncomplicated DD. A recent systematic review suggests the potential usefulness of fibre, rifaximin, mesalazine, and probiotics, and their possible combination in symptomatic uncomplicated DD treatment, but reliable controlled therapeutic trials are still lacking.

Research frontiers

Probiotics, prebiotics, and symbiotics may modify the gut microbial balance and changes in peri-diverticular bacterial flora likely play a role in the pathogenesis of diverticular microscopic inflammation and in generating abdominal symptoms in uncomplicated DD. Probiotic therapy is safe and potentially useful in the management of DD patients. Flortec[®] is a totally natural symbiotic agent, consisting of the synergistic combination of *Lactobacillus paracasei* (*L. paracasei*) B21060 (probiotic component) and arabinogalactan/xylooligosaccharides (prebiotic component), shown to be effective in relieving symptoms associated with irritable bowel syndrome and acute diarrhea. The therapeutic benefit of a symbiotic formulation in addition to a high dietary fibre intake in symptomatic uncomplicated DD remains to be defined.

Innovations and breakthroughs

In this study, for the first time the efficacy of a symbiotic formulation in addition to a high dietary fibre intake in symptomatic uncomplicated DD is investigated. All patients were instructed to follow a high-fibre diet containing at least 30 g daily intake of dietary fibre as well as a daily water intake of at least 1.5 L. For this purpose, all patients were given an information sheet regarding the content of dietary fibre in commonly consumed fruits, vegetables and cereals, and dietary counselling was performed.

Applications

The high-fibre diet alone is effective in relieving abdominal pain in patients with symptomatic uncomplicated DD. Adherence to diet should be monitored by dietary counselling. The combination of the high-fibre diet with a symbiotic preparation may improve the therapeutic response. Data of this pilot study need to be confirmed in other larger, placebo-controlled trials.

Terminology

Colonic diverticula is a wide-ranging condition running the spectrum from a symptomless to a severe, chronic, recurrent disorder, and has been classified in four clinical stages: (1) the development of diverticula (stage 1); (2) the symptom-free stage (stage 2); (3) the symptomatic uncomplicated diverticular disease (stage 3); and (4) the complicated diverticular disease (stage 4). Symbiotics are the synergistic combination of a probiotic component, as for example *L. paracasei* B21060, and a prebiotic component, as for example arabinogalactan and/or xylooligosaccharides.

Peer review

The article demonstrates that the combination of high-fibre diet and *L. paracasei* B21060 can relieve abdominal bloating as well as abdominal pain. The result is interesting and suggests that a high-fibre diet is effective in relieving abdominal symptoms in symptomatic uncomplicated diverticular disease. This treatment may be implemented by combining a high-fibre diet with Flortec®.

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Effects of the viability of *Lactobacillus rhamnosus* GG on rotavirus infection in neonatal rats

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Abstract

AIM: To study the effects of live and dead *Lactobacillus rhamnosus* GG (GG) on rotavirus infection in a neonatal rat model.

METHODS: At the age of 2 d, suckling Lewis rat pups were supplemented with either live or dead GG and the treatment was continued daily throughout the experi-

ment. At the age of 5 and 6 d the pups received oral rotavirus (RV) SA-11 strain. The pups were sacrificed at the age of 7 or 8 d by decapitation. The gastrointestinal tract was removed and macroscopic observations were done. The consistency of feces in the colon was classified using a four-tier system. RV was detected from the plasma, small intestine, colon and feces by real-time quantitative polymerase chain reaction (PCR).

RESULTS: In this neonatal rat model, RV induced a mild-to-moderate diarrhea in all except one pup of the RV-inoculated rats. RV moderately reduced body weight development from day 6 onwards. On day 7, after 2 d of RV infection, live and dead GG groups gained significantly more weight than the RV group without probiotics [36% ($P = 0.001$) and 28% ($P = 0.031$), respectively]. In addition, when compared with the RV control group, both live and dead GG reduced the weight ratio of colon/animal body weight to the same level as in the healthy control group, with reductions of 22% ($P = 0.002$) and 28% ($P < 0.001$), respectively. Diarrhea increased moderately in both GG groups. However, the diarrhea incidence and severity in the GG groups were not statistically significantly different as compared with the RV control group. Moreover, observed diarrhea did not provoke weight loss or death. The RV control group had the largest amount of RV PCR-positive samples among the RV-infected groups, and the live GG group had the smallest amount. Rats receiving live GG had significantly less RV in the colon ($P = 0.027$) when compared with the RV control group. Live GG was also more effective over dead GG in reducing the quantity of RV from plasma ($P = 0.047$).

CONCLUSION: Both live and dead GG have beneficial effects in RV infection. GG may increase RV clearance from the body and reduce colon swelling.

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Key words: Diarrhea; *Lactobacillus rhamnosus* GG; Neonatal rat; Rotavirus; Viability

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INTRODUCTION

Group A rotaviruses are the leading cause of acute gastroenteritis in children < 2 years of age and account annually for nearly 600 000 child deaths worldwide^[1]. Rotavirus-induced diarrhea causes severe dehydration and vomiting which can be fatal for malnourished babies. In developed countries rotavirus gastroenteritis causes a large economic burden with a substantial number of hospitalizations, doctor visits, and medical costs. Two vaccines are available to prevent RV infection, but their use may be limited by financial constraints especially in low-income countries.

The use of probiotic bacteria has gained considerable attention as a safe and accessible form of treatment for gastrointestinal diseases^[2,3]. *Lactobacillus rhamnosus* GG (GG), in particular, has been effective in reducing both duration^[3-6], and severity^[6,7] of rotavirus-induced diarrhea. The therapeutic capacity of GG against rotavirus gastroenteritis might be due to its ability to adhere to intestinal epithelial cells and compete for binding with the pathogens^[8,9], or displace bound pathogens^[10], stabilize and reinforce the mucosal barrier^[11-13], and stimulate the local antigen specific and nonspecific immune responses^[5,12,14]. However, the effect mechanisms of GG in rotavirus diarrhea are not completely understood.

Only a few studies have addressed the effects of inactivated probiotics in rotavirus diarrhea^[5,15]. When studying the effects of inactivated and live GG on acute RV diarrhea in children, both product forms equally promoted clinical recovery from diarrhea, but only live GG enhanced an IgA antibody response to RV^[5]. In mice, in contrast to live GG, heat-killed GG failed to protect animals from duration or severity of RV diarrhea^[15]. However, these studies did not include an untreated control group to allow comparison between the effects of product forms and RV.

In order to gain more understanding of the effect mechanisms of live and dead GG in RV-induced diarrhea, we compared their effects in the suckling rat RV SA-11 infection model with regard to parameters of infection severity such as weight gain, colon weight, con-

sistency of the feces, and also measured the amount of rotavirus in plasma, intestine, and feces.

MATERIALS AND METHODS

Animals

This study was approved by the Animal Care and Use Committee of the State Provincial Office of Southern Finland (license number ESAVI-2010-06221_Ym-23). Pregnant-specific pathogen-free Lewis rats were obtained from Harlan (Horst, The Netherlands) and they were allowed to give birth naturally in the test facility. The birth time of the pups was monitored twice a day. All pups were born on the same day within 12 h. Prior to all experiments, each litter was adjusted to 6 pups per dam to avoid biological variation due to litter size. The litters were randomly assigned to 4 experimental groups (4 dams with 6 pups each, $n = 24$): rats infected with RV SA-11 alone (RV control group); dead GG treated + RV SA-11 infected rats (dead GG group); live GG treated + RV SA-11 infected rats (live GG group); and minimum essential medium (MEM) control animals (healthy control group). The rat pups remained with their dams throughout the study. Control and inoculated groups were housed in the same individually ventilated Scantainer (Scanburg, Denmark), and each RV-infected group in its own Scantainer, in a normal rat cage (Makrolon III) with Aspen chips bedding (Tapvei Oy, Kaavi, Finland) and nest material (Aspen chips PM90L/R). The temperature was 22 ± 2 °C with relative humidity 50%-95%. Lighting was artificial, 12 h light and 12 h dark (18:00-06:00). Food (TEKLAD T.2916 IRR*; Irradiated Global 16%, Rodent Diet for mice and rats, Harlan) and deionized water were autoclaved and provided *ad libitum* from the day of the rats arrival until the completion of the experiments. The animals were identified individually by dorsal numbering.

Lactobacillus rhamnosus GG products

Viability was determined by plating the GG preparation after inactivation and no colony forming units (cfu) were detected. Both live and dead GG (ATCC 53103) were obtained from Valio (Valio Ltd, Helsinki, Finland). Prior use live GG stock (10^{11} cfu/mL) was aliquoted in de Man, Rogosa and Sharpe culture medium broth and stored at -20 °C. Powdered dead GG (killed at Valio Ltd, trade secret) at an equivalent cfu number of 10^{11} cfu/g of viable GG was maintained at room temperature. For the experiments live GG was thawed and dead GG weighed, and both were prepared daily in PBS at a concentration of 3×10^9 cfu/mL.

Virus propagation

Simian RV SA-11 strain was grown in a continuous cell line of rhesus monkey kidney cells, MA-104. The cells were cultivated in MEM containing 10% heat-inactivated fetal bovine serum supplemented with 2 mmol *L*-glutamine, penicillin and streptomycin in roller flasks in roller apparatus at 37 °C. When the cells had a

confluency of 70%-80%, they were inoculated from a stock containing 10^8 pfu/mL of plaque-purified rotaviruses. Before inoculation, RV stock was treated with 10-20 $\mu\text{g/mL}$ (final concentration) of trypsin (Sigma, St Louis, United States) for 30 min at 37 °C. RV stock in dilution 10^{-4} was added to each roller bottle. After incubation for 1 h, 30 mL of serum-free MEM with 1 $\mu\text{g/mL}$ of trypsin was added, and the cultivation was continued for 48 h at 37 °C. RV were harvested by freeze-thawing of cells for 3 times, cell debris was removed by low-speed centrifugation, supernatant was collected, divided into aliquots, and stored at -70 °C until use. RV titre was determined as 1.4×10^8 pfu/mL.

Animal experiments

The pups were weighed at fixed times daily before, and twice a day after, RV infection. At the age of 2 d, pups received a single daily 0.05 mL dose of either dead or live GG supplementation (1.5×10^8 cfu/pup). RV SA-11 was inoculated by plastic feeding tube in 3 separate doses to achieve the total amount of 10^8 pfu/pup as follows: at the age of 5 d pups received 2 RV doses (0.3 mL each), and the third dose at the age of 6 d (0.12 mL) in order to boost the RV infection. MEM containing 100 \times glutamine, penicillin 100 IU/mL, streptomycin 100 $\mu\text{g/mL}$ was used as a healthy control. After inoculations pups were returned to their dams and allowed to suckle.

Clinical indices and specimen collection

The pups were randomized to be exsanguinated from either 2 d or 3 d post-infection at the age of 7 d and 8 d. The blood samples were collected from all animals by decapitation into EDTA tubes (Venosafe™), and the plasma was obtained by centrifugation (10 min, 4000 rpm), and frozen at -20 °C within 1 h from sampling. The gastrointestinal tract was removed for macroscopic observations and specimen collection immediately after blood sampling. Small intestine was collected and weighed; colon tissue was collected and removed from its content by gently pushing along the tissue length by a spatula after which it was weighed. The feces were collected by carefully emptying the colon and rectum. Specimens were stored in dried ice until storing them at -80 °C. Consistency of feces was classified from 0-3 using a four-tier system: [0 = normal feces, 1 = slight diarrhea (feces is pale but solid); 2 = moderate diarrhea (feces is pale and semi-solid); 3 = strong diarrhea (feces is clearly wet)].

Sample processing

Plasma samples were thawed and viral RNA was extracted from 0.1 mL of sample with BioSprint® 96 One-For-All Vet-kit (Qiagen GmbH, Hilden, Germany), using the automated KingFisher mL purification system (Thermo Fisher Scientific, Vantaa, Finland) according to the manufacturer's instructions.

Frozen small intestine, colon, and feces were homogenized for nucleic acid extraction. Feces were processed on ice in 0.2 mL of 10% protease-inhibitor solution containing 1% bovine serum albumin, 10 mmol pefabloc

(Roche Applied Science, Mannheim, Germany), 100 $\mu\text{g/mL}$ aprotinin (Sigma-Aldrich, St. Louis, MO, United States) 100 $\mu\text{g/mL}$ leupeptin (Sigma-Aldrich) in Eagle minimum essential medium I (Gibco, Carlsbad, CA) supplemented with 5% fetal calf serum, and 20 mmol Hepes (pH 7.4). Suspensions were vortexed with sterile glass beads, centrifuged (10 min, 5000 rpm), and viral RNA was extracted from supernatants with E.Z.N.A.® Total RNA Kit (Omega Bio-Tek, Doraville, GA, United States) according to manufacturer's instructions.

Colon and the entire small intestine with its contents were homogenized with sterile glass rods, and 30 mg of homogenized tissue was added into 0.6 mL of RLT buffer (Qiagen) and incubated at 37 °C for 10 min in a water bath. The lysate was centrifuged with QIAshredder (Qiagen) (2 min, 12 000 $\times g$), and RNA was extracted with RNeasy Mini Kit (Qiagen) or BioSprint® 96 One-For-All Vet-kit (Qiagen) using the automated KingFisher mL purification system as above.

Detection of rotavirus

A total of 10 μL of the viral RNA was reverse transcribed into cDNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, United States) in a 20 μL reaction volume according to the manufacturer's instructions. real-time (RT) reaction was performed as described with few modifications^[16]. Depending on the sample material, RNA was first denatured for 5 min at 95 °C, and RT was performed by incubating the reaction mixture for 10 min at 25 °C, 120 min at 37 °C, and 5 min at 85 °C. The quantitative polymerase chain reaction (qPCR) protocol and the primers which target the VP7 gene of RV were designed according to the primers described by Li *et al*^[17]. Briefly, the qPCR reaction was carried out in 25 μL reaction mixtures consisting of 12.5 μL of 2 \times SYBR Premix Ex Taq™ (Takara, Dalian, China), 0.5 μL of each primer (20 $\mu\text{mol/L}$ final concentrations), 0.5 μL ROX Ref Dye II (50 \times), 4 μL of RV cDNA template, and 7 μL of sterile water (Sigma-Aldrich). The thermocycling profile included initial denaturation at 95 °C for 30 s, followed by 45 cycles of 95 °C for 5 s, 58 °C for 20 s, and 72 °C for 30 s. Finally, the melting curve analysis was performed at 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s. RNA isolated from cultured RV SA-11 samples was used as a positive control to establish the standard curve, and sterile water (Sigma-Aldrich) as a negative control. The samples were regarded RV SA-11 positive if the melting peak temperature was 83 ± 1.5 °C. The results were analyzed by comparing the cycle threshold (CT) values, which were inversely correlated with the amount of RV VP7 gene in the sample, i.e., the lower the CT value, the greater the amount of gene in sample.

Statistical analysis

Analysis of variance was applied to compare the groups with respect to weight gain and colon weight, and the results are given as means with standard error of the mean \pm SE. In cases of significant global *P*-values,

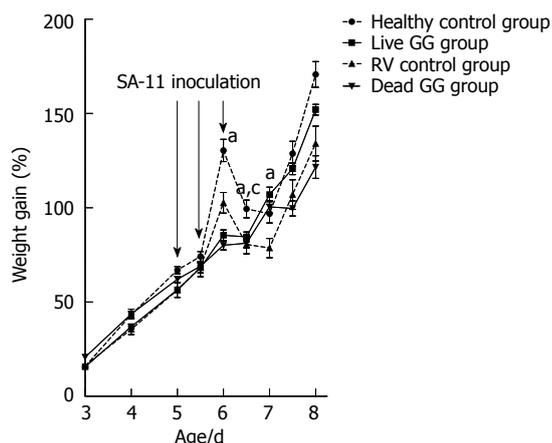


Figure 1 Percentage weight gain of the rats. The rat pups were weighed 1-2 times daily after day 2 during the experiment. On days 5 and 6, rotavirus (RV) was inoculated orally to the rats. Weight gain is expressed as percentual weight gain from the beginning of the experiment. ^a*P* < 0.05 vs RV control group; ^c*P* < 0.05 vs dead GG group.

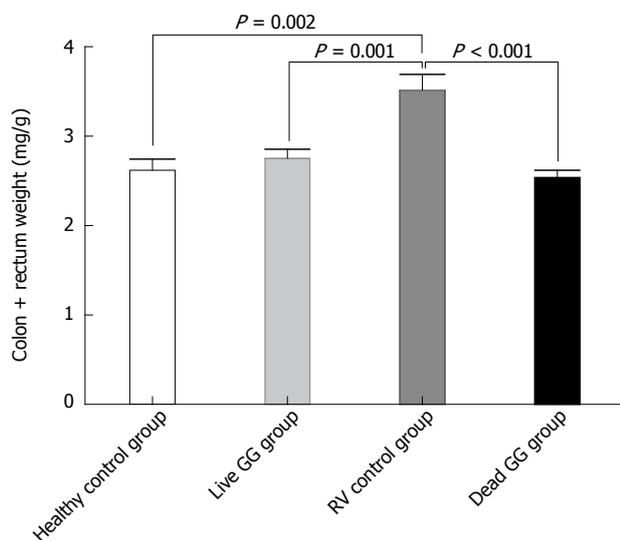


Figure 2 Weight of colon without feces. Results between groups are presented as the weight ratio of colon/animal. *P* = 0.002, *P* = 0.001, *P* < 0.001 vs rotavirus (RV) control.

multiple comparisons were performed and the *P*-values were Bonferroni corrected. RV diarrhea occurrence and severity between the study groups were analyzed using logistic regression analysis. Statistical differences in the *CT*-values between RV-infected groups were analyzed using Kruskal-Wallis test (global test) and Mann-Whitney *U*-test (pair-wise comparisons). *P*-values < 0.05 were considered statistically significant. The data were analyzed using PASW version 18.0 (SPSS Inc. Chicago, IL, United States).

RESULTS

Clinical investigations

Weight gain: The pups were weighed 1-2 times daily

Healthy control group (n = 5)	RV control group (n = 6)	Dead GG group (n = 6)	Live GG group (n = 6)
0	0	2	3
0	1	2	2
0	2	2	2
0	1	3	3
0	2	2	3
NA ¹	1	1	1

There was no statistically significant difference between groups. ¹Animal deceased during the experiment due to technical error in dosing. RV: Rotavirus; NA: Not analyzed.

Group	RV PCR-positive samples			
	Plasma	Small intestine	Colon	Feces
Healthy control group	0/5	0/5	0/5	0/5
RV control group	6/6	5/6	6/6	5/6
Dead GG group	6/6	4/6	6/6	4/6
Live GG group	6/6	2/6	6/6	3/6

Samples with cycle threshold (*CT*)-values < 45 were regarded as positive. RV: Rotavirus; PCR: Polymerase chain reaction.

during the experiment. There were no significant differences in body weight development before the virus inoculation between the study groups. RV moderately reduced body weight development from day 6 onwards (1 d after the infection) when compared with the pups receiving only MEM (Figure 1). RV did not severely compromise the condition of the pups. One pup died from the healthy group due to technical difficulties in dosing. The groups pre-colonized with live or dead GG had gained significantly more weight on day 7 than the RV group without probiotics [36% (*P* = 0.001) and 28% (*P* = 0.031), respectively].

Colon weight: Tissue samples were blindly collected and weighed at necropsy. In the large intestine, RV increased the weight of colon. Results between groups were compared by measuring the ratio of colon weight/body weight (Figure 2). When compared with the RV control group, both live and dead GG reduced the weight ratio of the colon to the same level as seen in the healthy control group, with reductions of 22% (*P* = 0.002) and 28% (*P* < 0.001), respectively.

Diarrhea: At the necropsy, diarrhea was determined in a blinded fashion by scoring the consistency of feces using the four-tier system from 0-3. RV induced a mild-to-moderate diarrhea in all except one of the RV-inoculated rats when compared with the healthy control group. In live and dead GG groups, diarrhea seemed to be moderately increased. However, the diarrhea incidence or severity in the groups was not statistically significant (*P* > 0.05) as compared with the RV control group (Table 1).

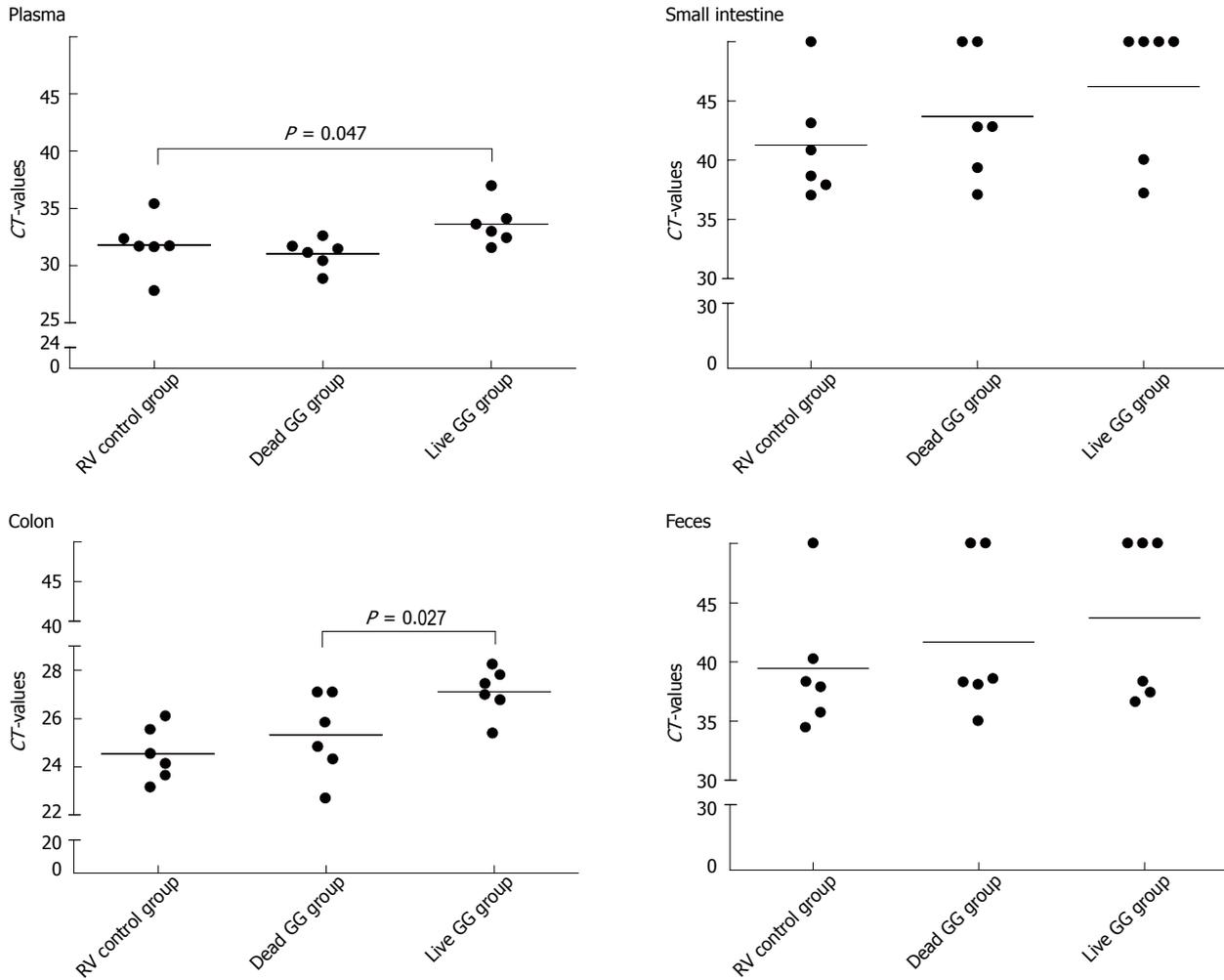


Figure 3 Rotavirus detection in plasma, small intestine, colon, and feces by real-time quantitative polymerase chain reaction. Limit of detection of the reaction was cycle threshold (CT) 45. The lines represent the mean of the CT-values. $P = 0.047$ vs RV control group, $P = 0.027$ vs dead GG group.

Rotavirus detection

The number of RV PCR-positive samples in the study groups is shown in Table 2. In the healthy control group, RV was detected from none of the samples. Overall, the RV control group had the largest amount of RV PCR-positive samples among the 3 groups, and live GG group the smallest amount. By comparing the CT values between the groups, we found that rats receiving live GG had significantly less RV VP7 gene in the colon ($P = 0.027$) when compared with the RV control group (Figure 3). Live GG was also more effective than dead GG in reducing the quantity of RV from plasma ($P = 0.047$).

DISCUSSION

In the present study conducted in a neonatal rat model, we characterized the effects of live and dead probiotic strain *Lactobacillus rhamnosus* GG on RV-induced diarrhea. We found that both groups receiving GG had smaller amounts of RV in the intestinal tissues and feces over the RV control group. In particular, live GG was effective in reducing the number of RV in the colon. Similar studies exist in mice, where live GG supplementation in

combination with antibodies reduced rhesus RV load in the small intestine^[15]. This increased RV clearance could be one of the effect mechanisms of GG in RV diarrhea, as it could shorten the duration of disease, as seen in clinical studies^[5,18,19]. Since RV is also capable of spreading systemically, and infecting extraintestinal tissues such as liver, kidney, and central nervous system^[20-23], the other potentially beneficial effect mechanism of GG against RV diarrhea might be its ability to inhibit the virus entering the blood circulation. Here, live GG appeared to reduce the quantity of RV in plasma.

Similar to other studies, RV SA-11 was effective in inducing diarrhea in the rat pups^[24,25]. Interestingly, we found that both live and dead GG seemed to slightly, though not significantly, increase diarrhea. *Lactobacillus* species in general seem to have an anti-diarrheal effect in clinical and *in vivo* studies^[5,6,26,27]. Especially in neonatal rats, *Lactobacillus casei* (*L. casei*) DN-114 001 strain in fermented milk decreased clinical signs of RV SA-11-induced diarrhea, reduced the number of RV antigens from the small intestine 48 h after infection, and reduced RV antigen load in the feces^[24]. Our results may be explained by the fact that nitric oxide (NO), which may

stimulate the enteric nervous system and induce water secretion into a luminal space further causing diarrhea^[28], is released by RV-infected enterocytes^[29], and GG also induces NO in macrophages^[30]. Enhanced diarrhea in the GG groups may further lead to the increased clearance of RV from the intestinal tissues by inhibiting adherence of RV, and “flushing” the virus from the body. However, we did not include a group receiving only GG, and cannot confirm whether the amount of GG could have an effect on the consistency of feces. On the other hand, another study did not report any changes in the feces in groups receiving probiotic supplements alone^[24].

Although the pups suffered from diarrhea, both groups receiving either live or dead GG gained more weight than the RV control group after RV inoculation. However, another probiotic strain, *L. casei* DN-114 001, failed to induce weight gain in a similar RV SA-11 rat model^[24], suggesting that the effect is strain specific. Interestingly, after day 6, the percentage weight gain was also reduced in the uninfected healthy control group. It is possible that a relatively large number of dosings during a short period of time partly inhibited rat pups to suckle milk from their dams.

RV induces inflammation and promotes tissue swelling by activating cytokine response of intestinal epithelial cells^[31]. RV-induced tissue swelling could this way increase weight of the colon. Interestingly, we found that both live and dead GG reduced colon weight. In the GG group this reduction may result from the GG's ability to stimulate the production of anti-inflammatory cytokines^[32]. These results further support the idea that GG might shorten the duration, and enhance the recovery from RV diarrhea.

The question of whether unviable and killed bacteria could have similar beneficial effects as live probiotic strains is contradicted. In clinical studies, heat-inactivated GG in children was unable to elicit local or systemic effects in rotavirus diarrhea^[5]. In addition, a heat-inactivated probiotic mixture including GG was ineffective against antibiotic-associated diarrhea when compared to equivalent live strains^[33]. In children with milk allergy, heat-inactivated GG treatment was associated with diarrhea^[34]. However, animal experiments conducted with unviable GG showed that the unviable form has beneficial effects against several inflammatory conditions such as arthritis and *Escherichia coli* lipopolysaccharide-induced inflammation in the lungs and liver of rats^[32,35]. This finding was possibly seen in our study as reduced colon weight. Nevertheless, live GG seemed to be more effective over dead GG in increasing the weight gain of rat pups after RV infection, and was more efficient in reducing the number of RV from plasma. The effects of dead bacteria, however, might depend on the method of inactivation. For instance, inactivation by heat or irradiation might disrupt the surface protein conformation of the bacteria, inhibiting the probiotic's ability to adhere to epithelial cell^[36]. In case the anti-diarrheal effects are due to secreted bioactive or antimicrobial peptides^[13,36,37], GG

needs to be viable.

In conclusion, only live GG decreased the number of RV in the colon of infected rat pups. However, dead GG had also some potential to alleviate RV infection possibly by reducing tissue swelling. The results provide new insights into aspects of the bacterial strain's viability, offering new possibilities to develop novel functional food matrices.

COMMENTS

Background

Group A rotaviruses are responsible for most cases of gastroenteritis in children under 2 years of age worldwide. Probiotics have gained an important role as adjuvant therapy in the treatment of acute diarrhea. Probiotic strain *Lactobacillus rhamnosus* GG (GG) in particular is known to reduce the duration of rotavirus-induced diarrhea in young children. However, it is unknown whether the viability of the strain plays a critical role in the probiotic's beneficial effects on diarrhea.

Research frontiers

The potential of unviable/inactivated/killed bacteria to relieve rotavirus (RV) gastroenteritis is not known. In this preliminary study, the authors explored the effects of both live and dead GG in RV infection in a neonatal rat model.

Innovations and breakthroughs

Recent clinical and animal studies have shown that GG relieves RV infection by shortening the duration of diarrhea, and reduces the amount of RV in intestinal tissues. In the present study the authors found only live GG reduced the amount of RV in intestinal tissues. However, the dead product form was found to have a potential to decrease RV diarrhea-induced weight reduction, and inhibit RV-induced colon swelling.

Applications

The results of this study indicate that the viable and dead forms of the bacterium have different favorable effects on RV infection. Dead product forms would have a great potential in the food industry by providing new product applications, increasing product shelf life, and reducing storage costs.

Terminology

Probiotic bacteria are defined as live microorganisms that have beneficial effects on human health. However, data regarding whether dead bacteria could have similar favorable health effects to live probiotic strains is limited.

Peer review

This study has investigated in experimental animals the effects of probiotics on rotavirus-induced diarrhea using *Lactobacillus* strain. They have used killed and live *Lactobacillus* strain in animals in which diarrhea was induced by rotaviruses. The findings are supportive of early observations of similar nature and are of clinical significance.

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Methane production and small intestinal bacterial overgrowth in children living in a slum

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Abstract

AIM: To analyze small intestinal bacterial overgrowth in school-aged children and the relationship between hydrogen and methane production in breath tests.

METHODS: This transversal study included 85 children residing in a slum and 43 children from a private school, all aged between 6 and 10 years, in Osasco, Brazil. For characterization of the groups, data regarding the socioeconomic status and basic housing sanitary conditions were collected. Anthropometric data was obtained in children from both groups. All children completed the hydrogen (H₂) and methane (CH₄) breath test in order

to assess small intestinal bacterial overgrowth (SIBO). SIBO was diagnosed when there was an increase in H₂ \geq 20 ppm or CH₄ \geq 10 ppm with regard to the fasting value until 60 min after lactulose ingestion.

RESULTS: Children from the slum group had worse living conditions and lower nutritional indices than children from the private school. SIBO was found in 30.9% (26/84) of the children from the slum group and in 2.4% (1/41) from the private school group ($P = 0.0007$). Greater hydrogen production in the small intestine was observed in children from the slum group when compared to children from the private school ($P = 0.007$). A higher concentration of hydrogen in the small intestine ($P < 0.001$) and in the colon ($P < 0.001$) was observed among the children from the slum group with SIBO when compared to children from the slum group without SIBO. Methane production was observed in 63.1% (53/84) of the children from the slum group and in 19.5% (8/41) of the children from the private school group ($P < 0.0001$). Methane production was observed in 38/58 (65.5%) of the children without SIBO and in 15/26 (57.7%) of the children with SIBO from the slum. Colonic production of hydrogen was lower in methane-producing children ($P = 0.017$).

CONCLUSION: Children who live in inadequate environmental conditions are at risk of bacterial overgrowth and methane production. Hydrogen is a substrate for methane production in the colon.

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Key words: Bacterial overgrowth; Breath test; Children; Colon; Hydrogen; Methane; Small intestine

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INTRODUCTION

Recent studies have identified small intestinal bacterial overgrowth (SIBO) as being involved in several clinical conditions, such as chronic constipation^[1], irritable bowel syndrome^[2,3] and short bowel syndrome^[4]. SIBO is also present in tropical/environmental enteropathy^[5,6].

SIBO is favored by environmental contamination and inadequate basic sanitation conditions^[7], and is often the result of ingesting food and/or water that is not appropriate for consumption^[8]. Thus, an excessive quantity of bacteria colonizes the upper intestinal tract, causing diarrhea^[9,10] and malnutrition^[5,10] due to nutrient malabsorption^[9,10]. A reduction in the absorption of carbohydrates, proteins, lipids and some vitamins can be observed, even in asymptomatic patients^[11]. Therefore, environmental enteropathy is a condition that may compromise child growth^[12].

The breath test is a noninvasive method that has been widely used in the diagnosis of SIBO^[10]. The breath test measures the production of hydrogen derived from the fermentation of lactulose by intestinal bacteria^[5,7,9,13,14]. In addition to hydrogen, methane may also be detected in the exhaled breath during the test. However, the significance of methane in the diagnosis of bacterial overgrowth in the small intestine is still controversial, and in general, it is not considered in the interpretation of the breath test^[5,7,13]. However, some studies^[1,2,9,14,15] have suggested that methane is also an indicator of bacterial overgrowth in the small intestine. A universal criterion for breath test interpretation has not been proposed.

The design of the present study was based on the fact that no other community study had used both hydrogen and methane as SIBO diagnostic criteria. In addition, the relationship between methane production, environmental conditions and the presence of SIBO was considered.

Considering the above rationale, the aim of the present study was to analyze SIBO using the lactulose hydrogen and methane breath test in school-aged children from two distinct socioeconomic strata, and to assess the relationship between intestinal production of hydrogen and methane, environmental conditions and SIBO.

MATERIALS AND METHODS

Design, study population and sample size

A transversal study was performed in the municipality of Osasco, in the state of Sao Paulo, Brazil. The study par-

ticipants consisted of two groups of children belonging to distinct socioeconomic strata.

The sample size was calculated taking into account a power of 80% and an alpha of 5%. Two aspects of the study were considered: (1) The prevalence of SIBO in the slum community was the first parameter determined. The population in this community included 1220 children aged between 6 and 10 years. The expected prevalence of SIBO was 35%^[5], with a maximum variation of 10%. Epi Info 3.4.3 software was used which indicated that 82 children in the slum group were needed to define the prevalence of SIBO; and (2) For comparison with the slum group, the necessary sample size of the private school group was estimated taking into account an expected prevalence of 35% for SIBO in the slum group and of 6% in the private school group^[5]. According to Epi Info 3.4.3, each group should have at least 36 children.

The first group was composed of children whose families lived in poor socioeconomic conditions and who resided in a slum in the vicinity of a landfill without adequate housing or basic sanitation conditions. To obtain a representative sample of the population, the children were selected at random by performing a survey of households in the slum area. Of the 100 children randomly selected, 85 agreed to participate in the study.

The second group was composed of children from a private school who belonged to socioeconomically advantaged families and had satisfactory housing and basic sanitation conditions. Of the 60 children whose parents or guardians showed an interest in participating in the study, 43 (71.6%) completed the study.

To be included in the study, the children had to be between 6 and 10 years of age and not have suffered from diarrhea for at least 30 d. The presence of clinical evidence that could mark a serious illness, such as heart disease, nephropathy, or neuropathy, was a criterion for exclusion from participation in the study. In addition, the use of antibiotics up to 15 d before the breath test was considered a criterion for exclusion from the study.

This project was evaluated and approved by the Research Ethics Committee of the Federal University of Sao Paulo. A signed informed consent form was obtained from the parents or guardians of each participant at the time of admission into the study.

Characterization of socioeconomic and basic sanitation conditions

A questionnaire was given to the parents and/or guardians of the children who participated in the study. Information regarding the presence of a water supply, a sewage system (destination of waste) and garbage collection was obtained.

Analysis of water contamination

An analysis of the water used for household consumption by the children in the slum group was performed. To determine the levels of total and fecal coliforms, the water was stored in 100 mL sterile plastic bags containing

a sodium thiosulfate tablet, which neutralizes the action of chlorine used in water treatment. The samples were transported and then incubated at 37 °C for 18 and 24 h, respectively, on the same day as the material was collected. Subsequently, the samples were analyzed using the Colilert Substrate method (IDEXX Laboratories, Maine, United States), which enumerates the levels of total and fecal coliforms (*Escherichia coli*) simultaneously in the same water sample, according to the manufacturer's instructions.

Anthropometric indicators

The weight and height of the children participating in the study were measured according to the recommendations of Jelliffe^[16]. To measure weight, a mechanical balance with a capacity of 150 kg and a sensitivity of 100 g was used. For height measurements, a portable vertical anthropometer with the capacity to measure up to 190 cm and a sensitivity of 0.1 cm was used.

Z-scores were calculated for weight-for-age, height-for-age, and body mass index (BMI), with adjustments for age and sex^[17]. The anthropometric indicators were calculated using the Epi Info version 3.4.3 program and the reference values from the National Center for Health Statistics^[18].

Hydrogen and methane breath test

The lactulose breath test was performed in the morning following oral hygiene using 0.05% chlorhexidine. The children fasted for a period of 12 h prior to the test.

Breath samples were collected using a non-rebreathing valve setup (QuinTron Instrument Co. Inc., Menomonee Falls, Wisconsin, United States). After collection of the fasting breath, 10 g of lactulose (Daiichi Sankyo, Sao Paulo, Brazil) was administered orally as a 10% aqueous solution. Subsequently, new breath samples were collected 15, 30, 45, 60, 90, 120, and 180 min after ingestion of lactulose.

The levels of hydrogen (H₂) and methane (CH₄) in the samples were simultaneously measured by gas chromatography using a 12i model QuinTron MicroLyzer unit (QuinTron Instrument Company, Milwaukee, Wisconsin, United States). Study participants were considered to exhibit SIBO when an increase in H₂ ≥ 20 ppm or CH₄ ≥ 10 ppm with respect to the fasting value was observed up to 60 min after the ingestion of lactulose^[1]. The study participants were treated as methane producers when the concentration of methane in the breath was higher than (or equal to) 3 ppm with respect to the concentration of methane in the environment^[19].

Information was also collected on the intestinal habits of the children from both groups, taking into account the existence of an association between the production of methane and evacuation disorders.

Statistical analysis

For data analysis, Sigma Stat 3.5 and Epi Info 3.4.3 software were used, setting 5% as the level to reject the null

Table 1 Demographic data, anthropometric indicators, and basic sanitation conditions of children from the slum and private school groups

	Slum (n = 85)	Private school (n = 43)	P value
Age (yr)	8.2 ± 1.4	8.4 ± 1.3	0.532 ¹
Gender (%)			
Male	48 (56.5)	37 (43.5)	0.061 ²
Female	16 (37.2)	27 (62.8)	
Z-score			
Age-weight	-0.56 ± 1.03	0.57 ± 1.18	< 0.001 ¹
Age-height	-0.31 ± 1.01	0.29 ± 1.14	0.003 ¹
BMI	-0.58 ± 1.06	0.56 ± 1.25	< 0.001 ¹
Access to public water network (%)	50 (58.8)	43 (100.0)	< 0.0001 ²
Access to public sewage (%)	8 (9.4)	43 (100.0)	< 0.0001 ²
Public collection of household garbage (%)	2 (2.4)	43 (100.0)	< 0.0001 ²

¹mean ± SD, Student's *t*-test; ²χ² test. BMI: Body mass index.

hypothesis.

RESULTS

Of the children studied, 85 were living in a slum and 43 were enrolled in a private school. Table 1 describes the demographic data, nutritional status indicators, and basic sanitation conditions of the studied population. There were no statistical differences in age or gender between the children in the two groups. The children in the slum group exhibited lower Z-score values for weight-for-age, height-for-age, and BMI when compared with children in the private school group. It was found that the majority of families in the slum did not have access to public service sewage or household garbage collection. Clandestine water supplies existed in 41.2% (35/85) of the households, and water analysis revealed the presence of total coliforms in 65 (77.4%) and fecal coliforms in 43 (51.2%) of the 84 samples analyzed.

During the study, three children did not perform the breath test; one child was from the slum group, and two children were from the private school group. SIBO was found in 30.9% (26/84) of the children in the slum group and in 2.4% (1/41) of the children in the private school group (χ² test, *P* = 0.0007). In the slum group, 65.4% (17/26) of the children with SIBO had increased hydrogen production with respect to the fasting value (H₂ ≥ 20 ppm), 23.1% (6/26) had increased methane concentrations with respect to the fasting value (CH₄ ≥ 10 ppm) and 11.5% (3/26) fulfilled both criteria for SIBO. In the private school group, the one child with SIBO fulfilled only the hydrogen criterion.

The demographic data, nutritional status indicators, and basic sanitation conditions of the children from the slum group with or without SIBO are shown in Table 2. No statistically significant differences for any of the variables analyzed (*P* > 0.05) were observed. It is important to emphasize that all the cases of SIBO were

Table 2 Demographic data, anthropometric indicators, and basic sanitation conditions of children with or without small intestine bacterial overgrowth from the slum group

	With SIBO (<i>n</i> = 26)	Without SIBO (<i>n</i> = 58)	<i>P</i> value
Age (yr)	8.3 ± 1.2	8.2 ± 1.5	0.817 ¹
Z-score			
Age-weight	-0.76 ± 1.05	-0.46 ± 1.02	0.213 ¹
Age-height	-0.38 ± 0.93	-0.28 ± 1.07	0.669 ¹
BMI	-0.80 ± 1.06	-0.46 ± 1.05	0.176 ¹
Water contamination (%)			
Fecal coliforms	21/26 (80.8)	44/57 ¹ (77.2)	0.594 ²
Total coliforms	13/26 (50.0)	30/57 ¹ (52.6)	0.941 ²
Access to public water network (%)	13 (50.0)	37 (63.8)	0.236 ²
Access to public sewage (%)	3 (11.5)	5 (8.6)	0.474 ³
Public collection of household garbage (%)	0 (0.0)	2 (3.4)	0.474 ³

¹mean ± SD, Student's *t*-test; ² χ^2 test; ³Exact Fisher test; ⁴Water samples analyzed in this group. BMI: Body mass index; SIBO: Small intestinal bacterial overgrowth.

asymptomatic.

The hydrogen concentrations (ppm) obtained by the lactulose breath test were analyzed for the areas under the individual curves. It was found that the children in the slum group exhibited greater (Student's *t*-test, *P* = 0.007) hydrogen production during the first hour of the test, which presumably originated from the small intestine, when compared with the children in the private school group (491.16 ± 369.05 ppm *vs* 314.45 ± 251.49 ppm per min, respectively). Between 60 min and 180 min of the test, the period during which hydrogen production occurs predominantly in the large intestine, the concentration of hydrogen in the breath of the children in the slum and private school groups were similar (4363.93 ± 1045.63 ppm *vs* 4275.0 ± 1390.55 ppm per min, respectively, *P* = 0.690) (Figure 1A).

Figure 1B shows the mean hydrogen concentrations (ppm) obtained from the breath tests of children with and without bacterial overgrowth in the slum group. A greater area under the curve for the small intestine was observed among the 26 children with SIBO compared with the 58 children without SIBO up to 60 min after the ingestion of lactulose (344.22 ± 185.23 ppm *vs* 818.94 ± 460.55 ppm per min; Student's *t*-test, *P* < 0.001). A similar response was observed for the colon during the 60 to 180 min of the test (4021.03 ± 711.73 ppm *vs* 5128.85 ± 1262.40 ppm per min; Student's *t*-test, *P* < 0.001).

Methane production was observed in 63.1% (53/84) of the children in the slum group and in 19.5% (8/41) of the children in the private school group (χ^2 test, *P* < 0.0001). The mean methane concentration remained relatively constant in both groups during the breath test and did not vary after lactulose ingestion. Among the 8 children in the private school group who were methane producers, 3 (37.5%) had intestinal constipation without fecal incontinence. There were no cases of constipation among the 53 children in the slum group who were methane producers (χ^2 test, *P* < 0.0001).

Figure 1C shows the mean methane concentrations (ppm) obtained from the breath tests of children with and without bacterial overgrowth in the slum group. In the small intestine, differences in the area under the curve were not observed among the 26 children with SIBO in relation to the 58 children without SIBO up to 60 min after the ingestion of lactulose (730.96 ± 829.56 ppm *vs* 576.72 ± 573.72 ppm per min; Student's *t*-test, *P* = 0.327). In addition, no significant difference was observed in the area under the curve for the colon during the 60 min to 180 min of the test (3835.34 ± 1159.71 *vs* 4324.04 ± 2053.62 ppm per min; Student's *t*-test, *P* = 0.168).

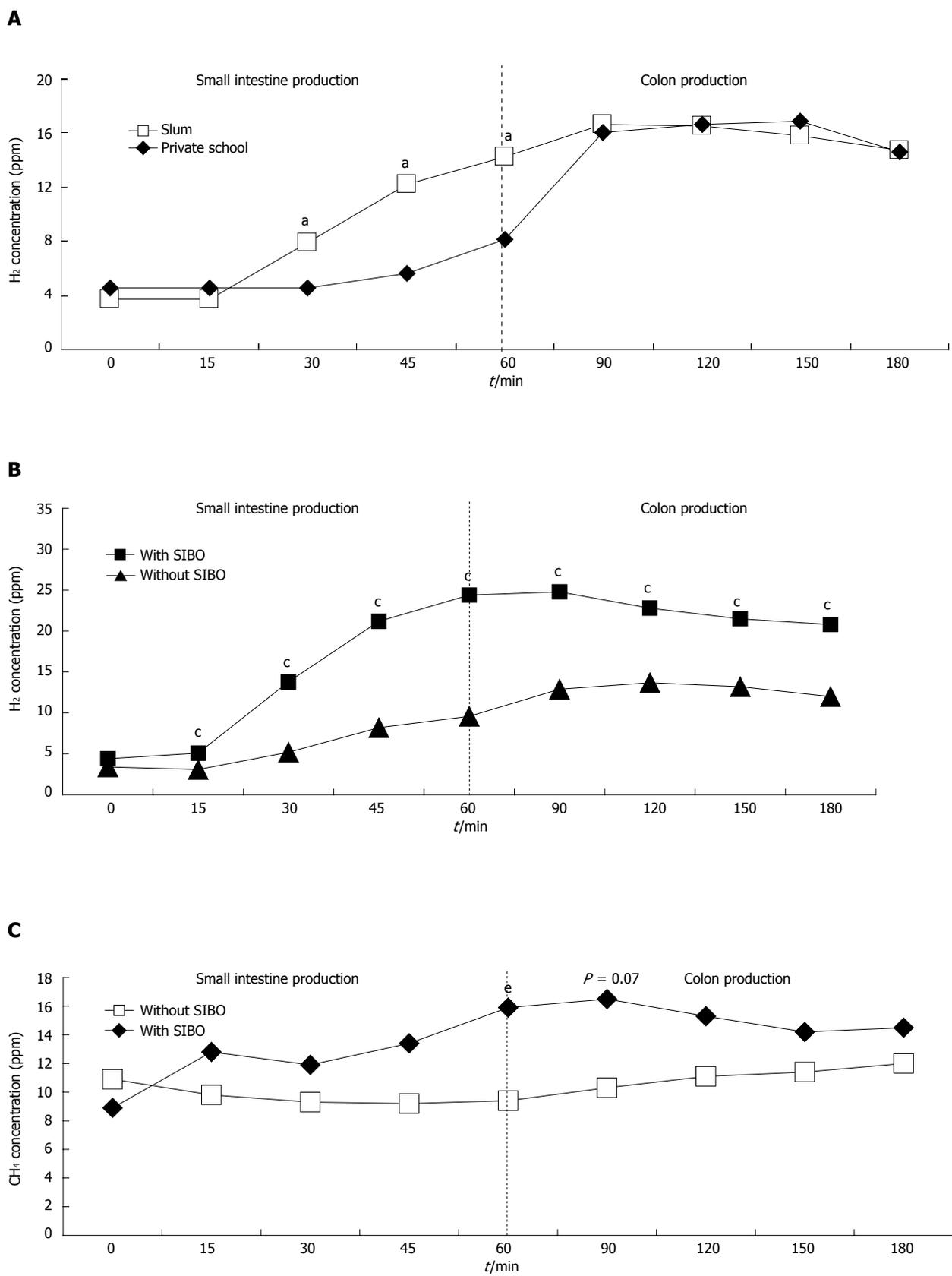
Methane production was observed in 38 (65.5%) of the 58 children who did not exhibit bacterial overgrowth and in 15 (57.7%) of the 26 children with bacterial overgrowth (χ^2 test, *P* = 0.658).

Figure 1D shows the concentration of hydrogen (ppm) in the breath for individual methane producers (*n* = 53) and non-producers (*n* = 31) in the slum group. An analysis of the area under the curve revealed that between 0 min and 60 min after lactulose ingestion, no difference existed in the levels of hydrogen production between the methane-producing and nonproducing children (448.16 ± 316.03 ppm *vs* 564.68 ± 441.40 ppm per min; Student's *t*-test, *P* = 0.164). In the period between 60 and 180 min of the test, less hydrogen production was observed among the methane-producing children (4157.55 ± 952.64 ppm per min) than among the methane non-producers (4716.77 ± 1117.13 ppm per min; Student's *t*-test, *P* = 0.017).

DISCUSSION

SIBO was found in 30.9% of the children in the slum group and in 2.4% of the children in the private school group. These values are similar to those previously reported in Brazil^[5] for children living in a slum and those attending a private health clinic (37.5% and 2.1% SIBO in each group, respectively). In Australia, SIBO was found in 27.2% of aboriginal children under 5 years old^[13]. These studies^[5,13] did not evaluate the methane concentration in the breath test. In the present study, 6 (23.1%) of the 26 children were diagnosed as having SIBO only by an increase in the breath methane level compared with the fasting values. Therefore, using the criteria that takes into account not only breath hydrogen but also breath methane, the diagnosis of SIBO may be more comprehensive. The higher frequency of bacterial overgrowth in the groups living in slums may be hypothetically explained by the different environmental and socioeconomic conditions to which they are exposed (Table 1).

Table 1 shows that the weight and height of children in the slum group were lower than those of the children in the private school group. This situation may be caused, at least in part, by the existence of digestive-absorptive abnormalities linked to bacterial overgrowth and tropical enteropathy^[5]. In addition, two factors may support the occurrence of the anthropometric deficit in the children in the slum group. The first factor corresponds to a diet



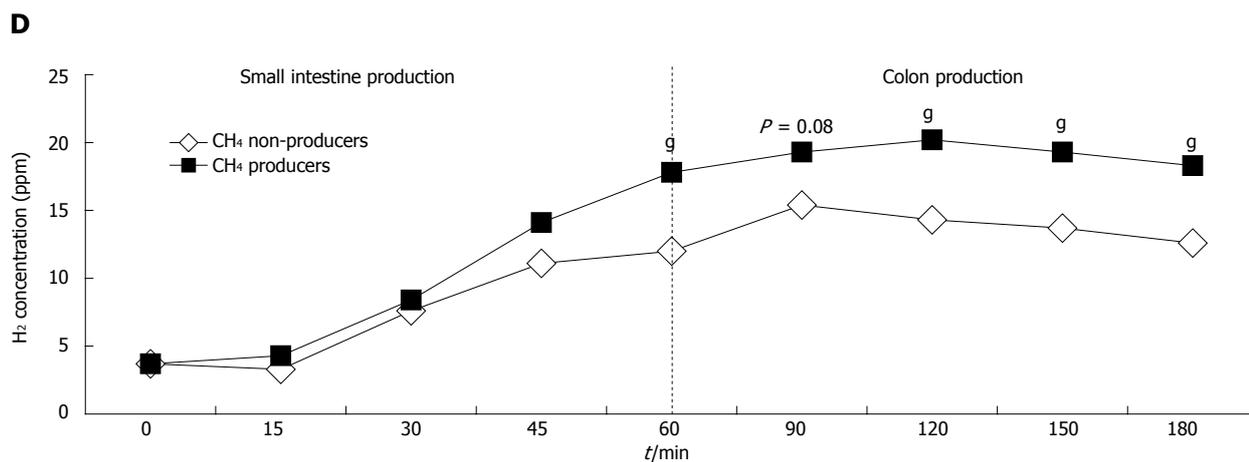


Figure 1 Small intestinal bacterial overgrowth in school-aged children and the relationship between hydrogen and methane production in breath test. A: Mean concentrations of hydrogen (ppm) in breath samples collected after fasting and at 15, 30, 45, 60, 90, 150 and 180 min after lactulose ingestion from children in the slum ($n = 84$) and private school ($n = 41$) groups; B: Mean concentrations of hydrogen (ppm) in breath samples collected after fasting and at 15, 30, 60, 90, 120, 150 and 180 min after lactulose ingestion from children in the slum group with ($n = 26$) and without ($n = 58$) small intestinal bacterial overgrowth (SIBO); C: Mean concentrations of methane (ppm) in breath samples collected after fasting and at 15, 30, 60, 90, 120, 150 and 180 min after lactulose ingestion from children in the slum group with ($n = 26$) and without ($n = 58$) SIBO; D: Mean concentrations of hydrogen (ppm) in breath samples collected after fasting and at 15, 30, 60, 90, 120, 150 and 180 min after lactulose ingestion from methane-producing ($n = 53$) and non-producing ($n = 31$) children in the slum group. Student's *t*-test; comparison between groups for collection time during the breath test (complementation of the analysis of area under the curve). ^a $P < 0.05$ vs the private school group; ^c $P < 0.05$ vs the slum group without SIBO in relation of the hydrogen production in breath test; ^b $P < 0.05$ vs the slum group without SIBO in relation of the methane production in breath test; ^g $P < 0.05$ vs the slum group non-methane producing in relation of the hydrogen production in breath test.

probably deficient in quality and quantity. Data from the food surveys showed that children in the slum group had less caloric intake, less protein consumption, less lipid consumption and less iron, calcium and vitamin A ingestion compared with children in the private school group (data not shown). The second factor corresponds to the inadequate basic sanitation conditions to which the children are exposed, exemplified by the high rates of water contamination, which may be directly related to recurrent infectious outbreaks and, consequently, to recurrent harm during physical development^[20].

In the lactulose breath test, the elevated concentrations of hydrogen observed in the breath of children in the slum group after 60 min of the test (Figure 1A) are consistent with the greater prevalence of bacterial overgrowth in these children.

One result of the present study that has yet to be explored in the literature involves the greater colonic production of hydrogen after the first hour of the breath test in the children in the slum group with SIBO (Figure 1B). It was found that these children, in addition to exhibiting greater hydrogen production in the small intestine up to 60 min after the start of the breath test, also showed elevated production of hydrogen from 60 to 180 min after the start of the test when compared with those without SIBO. This difference was found to be statistically significant with respect to the values corresponding to the area under the curve. It is likely that children with bacterial overgrowth possess greater quantities of fermenting bacteria, both in the small intestine and in the colon, when compared with children without SIBO who live in the same environmental conditions. Hydrogen excretion in the colon depends on fermentable substrates supplied by

the colonic bacteria^[21].

The presence of *Lactobacilli* and *Bifidobacteria* in the feces was also evaluated in this same group of children; however, no difference in the counts in the children with or without SIBO from the slum group was found (data not shown). Nevertheless, the number of *Lactobacilli* and *Bifidobacteria* colonies in the feces of children in the slum group was lower than that observed in the children in the private school group^[22,23]. There is currently no published information which relates SIBO with differences in the colonic microbiota.

Another interesting result of our study was the elevated prevalence of methane producers (63.1%) in the slum group. Considering the proximity of the slum to the municipal landfill, methane is produced by the intense anaerobic degradation of organic waste^[24], and methanogenic bacteria which may be present in the soil could potentially colonize the human intestine. These effects constitute an indication of the effects of the environment on the biological condition of individuals exposed to methane gas. Early age, close contact and poor personal hygiene may be factors explaining the high percentage of methane producers^[21] in the slum group.

Furthermore, there is concern about the association between the production of methane and the occurrence of diseases related to intestinal motility^[25,26]. It should be stressed that, in children, methane production has been related to intestinal constipation with fecaloma and fecal escape^[1,27,28], which are associated with a greater slow small intestine^[29] and colonic transit time^[28]. In our study, an association was not found between methane production and constipation in the children from the slum. However, 3 (37.5%) of the 8 children in the private

school group who were methane producers had intestinal constipation without fecal incontinence.

No differences were observed in the methane production between the children from the slum group with or without SIBO (Figure 1C), both in the colon and in the small intestine. Ingestion of non-absorbable disaccharide did not influence breath CH₄ excretion, different to that which occurred in H₂ excretion^[21].

The children in the slum group who were characterized as methane producers exhibited lower hydrogen concentrations in the colon when compared with the methane non-producers (Figure 1D); this result is similar to previously reported results in adults^[6]. Assuming that methane production is a good indicator of intracolonic metabolism^[19,21,30], this profile may be a consequence of the transformation of hydrogen into methane by methanogenic bacteria present in the colon^[14,30]. Methane is synthesized by bacteria in the intestine, where four mmols of hydrogen and one mmol of carbon dioxide create one mmol of methane and water^[10]. *Methanobrevibacter smithii* is the main methanogenic bacterium found in humans, and preferentially colonizes the left colon^[31]. Approximately 15% of individuals in the general population are producers of methane instead of hydrogen^[15], which is lower than the frequency observed in the slum group.

A previous study performed with adults^[14] raised the hypothesis that methane production may be responsible for the false-negative results obtained from studies on SIBO. In previous studies of SIBO in children, the concentration of methane in the breath was not determined. Despite the consistent absence in the literature of methane, in addition to hydrogen, as an indicator of SIBO, the data from our current study indicate that this possibility should be analyzed in future research.

In conclusion, in the present study, we observed a high prevalence of methane producers in children with or without SIBO who were exposed to poor living conditions. However, there was no direct relationship between the presence of SIBO and increased methane production in these children. Thus, the presence of breath methane seems to be a common condition in individuals exposed to inadequate environmental factors, as previously reported^[21]. Methane production appeared to be relatively constant during the course of the 3 h breath test. The value of the inclusion of a methane increment as an additional criterion for the diagnosis of SIBO should be evaluated in further studies. With respect to the use of hydrogen as a substrate for methane production in the colon, our hypothesis was confirmed, based on our observation regarding increased hydrogen production in the colon of methane non-producers in the slum.

COMMENTS

Background

Small intestine bacterial overgrowth (SIBO) is a clinical disorder characterized by an excessive quantity of bacteria in the upper intestinal tract. SIBO occurrence is common when associated with environmental enteropathy. Poverty

associated with ingestion of contaminated water and foods are involved in the etiology of this disease. Some of the consequences of SIBO are diarrhea and malnutrition, however, asymptomatic cases are observed. Breath tests are non-invasive tests used in the diagnosis of SIBO.

Research frontiers

In breath tests, hydrogen and methane can be detected. Both gases originate from bacterial fermentation. However, the relationship between methane production and SIBO diagnosis is still unclear.

Innovations and breakthroughs

This is the first study in which methane production was observed in individuals residing in a slum area. In the present study, high methane production was observed in children from a slum area with or without a diagnosis of SIBO. Thus, in addition to literature data relating methane production with severe constipation, methane can also be characterized as an indicator of environmental contamination. These results confirm the hypothesis that hydrogen is used as a substrate for methane production in the colon.

Applications

The results of this study suggest that the respiratory tests, characterized by the production of hydrogen and methane, can be performed in the research of SIBO in individuals exposed to unsanitary/unhealthy environments.

Terminology

SIBO: Clinical disorder characterized by the presence of contaminating bacteria in the small intestine; Environmental enteropathy: Syndrome characterized by a set of nonspecific changes, functional and/or morphological, in the small intestine associated with or without gastrointestinal symptoms. This is a clinical condition associated with environmental contamination; Breath test: The test consists of administering a carbohydrate which is degraded and metabolized by bacteria in the intestine, producing an increase in hydrogen levels in expired air. Others gases can be detected in the expired air, for example, methane.

Peer review

This is an original study looking for methane and hydrogen expiration in two children communities, as a token of SIBO.

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Efficacy of endoluminal gastroplication in Japanese patients with proton pump inhibitor-resistant, non-erosive esophagitis

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medication, patients' symptoms and quality of life (QOL) were assessed using the questionnaire for the diagnosis of reflux disease, the frequency scale for symptoms of gastro-esophageal reflux disease (FSSG), gastrointestinal symptoms rating scale, a 36-item short-form. In addition, 24-h esophageal pH monitoring or 24-h intraesophageal pH/impedance (MII-pH) monitoring was performed. The Bard EndoCinch™ was used for ELGP, and 2 or 3 plications were made. After ELGP, all acid reducers were temporarily discontinued, and medication was resumed depending on the development and severity of symptoms. Three mo after ELGP, symptoms, QOL, pH or MII-pH monitoring, number of plications, and PPI medication were evaluated. Further, symptoms, number of plications, and PPI medication were evaluated 12 mo after ELGP to investigate long-term effects.

RESULTS: The mean FSSG score decreased significantly from before ELGP to 3 and 12 mo after ELGP (19.1 ± 10.5 to 10.3 ± 7.4 and 9.3 ± 9.9 , $P < 0.05$, respectively). The total number of plications decreased gradually at 3 and 12 mo after ELGP (2.4 ± 0.8 to 1.2 ± 0.8 and 0.8 ± 1.0 , $P < 0.05$, respectively). The FSSG scores in cases with no remaining plications and in cases with one or more remaining plications were 4.4 and 2.7, respectively, after 3 mo, and 2.0 and 2.8, respectively, after 12 mo, showing no correlation to plication loss. On pH monitoring, there was no difference in the percent time pH < 4 from before ELGP to 3 mo after. Impedance monitoring revealed no changes in the number of reflux episodes or the symptom index for reflux events from before ELGP to 3 mo after, but the symptom sensitivity index decreased significantly 3 mo after ELGP (16.1 ± 12.9 to 3.9 ± 8.3 , $P < 0.01$). At 3 mo after ELGP, 6 patients (31.6%) had reduced their PPI medication by 50% or more, and 11 patients (57.9%) were able to discontinue PPI medication altogether. After 12 mo, 3 patients (16.7%) were able to

Abstract

AIM: To evaluate the efficacy, safety, and long-term outcomes of endoluminal gastroplication (ELGP) in patients with proton pump inhibitor (PPI)-resistant, non-erosive reflux disease (NERD).

METHODS: The subjects were NERD patients, diagnosed by upper endoscopy before PPI use, who had symptoms such as heartburn or reflux sensations two or more times a week even after 8 wk of full-dose PPI treatment. Prior to ELGP, while continuing full-dose PPI

reduce the amount of PPI medication by 50% or more, and 12 patients (66.7%) were able to discontinue PPI medication altogether. A high percentage of cases with remaining plications had discontinued PPIs medication after 3 mo, but there was no difference after 12 mo. No serious complications were observed in this study.

CONCLUSION: ELGP was safe, resulted in significant improvement in subjective symptoms, and allowed less medication to be used over the long term in patients with PPI-refractory NERD.

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Key words: Endoluminal gastroplication; Esophageal pH; Gastro-esophageal reflux disease; Non-erosive reflux disease; Proton pump inhibitor-resistant

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Tokudome K, Funaki Y, Sasaki M, Izawa S, Tamura Y, Iida A, Ogasawara N, Konagaya T, Tokura Y, Kasugai K. Efficacy of endoluminal gastroplication in Japanese patients with proton pump inhibitor-resistant, non-erosive esophagitis. *World J Gastroenterol* 2012; 18(41): 5940-5947 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v18/i41/5940.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i41.5940>

INTRODUCTION

Non-erosive reflux disease (NERD) refers to a syndrome that is characterized by complaints of reflux symptoms such as heartburn without endoscopic evidence of mucosal injury^[1]. It accounts for more than half of gastroesophageal reflux disease (GERD) cases in Japan, as it does in Europe and the United States^[2]. NERD and erosive GERD cannot be distinguished by the severity of symptoms or their frequency, and they are equally characterized by compromised quality of life (QOL)^[3,4]. Proton pump inhibitors (PPIs), which are first-line drugs for GERD, are less effective for NERD than they are for erosive GERD^[5]. NERD symptoms are often not amenable to treatment with oral medication. It was recently proposed that NERD is not just a milder form of reflux disease but a group of symptoms with pathophysiologic mechanisms that differ from those of erosive GERD^[6]. It has been reported that a number of etiologies may contribute to the symptoms of heartburn, including motor events, reflux of nonacidic gastric contents, minute changes in intraesophageal pH, visceral hypersensitivity, and emotional or psychological abnormalities^[7-9]. However, many of the details involved in its mechanism remain unknown. The recent use of ambulatory multichannel intraluminal impedance-pH (MII-pH) monitoring has made it possible to learn about various symptom-inducing factors that could not be determined by conventional pH monitoring alone^[10], and it has elucidated the patho-

physiology involved in PPI-refractory NERD. It is now believed that factors other than gastroesophageal reflux or non-acid reflux are involved in its pathophysiology^[11].

The surgical treatment of erosive GERD originated with Allison *et al*^[12] in 1951. Laparoscopic fundoplication (Nissen fundoplication or Toupet fundoplication) is now widely used and is reportedly useful over the long-term^[13,14]. However, its inherent invasive nature as a surgical procedure remains problematic. In response to the inconvenience and non-compliance associated with drug use, as well as the complications associated with laparoscopic surgery, a number of endoscopic techniques have been developed since 2000, primarily in Europe and the United States, as less invasive but effective methods. In Japan, only endoluminal gastroplication (ELGP) using the Bard EndoCinchTM (C. R. Bard, Murray Hill, NJ, United States) has been covered by national health insurance (K667-3: 12 000 points). This procedure permits less invasive fundoplication to be accomplished using an endoscope, and it has been reported to be useful over the long term^[15]. However, it is used in cases in which PPI treatment is effective. The efficacy of endoscopic treatment in PPI-resistant GERD not amenable to treatment with oral medication has not yet been sufficiently studied.

Until recently, NERD has been assumed to be milder than erosive GERD, and NERD has not been considered suitable for surgical treatment. However, surgical fundoplication is now performed in cases of NERD not amenable to drug treatment, and its efficacy is becoming increasingly clear^[16,17]. However, the efficacy of endoscopic treatment in NERD patients has not been studied. Therefore, the efficacy, safety, and long-term course of ELGP as endoscopic treatment in patients with PPI-resistant NERD, who are the most intractable of NERD patients, were studied.

MATERIALS AND METHODS

This was a prospective study conducted at Aichi Medical University Hospital. Nineteen subjects were enrolled between March 2005 and June 2009 (14 males and 5 females; average age 53.0 ± 4.3 years). The study was approved by the Aichi Medical University School of Medicine Ethics Review Board and was conducted with the written informed consent of patients who had been given a full explanation of the study. The subjects were NERD patients, diagnosed by upper endoscopy before PPI use, who had symptoms such as heartburn or reflux sensations two or more times a week even after 8 wk of full-dose PPI-treatment (i.e., 30 mg of lansoprazole, 20 mg of rabeprazole, or 20 mg of omeprazole per day). The following exclusion criteria were used: < 18 years of age; hiatal hernia (≥ 2 cm); erosive GERD (Los Angeles Grade A or higher) before PPI use; Barrett's esophagus; gastroesophageal varices; past history of gastroesophageal surgery; gross obesity (body mass index > 40 kg/m²); esophageal stenosis; dysphagia; and primary esophageal motility disorders diagnosed by intraesophageal manom-

etry (Polygraf ID, Sierra Scientific, Los Angeles, CA, United States).

Interview and tests

Prior to ELGP, while continuing full-dose PPI medication, patients were assessed for reflux symptoms based on the questionnaire for the diagnosis of reflux disease (QUEST)^[18] and the frequency scale for symptoms of GERD (FSSG)^[19]. The gastrointestinal symptoms rating scale (GSRS) was used as an indicator of gastrointestinal symptoms, and 36-item short-form (SF-36)^[20] interview was conducted as an assessment of the activity index. In addition, 24-h esophageal pH monitoring (Digitrapper MK III, Synectics Medical, Los Angeles, CA, United States) or 24-h transesophageal pH/impedance monitoring (Sleuth[®] multi-impedance pH monitoring system, Sandhill Scientific, Highlands Ranch, CO, United States)^[10] was performed. After ELGP, all acid reducers were temporarily discontinued, and medication was resumed depending on the development and severity of symptoms.

Reflux symptoms were assessed by the QUEST and FSSG interviews at 3 and 12 mo after ELGP. The GSRS and SF-36 interviews, as well as 24-h esophageal pH monitoring or 24-h intraesophageal pH/impedance monitoring, were performed after 3 mo. Analysis was performed using the automatic analysis program BioVIEW Analysis[®] (version 5.3.4; Sandhill Scientific, Inc.)^[21] to compare the symptom index (SI), the symptom sensitivity index (SSI), number of reflux episodes, and number of symptom events. SI is defined as the number of symptoms associated with reflux divided by the total number of symptoms during 24 h, and it primarily assesses the specificity of a patient's reflux symptoms; it is considered positive if more than 50% of the symptoms are associated with reflux^[22]. SSI is defined as the number of reflux events associated with symptoms divided by the total number of reflux events in 24 h, and it quantifies the subject's sensitivity for reflux; it is considered positive if more than 10% of the reflux events are associated with symptoms^[23]. Upper endoscopy was also performed 3 and 12 mo after ELGP to compare the number of remaining plications.

ELGP method

The Bard EndoCinch[™] (C. R. Bard) was used for endoluminal gastroplication. After the esophagus had been examined by routine endoscopy, an endoscope with a capsule-shaped plication device (with a side hole) mounted at the tip was brought to the level of the squamocolumnar junction through the over-tube, where the side hole was brought into close contact to draw the mucosa into the capsule with at least 400 mmHg of air suction. After it had been confirmed that all tissue had been drawn in, a puncture needle with 3-0 nonabsorbable suture attached (suture tag), which had been inserted into the biopsy channel, was passed through. The suction pressure was released, and the capsule was carefully rotated away from the stitches side. A suture tag was again set up in the endoscope, and a second set of stitches was placed follow-

Table 1 Patients' baseline characteristics

Characteristics	Value
Patients (n)	19
Age, yr (range)	53.0 ± 4.3 (25-82)
Sex (male:female)	14:5 (73.7%:26.3%)
Body mass index (kg/m ²) (range)	22.3 ± 0.4 (21.5-23.5)
Hiatal hernia, n (%)	18 (94.7)
PPI medication, n (%)	19 (100)

PPI: Proton pump inhibitor.

ing the same procedure at a position rotated 30 to 60 degrees away from the first set of stitches. The two sutures made a plication using a suturing device (knotting device) that had been inserted into the biopsy channel of a separate endoscope, and plication was completed by plicating the tissue in the form of a pouch. The second and third plications were performed in either a linear or circumferential manner, or a combination of the two, depending on the available area within the gastro-esophageal junction and position preference^[23-27].

Statistical analysis

Data are shown as the means ± SD. Analysis was based on Wilcoxon's signed-rank test, the Kruskal Wallis test, and the Steel-Dwass or χ^2 test. A significant difference was defined as $P < 0.05$.

RESULTS

Table 1 shows the characteristics of the 19 subjects enrolled in this study. One patient died of an accident during long-term follow-up. Therefore, only 18 subjects completed long-term follow-up for 12 mo. The QUEST questionnaire and FSSG questionnaire were each collected from 19 (100%) and 12 (66.7%) subjects 3 and 12 mo after ELGP, respectively. Ten subjects consented to GSRS, SF-36, and 24-h intra-esophageal pH/impedance monitoring. Six patients failed to attend for endoscopy at 12 mo. No serious complications were observed in this study. Minor hemorrhage due to mucosal injury during the ELGP procedure was observed in only 2 cases.

The mean QUEST score did not change, but the mean FSSG score decreased significantly, from before ELGP to 3 and 12 mo after. The total number of plications decreased significantly at 3 and 12 mo after ELGP (Table 2).

On 24-h esophageal pH monitoring, there was no difference in the percent time pH < 4 from before ELGP to 3 mo after. Impedance monitoring revealed no changes in the number of reflux episodes or the symptom index (SI) for reflux events from before ELGP to 3 mo after, but the number of symptom events and SSI decreased significantly 3 mo after ELGP (Table 3, Figure 3).

At 3 mo after ELGP, 2 patients were still on full-dose PPI (10.5%), 6 patients had reduced their PPI medication by 50% or more (31.6%), and 11 patients were able to discontinue PPI medication altogether (57.9%). After 12 mo, 2 patients were still on full-dose PPI (11.1%), 3 pa-

Table 2 QUEST score, frequency scale for symptoms of the gastro-esophageal reflux disease score, and plication count after endoluminal gastroplication

	Baseline (<i>n</i> = 19)	3 mo (<i>n</i> = 19)	12 mo (<i>n</i> = 12)
QUEST score	5.7 ± 4.1	4.2 ± 4.5	2.5 ± 4.2
FSSG score	19.1 ± 10.5	10.3 ± 7.4 ^a	9.3 ± 9.9 ^a
Plication count	2.4 ± 0.8	1.2 ± 0.8 ^a	0.8 ± 1.0 ^a

^a*P* < 0.05 *vs* baseline by Kruskal Wallis test plus Steel-Dwass test. QUEST: Questionnaire for the diagnosis of reflux disease; FSSG: Frequency scale for symptoms of gastro-esophageal reflux disease.

Table 3 Twenty-four hour intra-esophageal ambulatory multichannel intraluminal impedance-pH monitoring data at baseline and 3 mo after endoluminal gastroplication

	<i>n</i>	Baseline	3 mo
24-h intra-esophageal time pH < 4 (%)	18	4.1 ± 4.0	10.4 ± 19.2
Numbers of reflux events, %	10	100.3 ± 16.1	79.5 ± 13.5
Symptom index (SI), %	10	70.9 ± 9.2	79.0 ± 10.1
Symptom sensitivity index (SSI), %	10	16.1 ± 12.9	3.9 ± 8.3 ^b
Number of symptom events, %	10	27.2 ± 5.9	7.3 ± 5.2 ^b

^b*P* < 0.01 *vs* 3 mo after endoluminal gastroplication by Wilcoxon's signed rank test.

tients had reduced their PPI medication by 50% or more (16.7%), and 12 patients were able to discontinue PPI medication altogether (66.7%) (Figure 1).

The GSRS total score, reflux score, abdominal pain score, and indigestion score had improved significantly from before ELGP to 3 mo after. Prior to ELGP, the SF-36 scores were all below the national standard scores. After 3 mo, physical functioning and overall health had improved significantly (Table 4).

Three months after ELGP, one or more plications remained in 80%, and two or more remained in 40%. After 12 mo, one or more plications remained in 43%, and it was confirmed that the sloughing off of plications was followed by scar formation in 75%. Therefore, the correlation of medication dose to loss of antireflux function and symptoms induced by the sloughing off of plications was studied. The FSSG score in cases with no remaining plications and in cases with one or more remaining plications was 4.4 and 2.7, respectively, after 3 mo, and 2.0 and 2.8, respectively, after 12 mo, revealing no correlation to plication loss (Figure 2A). After 3 mo, 33.3% and 66.7%, respectively, had discontinued PPI medication, whereas 83.3% had done so in both groups after 12 mo. A high percentage of cases with remaining plications had discontinued PPIs medication after 3 mo, but there was no difference after 12 mo (Figure 2B).

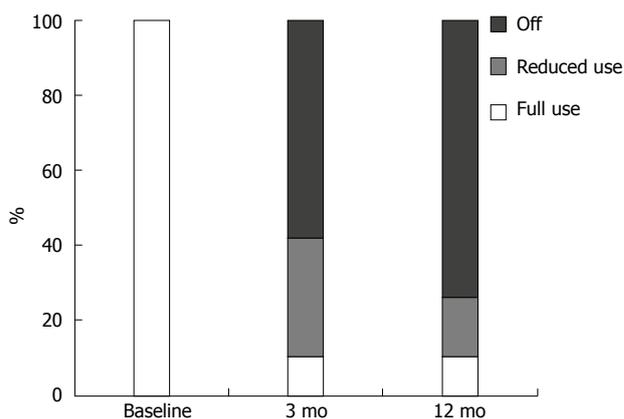
DISCUSSION

This is the first report showing that ELGP is useful in improving symptoms in Japanese PPI-resistant NERD

Table 4 Gastrointestinal symptoms rating scale and 36-item short-form at baseline and 3 mo after endoluminal gastroplication

	Baseline (<i>n</i> = 10)	3 mo (<i>n</i> = 10)
GSRS scale		
Overall	2.5 ± 0.8	1.8 ± 0.4 ^a
Acid reflux	3.3 ± 1.5	2.1 ± 0.7 ^a
Abdominal pain	2.7 ± 1.4	1.8 ± 0.9 ^a
Indigestion	2.6 ± 1.0	1.6 ± 0.8 ^a
Diarrhea	1.8 ± 0.9	1.4 ± 0.6
Constipation	2.3 ± 1.3	1.9 ± 1.3
SF-36 scale		
Physical functioning	46.6 ± 15.8	53.7 ± 3.8 ^a
Role physical	36.9 ± 20.0	48.7 ± 15.7
Bodily pain	40.5 ± 16.7	52.7 ± 8.3
General health	43.2 ± 7.8	48.8 ± 6.2 ^a
Vitality	42.9 ± 12.7	50.6 ± 6.7
Social functioning	44.0 ± 12.6	47.2 ± 9.9
Role emotional	37.2 ± 19.4	47.7 ± 13.7
Mental health	46.3 ± 11.8	43.3 ± 11.6

^a*P* < 0.05 *vs* 3 mo after endoluminal gastroplication by Wilcoxon's signed rank test. GSRS: Gastrointestinal symptoms rating scale; SF-36: 36-item short-form.

**Figure 1** Rate of discontinuation or reduction by more than 50% of proton pump inhibitor use.

patients, reducing their PPI dose and improving their QOL. Surgical fundoplication has been shown to be effective in reducing esophageal reflux in erosive GERD, as well as in NERD. Omura *et al*^{16]} have reported that, after laparoscopic fundoplication in 21 NERD patients with acid or bilirubin reflux, all subjects experienced improvement in symptoms of heartburn and were able to discontinue or reduce PPI medication. Broeders *et al*^{17]} performed Nissen fundoplication in 96 NERD patients and 117 erosive GERD patients, with improvement in symptoms in 89% and 96%, respectively, after 5 years. In addition, there were no differences between the two groups in terms of the effect in reducing PPI medication, improving QOL score, and reducing acid exposure times, suggesting the long-term efficacy of fundoplication. The efficacy of surgical fundoplication thus continues to be established in GERD, as well as in NERD. However,

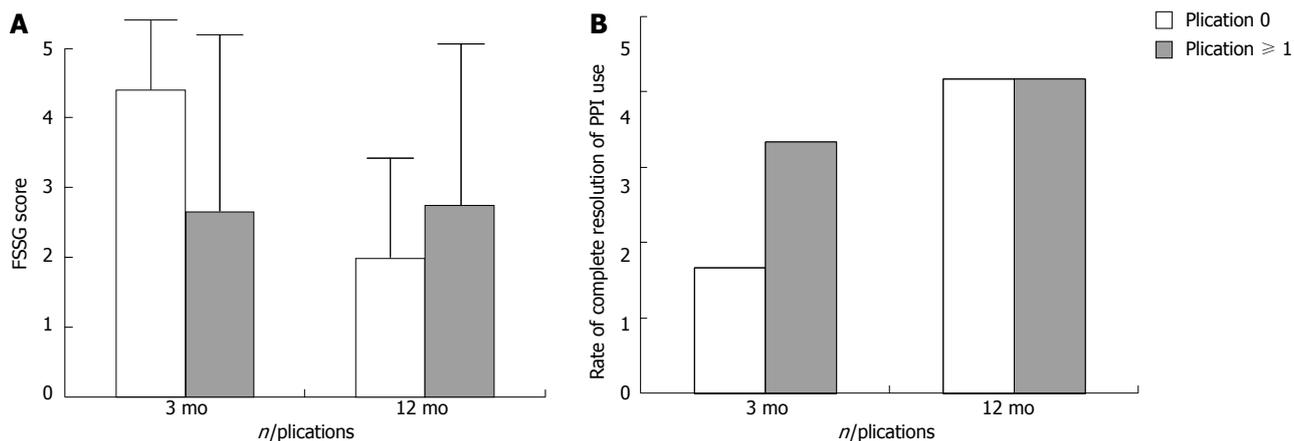


Figure 2 Relationship with the plication number and symptom or proton pump inhibitor use. A: Relationship between the number of plications and the score of the frequency scale for symptoms of gastro-esophageal reflux disease. There is no significant difference between the two groups (remaining plications 0 vs one or more) after 3 or 12 mo after endoluminal gastroplication (ELGP); B: Relationship between the number of plications and the rate of proton pump inhibitor (PPI) use. The group with one or more remaining plications shows a tendency to reduced PPI use 3 mo after ELGP ($P = 0.07$). However, there is no significant difference between the groups after 12 mo of ELGP.

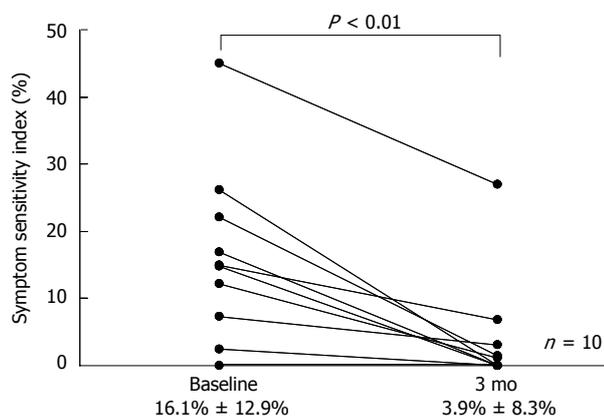


Figure 3 Symptom sensitivity index transition at baseline and 3 mo after endoluminal gastroplication.

it has been reported that surgical fundoplication is associated with complications approximately 10% of the time, and that dysphagia lasting 3 mo or longer has been observed^[28]. The procedure is also associated with an operative mortality of about 0.5% to 0.7%^[29], which may be considered a problem for the treatment of a benign disease. There are also no differences in the long-term (140 mo) effect compared to drug treatment^[30], and the cost is higher compared to 10 years of treatment with omeprazole^[31]. It was in light of this background that endoscopic treatments were proposed as less invasive but effective treatment methods^[32,33]. Endoscopic fundoplication is an extremely safe method among such treatments, and its efficacy in erosive GERD is also being studied^[34,35].

The results of 6, 12, and 24 mo of observation have been reported by Filipi *et al.*^[36], Mahmood *et al.*^[25], and Chen *et al.*^[26] in typical clinical studies of ELGP. All reported significant improvements in heartburn and reflux scores, as well as a significant reduction in PPI medication, and these effects lasted as long as 24 mo. In the only randomized, double-blind, comparative study, which was

conducted by Schwartz *et al.*^[37] in 2007, 60 patients with GERD were assigned to an EndoCinch group, a sham treatment group, and an observation group, each composed of 20 subjects, and the subjects were assessed at 3, 6, and 12 mo. After 3 mo, the active treatment group showed a significant reduction in medication and improvement in GERD symptoms and QOL compared to the sham treatment group, and these effects lasted as long as 12 mo. However, there was no difference in esophageal acid exposure between the treated and sham groups, and 29% of the cases required re-treatment. In the only report of a clinical study in Japan, 48 patients with erosive GERD underwent ELGP and were followed-up for 2 years. On 24-h pH monitoring, there was improvement in the percent time pH < 4 from 23.3% ± 26.3% to 10.4% ± 9.6%, as well as 80% improvement from a Los Angeles endoscopic classification of Grade A, B, or C to Grade O. Overall, 66% discontinued PPIs or H₂RA medication, 76% discontinued at least 50% of their medications, and 54% to 66% experienced complete resolution of GERD symptoms^[15]. Meanwhile, Arts *et al.*^[27] performed ELGP in 20 PPI-resistant GERD patients, and reported that 13 and 6 patients were able to discontinue PPI medication 3 and 12 mo later, respectively, and pH monitoring also revealed normalization. These results showed that this treatment method could be indicated for PPI-resistant patients, but there have thus far not been any reports involving PPI-resistant NERD patients.

As previously reported, the mechanism of ELGP is believed to involve a decrease in esophageal acid exposure^[15]. However, in the present study, 24-h intraesophageal pH monitoring revealed mild worsening rather than improvement in the percent time pH < 4 after ELGP. Although it can be said that this was caused by the fact that pH was monitored prior to ELGP while the patients were on full-dose PPI, the fact that symptoms improved and that the rate of PPI medication decreased in patients with GERD symptoms despite being on PPI medication

suggests that factors other than esophageal acid exposure are involved in the development of symptoms in PPI-resistant NERD patients. ELGP involves the endoscopic formation of folds, which are believed to physically prevent reflux through bosselation at the gastroesophageal junction. However, 24-h intraesophageal pH impedance monitoring in the present study did not reveal significant decreases in the percent time pH < 4 or number of episodes of reflux before and after ELGP. Although the possibility cannot be ruled out that esophageal reflux was not adequately controlled because of the spontaneous sloughing off of plications 3 mo after ELGP, it has also been reported elsewhere that pH monitoring revealed no changes before and after surgery^[36,37], and many questions remain regarding the relationship between esophageal acid exposure and the development of symptoms.

In the present study, the number of symptom events and SSI decreased significantly, regardless of the lack of change in the number of episodes of reflux and SI (Figure 3). These results mean that the specificity of a NERD patient's reflux symptoms did not change, but the subject's sensitivity for reflux decreased after surgery. This suggests that factors other than esophageal reflux are involved in the development of the symptoms of NERD, and the presence of esophageal hyperesthesia may be one such factor. Patients with GERD experience proximal esophageal reflux more than normal individuals, but the incidence of proximal esophageal symptoms is significantly higher in NERD patients than in GERD patients^[38]. In addition, the time for symptoms to develop during 10 min of intraesophageal hydrochloric acid infusion, the intensity of symptoms, and the sensitivity index are significantly higher in NERD patients than in normal individuals, patients with reflux esophagitis, and patients with Barrett's esophagus^[39].

Animal studies have confirmed fibrosis near plications and local thickening of smooth muscle of the gastroesophageal junction^[40,41]. It has thus been postulated that sensory nerves densely distributed in the gastroesophageal junction might be damaged by plication, resulting in a loss of sensitivity^[42]. The lack of apparent differences in symptom scores between cases in which plications completely disappeared and cases in which they remained suggests the involvement of esophageal hyperesthesia in the pathology of PPI-resistant NERD. Mechanisms in which tissue degeneration and scar formation in the gastroesophageal junction result in changes in compliance and internal pressure, as well as transient lower esophageal sphincter relaxation, have also been considered^[43].

GSRs has found overlap in various gastrointestinal symptoms and decreases in the scores of all items in the SF-36, and decreases in health-related quality of life (HR-QOL) have been confirmed in PPI-refractory NERD patients. In the present study, ELGP was found to improve HR-QOL in PPI-resistant NERD patients, and the long-term effects were confirmed not only at 3 mo after surgery, but also at 12 mo, showing no recurrence of GERD symptoms and a decrease in PPI use. These find-

ings suggest that HR-QOL was maintained.

Montgomery *et al.*^[44] conducted a randomized, comparative study of ELGP and placebo treatment in 46 patients with erosive GERD. After 3 mo, there was significant improvement in heartburn symptoms and PPI use in the ELGP group, but it was reported that the differences from the sham treatment group disappeared after 12 mo. Overall, 71% and 67% of the plications remained after 3 and 12 mo, respectively, and the attenuation of the effects was attributed to the decrease over time in the percentage of remaining plications^[45,46]. On the other hand, while an apparent loss of plications over time was observed in the present study of PPI-resistant NERD patients, there was virtually no recurrence of symptoms, and patients were able to wean themselves off of medication over the long term. It is therefore possible that ELGP is more useful in NERD, particularly PPI-resistant NERD that is not amenable to oral treatment, than in erosive GERD. The results also suggest differences in the pathology of erosive GERD and NERD.

Most endoscopic treatment in GERD to date has been in patients for whom oral treatment was effective, but the present study was unique in that it looked at cases not amenable to oral treatment and demonstrated the efficacy and safety of the treatment. However, the study suffered from several shortcomings. The first is the lack of any placebo treatment as a control, and the limited number of cases. In actuality, no sham groups were established in many preceding studies of endoscopic treatment^[15,25-27,36,47]. It is often difficult to establish a rigorous model, because the frequent development of complications in active treatment groups hinders blinding, and because increased efficacy is also sometimes observed in sham groups. In the future, it will be necessary to conduct a blinded, randomized, comparative study with a sufficient sample size in order to confirm the efficacy of ELGP treatment in PPI-resistant NERD patients suggested in this study. Second, the 12-mo observation period was inadequate, and it will be necessary to study efficacy for a longer period of time from medical and economic perspectives. It is also possible that the improvement in symptoms by ELGP in PPI-resistant NERD patients results from a variety of mechanisms, including esophageal hyperesthesia, in addition to the physical action of preventing reflux, and further study from that perspective is also necessary.

Despite the many limitations of this study, this is the first report to show endoscopic treatment to be highly effective and safe in PPI-resistant NERD patients who are not amenable to oral treatment and who suffer from significantly compromised QOL. With the accumulation of evidence in the future, it is possible that there will be new indications for endoscopic therapy, including post-gastrectomy GERD and complementary therapy until surgery or postoperative salvage for GERD.

In conclusion, in this study, ELGP significantly improved subjective symptoms and reduced long-term oral medication in Japanese PPI-resistant NERD patients, and it appears to be a safe and useful method of treatment.

COMMENTS

Background

Non-erosive reflux disease (NERD) refers to a syndrome that is characterized by complaints of reflux symptoms such as heartburn without endoscopic evidence of mucosal injury. The severity and frequency of NERD symptoms is similar to erosive gastroesophageal reflux disease (GERD) and is often not amenable to treatment with oral medication. Especially, PPI-resistant NERD is the most intractable NERD patients. Instead of medication therapy, the surgical treatment of erosive GERD, laparoscopic fundoplication, has been developed. On the other hand endoscopic techniques have been developed recently as less invasive but effective methods for erosive GERD.

Research frontiers

Endoscopic treatments were proposed as less invasive but effective treatment methods for GERD. Especially, endoluminal gastroplication (ELGP) is an extremely safe method among such treatments and its efficacy in erosive GERD is also being studied. Most endoscopic treatment in GERD to date has been in patients for whom oral treatment was effective, but the present study was unique in that it looked at cases not amenable to oral treatment and demonstrated the efficacy and safety of the treatment.

Innovations and breakthroughs

Before and after ELGP, patients' symptoms, quality of life (QOL) and 24-h intra-esophageal pH/impedance (MII-pH) monitoring were assessed. After ELGP, the symptoms, QOL and esophageal sensitivity improved significantly. Furthermore, 66.7% patients were able to discontinue proton pump inhibitor (PPI) medication. This is the first report to show endoscopic treatment to be highly effective and safe in PPI-resistant NERD patients who are not amenable to oral treatment and who suffer from significantly compromised QOL.

Applications

With the accumulation of evidence in the future, it is possible that there will be new indications for endoscopic therapy, including postgastrectomy GERD and complementary therapy until surgery or postoperative salvage for GERD.

Terminology

NERD: It is a syndrome that is characterized by complaints of reflux symptoms such as heartburn without endoscopic evidence of mucosal injury; ELGP is a recently introduced endoscopic therapy for GERD refractory to medical therapy. This novel approach involves the insertion of an endoscopic suturing device into the esophagus to create partial-thickness, internal gastric plications that serve as an anti-reflux barrier.

Peer review

This is a good clinical study in which authors analyze the endoscopic therapy effects for PPI-resistant NERD patients who are not amenable to oral treatment. The results are interesting and suggest that this endoscopic technique create a new indication for NERD therapy, especially PPI-resistant NERD who are the most intractable of NERD patients.

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Electrogastrography associated with symptomatic changes after prokinetic drug treatment for functional dyspepsia

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Abstract

AIM: To evaluate the effect of prokinetic drugs on electrogastrography (EGG) parameters according to symptomatic changes in patients with functional dyspepsia (FD).

METHODS: Seventy-four patients with FD were prospectively enrolled in this study between December 2006 and December 2010. We surveyed the patients using a questionnaire on dyspeptic symptoms before and after an 8-wk course of prokinetic drug treatment. We also measured cutaneous pre-prandial and post-prandial EGG recordings including percentage of gastric waves (normogastria, bradygastria, tachygastria), dominant frequency (DF), dominant power (DP), dominant frequency instability coefficient (DFIC), dominant power instability coefficient (DPIC), and the ratio of post-prandial to fasting in DP before and after the 8-wk course of prokinetic drug treatment.

RESULTS: Fifty-two patients (70%) achieved symptomatic improvement after prokinetic drug treatment.

Patients who had normal gastric slow waves showed symptom improvement group after treatment. Post-prandial DF showed a downward trend in the symptom improvement group, especially in the itopride group. Post-prandial DP was increased regardless of symptom improvement, especially in the itopride group and mosapride group. Post-prandial DFIC and DPIC in the symptom improvement group were significantly increased after the treatment. The EGG power ratio was increased after treatment in the symptom improvement group (0.50 ± 0.70 vs 0.93 ± 1.77 , $P = 0.002$), especially in the itopride and levosulpiride groups.

CONCLUSION: Prokinetics could improve the symptoms of FD by regulating gastric myoelectrical activity, and EGG could be a useful tool in evaluating the effects of various prokinetics.

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Key words: Electrogastrography; Functional dyspepsia; Itopride; Mosapride; Levosulpiride

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INTRODUCTION

Electrogastrography (EGG) is a noninvasive technique for recording gastric myoelectrical activity using electrodes on the abdominal wall overlying the stomach. EGG has been used as a diagnostic tool to determine the mechanism of symptom generation in patients who have dyspeptic symptoms, including nausea, vomiting, post-prandial fullness, bloating, and early satiety, due to gastric motility disorders and abnormal gastric myoelectrical activity^[1]. The EGG records the rhythms of gastric slow waves, which provide information on the velocity and propagations of gastric contractions. The previous studies showed the associations of tachyarrhythmia with absent antral contractions, and bradyarrhythmia with strong or absent antral contractions^[2]. Gastric dysrhythmia including bradygastria and tachygastria is observed in 31%-69% of patients with functional dyspepsia (FD), and several gastric rhythm abnormalities were described in patients with diabetic gastroparesis and motion sickness^[3-6]. EGG also records the gastric myoelectrical activities which show the amplitude of gastric contraction. The amplitude increases in the post-prandial state in healthy populations (90%-95%) and a lack of an increase is believed to reflect decreased gastric motor activity^[7].

Prokinetic drugs are used to treat FD by potentially enhancing gastrointestinal motility and accelerating gastric emptying. Several prokinetic drugs, such as cisapride and domperidone, are known to correct dysrhythmias and symptoms in patients with gastroparesis and dyspepsia^[8,9]. Recently, prokinetic drugs, such as itopride hydrochloride, mosapride citrate, and levosulpiride, were used widely for treatment of upper gastrointestinal motility disease, but the clinical utility of changes in EGG parameters after treatment with these prokinetics in patients with FD symptoms has not been well established^[10].

This prospective study was conducted to evaluate the effect of itopride hydrochloride, mosapride citrate, and levosulpiride on EGG parameters according to symptomatic changes in patients with FD.

MATERIALS AND METHODS

Patients

This study was a prospective study approved by the Institutional Review Committee of Yonsei University Health System and was conducted in compliance with the Declaration of Helsinki. All patients were fully informed of the purposes of the study and written informed consent was obtained from all patients prior to participation.

We reviewed patients who visited the Gangnam Severance Hospital, Yonsei University, South Korea for dyspeptic symptoms between December 2006 and December 2010. Patients with symptoms meeting the Rome III criteria for FD underwent the following procedures^[11]: an interview on medical history, physical examination,

hematologic and chemical evaluations, upper esophagogastro-duodenoscopy or an upper gastrointestinal series, before taking prokinetic drugs. Exclusion criteria included patients (1) who had organic or metabolic diseases (i.e., diabetes mellitus, liver cirrhosis); (2) who had gastrointestinal diseases which had associated dyspeptic symptoms such as inflammatory bowel disease, cancer and ulcers; (3) who had a history of abdominal surgery; and (4) who were taking drugs which could affect gastrointestinal motility, including other prokinetics, cholinergic/anticholinergic agents, and antidepressive agents, for at least 4 wk prior to study start.

Method

Protocol for drug administration: A prokinetic drug was administered after patients completed the questionnaires on FD and baseline EGG recordings were completed. The patients were assigned to one of 3 groups based on the type of treatment drug: itopride hydrochloride (Ganaton[®], Choogwae Pharma, South Korea) ($n = 24$), mosapride citrate (Gasmotin[®], Daewoong Pharma, South Korea) ($n = 28$), and levosulpiride (Levopride[®], SK Chemical Life Science, South Korea) ($n = 22$). Itopride hydrochloride (50 mg tablet), mosapride citrate (5 mg tablet), and levosulpiride (25 mg tablet) were administered to patients in each group 3 times a day in the post-prandial state for 8 wk, and drugs which could affect gastrointestinal function were not allowed to be used throughout the study.

Questionnaires for functional dyspepsia: Symptoms of epigastric pain, epigastric burning, post-prandial fullness, early satiety, post-prandial bloating, and post-prandial nausea or excessive belching were scored in accordance with the following scheme: 0 = none, 1 = mild (symptoms could be ignored if the patient did not think about it), 2 = moderate (symptoms could not be ignored but did not influence daily activities), 3 = severe (symptoms influenced daily activities)^[12]. For each patient, the total symptom severity score was the sum of the 6 symptom scores (minimum 0 to maximum 18). The frequency of dyspeptic symptoms also described above was scored in accordance with the following scheme: 0 = none, 1 = once or twice a month, 2 = once or twice a week, 3 = more than 3 times a week. These scores were added to yield the total symptom frequency score (minimum 0 to maximum 18). The questionnaires were completed again after 8-wk treatment.

Electrogastrography: EGG (Digitrapper EGG; Synetics Medical Inc, Stockholm, Sweden) was used to record gastric myoelectrical activity with low and high cutoff frequencies of 1 and 10 cpm, respectively. After an overnight fast, EGG recordings were obtained in the morning for 30 min in the fasting state and for another 30 min after a test meal at baseline before treatment. This procedure was repeated after 8-wk treatment. To

Table 1 Patient demographics and pattern of dysrhythmia according to symptom improvement

	Total patients (n = 74)		Symptom improvement (n = 52)		Symptom resistance (n = 22)	
Male:female (n)	26:48		18:34		8:14	
Age (range), yr	51.7 (19-70)		53.5 (27-70)		47.6 (19-70)	
Height	165.2 ± 12.4		166.3 ± 11.6		164.9 ± 10.1	
Weight	61.3 ± 9.7		62.3 ± 9.1		58.8 ± 10.2	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Symptom severity score	8.09 ± 0.43	5.51 ± 0.46 ^a	8.90 ± 0.46	4.52 ± 0.47 ^a	6.18 ± 0.85	7.86 ± 0.92
Symptom frequency score	9.27 ± 0.49	6.81 ± 0.54 ^a	9.96 ± 0.54	5.79 ± 0.60 ^a	7.64 ± 1.01	9.23 ± 0.97
Gastric dysrhythmia (pre-prandial)						
Bradygastria	16 (21.6)	11 (14.9)	9 (17.3)	7 (13.5)	6 (27.3)	4 (18.2)
Normogastria	33 (44.6)	52 (70.3) ^a	26 (50.0)	37 (71.2) ^a	9 (40.9)	15 (68.2)
Tachygastria	25 (33.8)	11 (14.9) ^a	17 (32.7)	8 (15.4) ^a	7 (31.8)	3 (13.6)
Gastric dysrhythmia (post-prandial)						
Bradygastria	17 (23.0)	10 (13.5)	10 (19.2)	6 (11.5)	4 (18.2)	4 (18.2)
Normogastria	33 (44.6)	45 (60.8)	26 (50.0)	37 (71.2) ^a	11 (50)	13 (59.1)
Tachygastria	24 (32.4)	19 (25.7)	16 (30.8)	14 (26.9)	7 (31.8)	5 (22.7)

^a*P* < 0.05 vs pre-treatment group. Data are presented by mean ± SD or *n* (%).

reduce the resistance between electrode and skin, hair was shaved and skin abraded with prepping paste (OMNI PREP[®], D.O. Weaver & Co. United States) on the abdomen, and conductive cream (Signa Creme[®], Parker Laboratories, United States) was applied to the skin. Two electrodes were placed on the abdomen, one midway between the xyphoid process and umbilicus, and the other 5 cm to the left, just below the costal margin. A reference electrode was placed on the right side of the abdomen. These electrodes were connected to a Digitrapper EGG recording devices. The patients were in a sitting position leaning 45° in a comfortable chair. The test meal was composed of solid food (rice rolled up in dried seaweed with orange juice, 500 kcal). The EGG data were uploaded into a personal computer and analyzed by a software program (Polygram for Windows, version 6.40, Synetics Medical Inc, Stockholm, Sweden).

EGG recordings were analyzed to derive the following parameters: (1) percentage of normal gastric waves (2.0-4.0 cpm), bradygastric waves (1.0-2.0 cpm), and tachygastric waves (4.0-10.0 cpm); (2) dominant frequency (DF); (3) dominant power (DP); (4) dominant frequency instability coefficient (DFIC, %); (5) dominant power instability coefficient (DPIC, %); and (6) the ratio of post-prandial to fasting in DP. A percentage of normal slow wave frequency of more than 70% was defined as normal.

Statistical analysis

The patients were classified into 2 groups: a symptom improvement group if symptom severity and frequency scores decreased after treatment with prokinetic drugs; and a symptom resistance group if symptom severity and frequency scores increased or were unchanged after treatment. EGG parameters at baseline were compared with post-treatment EGG parameters, according to symptomatic improvement and types of prokinetic

drugs used in this study.

Demographic data, questionnaire scores and parameters recorded in EGG were statistically analyzed by the paired Student *t* test and Fisher's exact test using SPSS 17.0. Data are expressed as the mean ± SE and a *P*-value < 0.05 was considered significant.

RESULTS

This study included 74 patients (26 men, 48 women: median age 51.7 years, range: 19-70 years). After 8 wk of prokinetic drug treatment, 52 patients (70%) showed symptomatic improvement, while 22 patients (30%) had no improvement or aggravated symptoms. There were no significant demographic differences between patients with improved symptoms and those without improvement (Table 1). There were no significant differences in demographics, and symptom improvement rate among the itopride hydrochloride group, the mosapride citrate group, and the levosulpiride group (Table 2).

Symptom scores for functional dyspepsia

The mean symptom severity score for all patients was 8.09 ± 0.43 at baseline vs 5.51 ± 0.46 post-treatment (*P* < 0.05). Symptom severity scores were significantly decreased in the symptom improvement group, while there were no significant changes in the symptom resistance group. Symptom severity scores were significantly decreased after all prokinetic drugs (Table 2).

The mean symptom frequency score of all patients was 9.27 ± 0.49 at baseline and 6.81 ± 0.54 after treatment (*P* < 0.05). Symptom frequency scores were significantly decreased in the symptom improvement group, while there were no significant changes in the symptom resistance group. Symptom severity scores were decreased after all prokinetic drugs, but significant differences were shown only in the itopride hydrochloride

Table 2 Demographic and treatment success of patients and pattern of dysrhythmia according to prokinetic drugs

	Itopride (<i>n</i> = 24)		Mosapride (<i>n</i> = 28)		Levosulpiride (<i>n</i> = 22)	
Male:female (<i>n</i>)	5:19		10:18		11:11	
Age (range), yr	49.8 (30-64)		49.6 (19-70)		56.6 (39-70)	
Height	166.1 ± 10.1		163.0 ± 11.0		167.1 ± 13.2	
Weight	60.8 ± 7.7		62.7 ± 11.5		61.3 ± 10.5	
Symptom improvement	18 (75)		17 (61)		17 (77)	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Symptom severity score	7.96 ± 0.84	5.00 ± 0.86 ^a	7.61 ± 0.64	5.46 ± 0.79 ^a	8.86 ± 0.76	6.14 ± 0.76 ^a
Symptom frequency score	9.21 ± 0.92	6.08 ± 0.99 ^a	8.86 ± 0.78	6.86 ± 0.89	9.86 ± 0.83	7.55 ± 0.96
Gastric dysrhythmia (pre-prandial)						
Bradygastria	5 (20.8)	4 (16.7)	6 (21.4)	4 (14.3)	4 (18.2)	3 (13.6)
Normogastria	8 (33.3)	17 (70.8) ^a	15 (53.6)	18 (64.3)	12 (54.5)	15 (68.2)
Tachygastria	11 (45.8)	3 (12.6) ^a	7 (25)	6 (21.4)	6 (27.3)	4 (18.2)
Gastric dysrhythmia (post-prandial)						
Bradygastria	5 (20.8)	3 (12.5)	6 (21.4)	6 (21.4)	3 (13.6)	1 (4.5)
Normogastria	11 (45.8)	14 (58.3)	12 (42.9)	16 (57.1)	14 (63.6)	15 (68.2)
Tachygastria	8 (33.3)	7 (29.2)	10 (35.7)	6 (21.4)	5 (22.7)	6 (27.3)

^a*P* < 0.05 vs pre-treatment group. Data are presented by mean ± SD or *n* (%).

group (Table 2).

Parameters of EGG recording

Patients who had gastric dysrhythmia: After prokinetic treatment, the number of patients who had normal gastric slow waves was increased in the symptom improvement group and in the itopride treatment group. In particular, the number of patients who had tachygastria were decreased in the symptom improvement group and in the itopride treatment group (Tables 1 and 2).

Percentage of gastric slow waves: The pre-prandial percentage of gastric slow waves was 64.99% ± 2.93% for normal, 14.01% ± 1.93% for bradygastria, and 18.73% ± 2.24% for tachygastria at pre-treatment (Figure 1A). At the end of the 8-wk treatment, the percentage of pre-prandial gastric slow waves was 68.47% ± 2.54% for normal, 16.12% ± 2.94% for bradygastria, and 15.09% ± 1.71% for tachygastria. Dysrhythmia did not show significant changes regardless of symptom improvement. The itopride treatment group showed significant decreases in pre-prandial tachygastria, but there were no significant changes in the mosapride and levosulpiride treatment groups. The percentage of post-prandial gastric slow waves was 63.08% ± 2.25% for normal, 16.52% ± 2.22% for bradygastria and 20.09% ± 2.05% for tachygastria at pre-treatment. At the end of prokinetic treatment, the percentage of post-prandial gastric slow waves was 63.87% ± 2.25% for normal, 16.52% ± 1.92% for bradygastria, and 20.09% ± 19.48% for tachygastria (Figure 1B). There were no significant changes regardless of symptom improvement, nor were there any significant changes among the itopride, mosapride, and levosulpiride treatment groups.

Dominant frequency and dominant power: Pre-pra-

ndial DF showed no significant changes regardless of symptom improvement or type of prokinetic drug (Figure 2). Post-prandial DF was decreased after treatment in the symptom improvement group and especially in the itopride treatment group. Pre-prandial DP showed no significant changes regardless of symptom improvement or prokinetic drug after treatment. Post-prandial DP was increased regardless of symptom improvement especially in the itopride group (19.34 ± 6.08 at baseline vs 42.49 ± 6.13 after treatment, *P* = 0.010) and mosapride group (24.04 ± 6.47 at baseline vs 56.24 ± 11.83 after treatment, *P* = 0.020).

Dominant frequency instability coefficient and dominant power instability coefficient: Pre-prandial DFIC and DPIC after treatment were not changed regardless of symptom improvement and type of prokinetic drug (Figure 2). Post-prandial DFIC and DPIC were significantly increased after treatment (74.29% ± 24.45% vs 82.69% ± 27.05%, *P* = 0.035) in the symptom improvement group, but there was no significant differences between the prokinetics.

Power ratio: After treatment, the EGG power ratio was increased in the symptom improvement group (0.64 ± 0.07 vs 1.23 ± 0.16, *P* = 0.002), especially in the levosulpiride treatment group (Figure 3).

DISCUSSION

FD is a common clinical syndrome characterized by pain or discomfort in the upper abdomen without any identifiable structural or biochemical abnormality. The pathophysiology of FD involves various mechanisms, including delayed gastric emptying, impaired accommodation in the proximal stomach, and increase duodenal

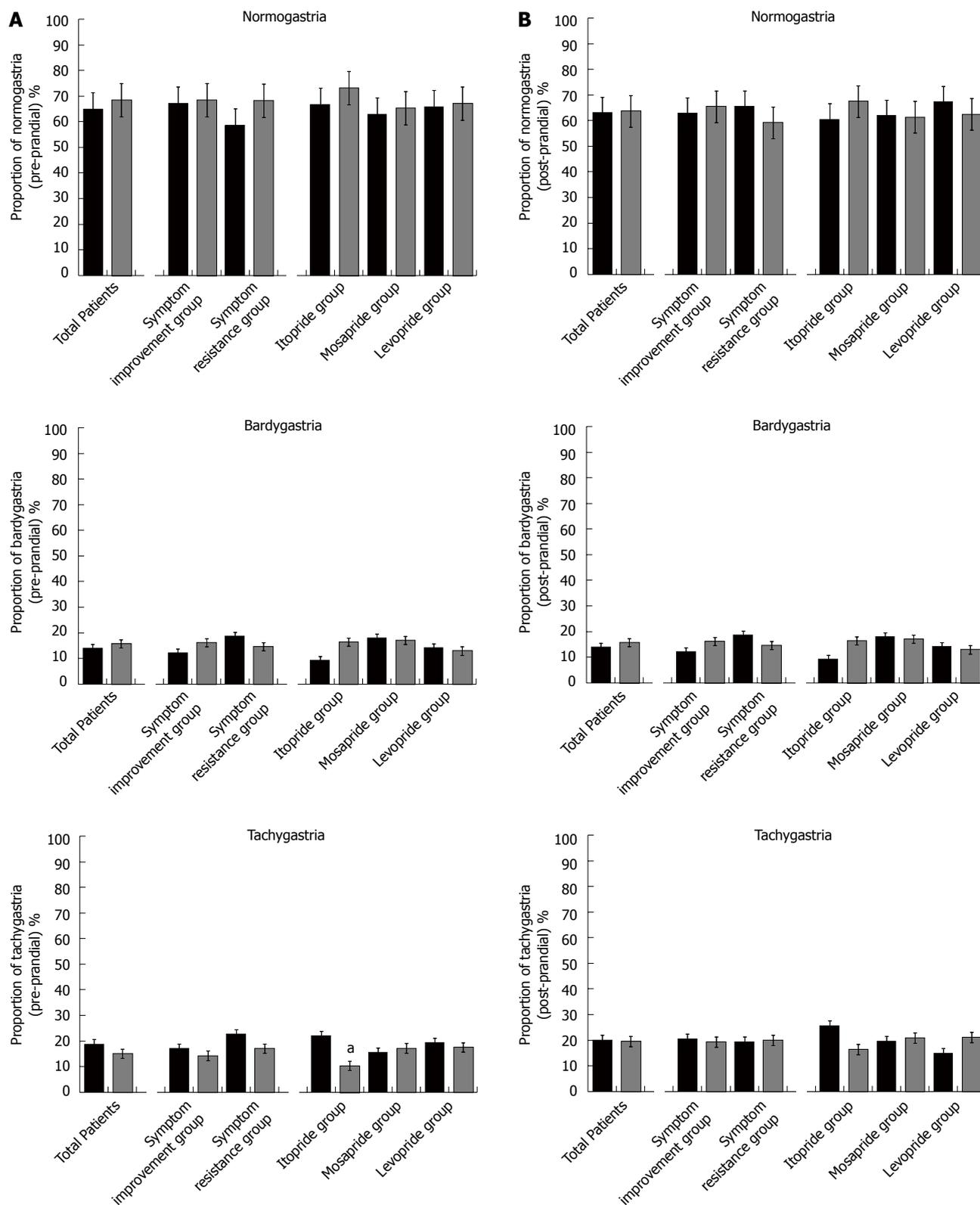


Figure 1 Proportion of gastric slow waves on electrogastrography. A: Pre-prandial; B: Post-prandial. ^a*P* < 0.05 vs pre-treatment.

sensitivity to lipid or acid, and pathologic factors include genetic susceptibility, *Helicobacter pylori* (*H. pylori*) infection, and psychological factors^[13]. There has been no single available therapy for FD due to the heterogeneity of the symptoms and various mechanisms and pathologic

factors. Accordingly, a wide variety of treatment methods have been used for FD such as dietary and lifestyle modification, *H. pylori* eradication, antacids, mucosal protectants, prokinetics, and psychological and complementary therapy^[13].

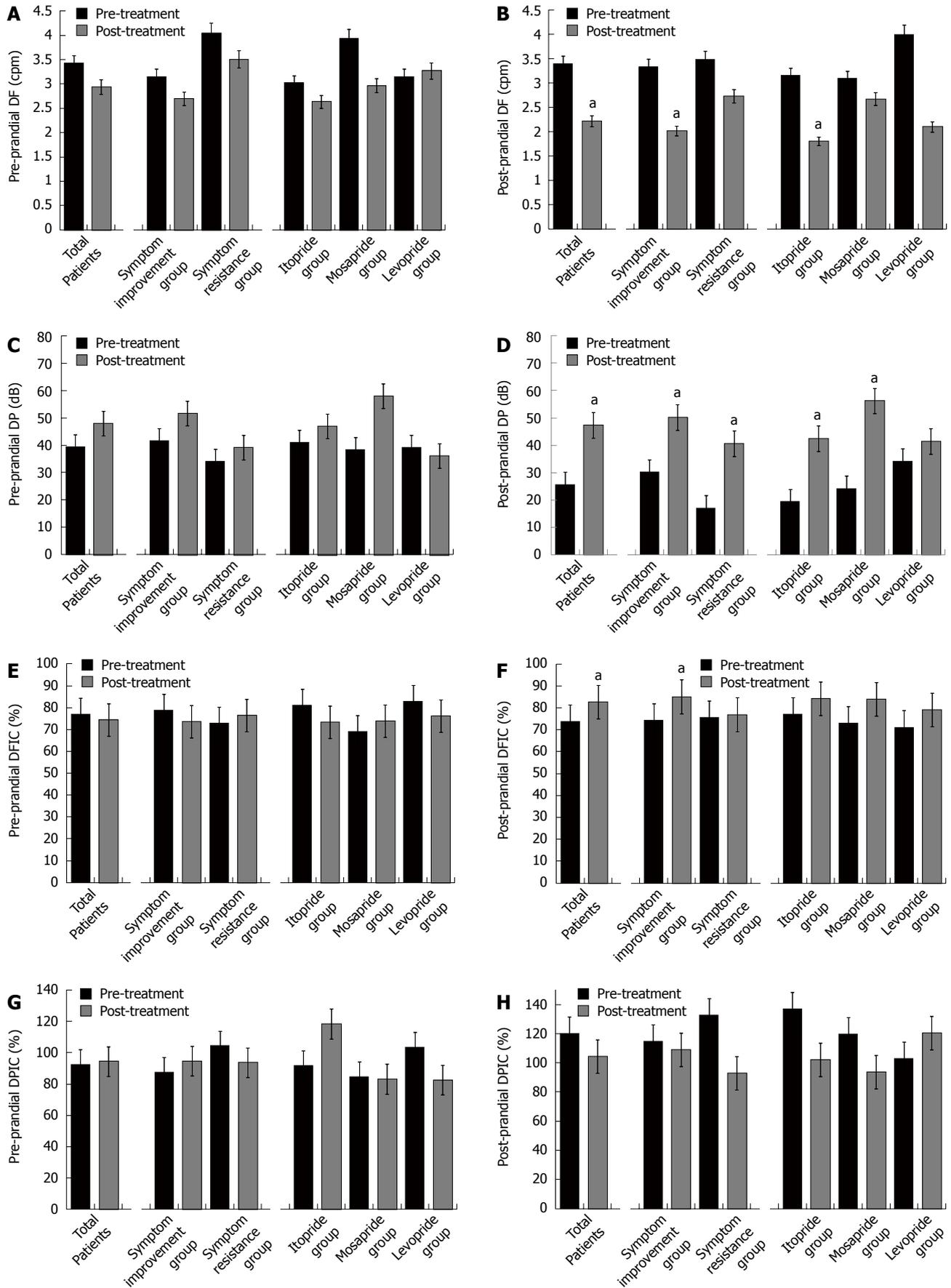


Figure 2 Changes in dominant frequency and dominant power, dominant frequency instability coefficient and dominant power instability coefficient after prokinetic treatment. A: Dominant frequency (DF) in pre-prandial electrogastronomy (EGG); B: DF in post-prandial EGG; C: Dominant power (DP) in pre-prandial EGG; D: DP in post-prandial EGG; E: Dominant frequency instability coefficient (DFIC) in pre-prandial EGG; F: DFIC in post-prandial EGG; G: Dominant power instability coefficient (DPIC) in pre-prandial EGG; H: DPIC in post-prandial EGG. ^a $P < 0.05$ vs pre-treatment.

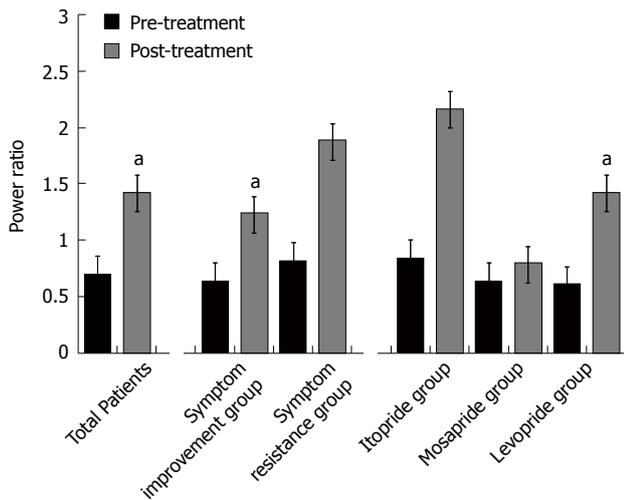


Figure 3 Power ratio before and after the 8-wk course of treatment. ^a*P* < 0.05 vs pre-treatment.

Abnormal gastric motility such as delayed gastric emptying or uncoordinated antral contraction is common in functional dyspepsia^[14,15]. Gastrointestinal motor dysfunctions can be assessed by gastric emptying scan and/or manometry, and gastric myoelectrical abnormalities can be detected by noninvasive cutaneous EGG. EGG as a diagnostic technique has been frequently used for the detection of gastric dysrhythmia in patients with nausea, vomiting and other dyspeptic symptoms. Several previous studies have shown a positive correlation between abnormal EGG and delayed gastric emptying^[16-18].

The most common abnormal EGG finding is dysrhythmia, low EGG power ratio and high instability coefficient^[19-22]. The percentage of patients who had gastric dysrhythmia (percent of normal slow waves < 70%) were 55.4% at pre-prandial and post-prandial periods in our study. This data was similar to previous studies which reported dysrhythmias in 31%-69% of functional dyspepsia cases^[4]. However, we did not find any significant difference in the percentage of gastric slow waves between the symptom improvement group and the symptom resistance group after treatment and there were no correlations between gastric dysrhythmia and symptom severity or symptom frequency either. This could be because FD symptoms are caused by different abnormalities, for example, impaired gastric accommodation (vagally and nitrergically mediated mechanisms) may cause symptoms but this has little to do with gastric slow waves^[23].

Prokinetics such as cisapride (5-HT₄ agonist/weak 5-HT₃ antagonist) and domperidone (D₂ antagonist) have been shown to improve gastric dysrhythmia in patients with diabetic gastroparesis, whereas low dose erythromycin was reported to have no effects on dysrhythmia^[24-27]. Few studies showed that mosapride improved the gastric dysrhythmia and power ratio. In our study, itopride, mosapride and levopride showed

improvements in gastric dysrhythmia in the pre-prandial state, but significant differences were shown only with itopride^[28,29].

The DF reflects the regularity of gastric slow waves and the DP reflects the amplitude of gastric slow waves. However, the relationship of DF and DP with functional dyspepsia was not clear^[30]. Our data showed a decrease in post-prandial DF in the symptom improvement group, and post-prandial DP was high regardless of symptom improvement. Itopride significantly decreased post-prandial DF, and both itopride and mosapride increased post-prandial DP. According to this study, prokinetics might improve the symptoms of FD by improvement in dysrhythmic gastric movement which is represented by decreased DF, and by activating gastric movement which is represented by increased DP.

DPIC increases during antral contractions, and DFIC increases during pregnancy and in patients with gastroesophageal reflux disease. Previous studies showed that pediatric patients who have dyspeptic symptoms reported a high instability coefficient, however, there was not enough data showing the relationship between the DPIC/DFIC and clinical symptoms in FD patients clearly^[31-33]. Our data showed increased DPIC/DFIC in the symptom improvement group after prokinetic drug treatment^[34]. Increased DPIC/DFIC might be due to the increased variability of changes in gastric movement activated by prokinetics.

The EGG power ratio increases after an appropriate test meal in normal subjects, and decreases in gastroparesis and FD patients^[1]. The EGG power ratio increased in responders after prokinetic treatment with itopride and levosulpiride, but not with mosapride in our study. The EGG power ratio is believed to be associated with gastric contractility; the increase in the EGG power ratio observed in this study reflected an increase in gastric contractions. This data is in agreement with previous studies in that prokinetics, especially levosulpiride, increased gastric contractions or gastric emptying^[35].

In summary, dysrhythmia was recorded about half of the time in FD patients, and prokinetic treatment successfully improved symptoms. The symptom improvement group showed decreased post-prandial DF and increased post-prandial DP, DFIC/DPIC and power ratio after treatment with prokinetics. Itopride improved gastric dysrhythmia, decreased post-prandial DF, and increased post-prandial DP; mosapride increased post-prandial DP and levosulpiride increased the EGG power ratio.

The mechanism of prokinetics on gastric electrical activity could be (1) to stabilize the gastric slow waves which is represented by an improvement in gastric dysrhythmia and a decrease in post-prandial DF; and (2) to increase gastric motility which is represented by an increase in post-prandial DP and in the EGG power ratio by activating gastric movements which is represented by increased DPIC/DFIC.

In conclusion, the findings of this study suggest that prokinetics could improve the symptoms of FD by regulating gastric myoelectrical activity, and the EGG could be a useful tool to evaluate the effects of various prokinetics.

COMMENTS

Background

Electrogastrography (EGG) abnormalities are frequently observed in patients with functional dyspepsia (FD). However, changes in EGG parameters after treatment with prokinetics according to symptom improvement have not been well investigated.

Research frontiers

Prokinetic drugs are used in functional dyspepsia to enhance gastrointestinal motility and correct dysrhythmias in FD patients. In this study, the authors observed that prokinetics could improve the symptoms of FD by regulating gastric myoelectrical activity.

Innovations and breakthroughs

Prokinetics successfully improved symptoms of FD, but the improvement did not seem to be correlated with any of the EGG parameters. Instead, there were some unique changes in EGG parameters according to the prokinetic drug. This study suggests that different prokinetics may have different mechanisms of action in regulating gastric myoelectrical activity, and the EGG could be a useful tool in evaluating the effects of various prokinetics.

Applications

There was controversy in the significance of EGG as diagnostic tool in FD due to the lack of data and standardized methodology. By understanding the changes in EGG parameters, this study might indicate a future strategy for EGG in evaluating the improvement in FD after prokinetic drug treatment. This study is an important basis for future experiments using EGG in pharmacology.

Terminology

EGG represents gastric myoelectrical activity. Dysrhythmia (bradygastria, tachygastria) reflect uncoordinated antral contraction, and the power ratio reflects gastric contractions. Dominant frequency reflects the regularity of gastric slow waves and dominant power reflects the amplitude of gastric slow waves. Dominant power instability coefficient increases during antral contractions and, dominant frequency instability coefficient increases during pregnancy.

Peer review

The authors tried to clarify the relation of EGG and FD symptoms and found the symptom improvement group after prokinetics therapy showed decreased post-prandial dominant frequency and increased dominant frequency instability coefficient/dominant power instability coefficient and increased power ratio.

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Comparative study of rendezvous techniques in post-liver transplant biliary stricture

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Abstract

AIM: To investigate the usefulness of a new rendezvous technique for placing stents using the Kumpe (KMP) catheter in angulated or twisted biliary strictures.

METHODS: The rendezvous technique was performed in patients with a biliary stricture after living donor liver transplantation (LDLT) who required the exchange of percutaneous transhepatic biliary drainage catheters for inside stents. The rendezvous technique was performed using a guidewire in 19 patients (guidewire group) and using a KMP catheter in another 19 (KMP catheter group). We compared the two groups retrospectively.

RESULTS: The baseline characteristics did not differ between the groups. The success rate for placing inside

stents was 100% in both groups. A KMP catheter was easier to manipulate than a guidewire. The mean procedure time in the KMP catheter group (1012 s, range: 301-2006 s) was shorter than that in the guidewire group (2037 s, range: 251-6758 s, $P = 0.022$). The cumulative probabilities corresponding to the procedure time of the two groups were significantly different ($P = 0.008$). The factors related to procedure time were the rendezvous technique method, the number of inside stents, the operator, and balloon dilation of the stricture ($P < 0.05$). In a multivariate analysis, the rendezvous technique method was the only significant factor related to procedure time ($P = 0.010$). The procedural complications observed included one case of mild acute pancreatitis and one case of acute cholangitis in the guidewire group, and two cases of mild acute pancreatitis in the KMP catheter group.

CONCLUSION: The rendezvous technique involving use of the KMP catheter was a fast and safe method for placing inside stents in patients with LDLT biliary stricture that represents a viable alternative to the guidewire rendezvous technique.

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Key words: Rendezvous; Biliary stricture; Liver transplantation; Endoscopic retrograde cholangiography; Percutaneous transhepatic biliary drainage

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INTRODUCTION

Biliary strictures develop in approximately 30% of patients after living donor liver transplantation (LDLT) and 50%-70% of biliary strictures can be treated by endoscopic retrograde cholangiopancreatography (ERCP)^[1,2]. However, percutaneous transhepatic biliary drainage (PTBD) is recommended for patients in whom ERCP has failed^[3]. Because of complications associated with the use of PTBD catheters such as pain, leakage, or infection, replacing PTBD catheters with inside stents by ERCP is required in many patients with PTBD catheters. When an angulated or twisted biliary stricture interrupts passage of a guidewire over the stricture, it is difficult to replace the PTBD catheter with inside stents by ERCP^[1,4,5]. The rendezvous technique can be used to overcome this difficulty.

The rendezvous procedure combines the endoscopic technique with percutaneous transhepatic cholangiography (PTC) to facilitate cannulation of the bile duct in cases where previous endoscopic attempts have failed^[6-9]. This combined technique increases the success rate of biliary tract cannulation and facilitates the diagnosis and treatment of biliary tract disorders^[10-12]. We previously reported that the rendezvous technique allows for successful placement of inside stents in angulated or twisted biliary strictures after LDLT^[13]. In the classic rendezvous technique, a guidewire is used for an endoscopic approach to the bile duct. However, manipulation of the guidewire is difficult and somewhat cumbersome, and kinking or breakage of the guidewire can occur^[14]. The modified rendezvous technique involves pushing the guidewire from the common bile duct into inside the lumen of an ERCP cannula outside the ampulla in the duodenum^[14]. It is also often difficult to push the guidewire inside the lumen of the ERCP cannula.

We attempted to resolve these problems by using a Kumpe (KMP) catheter (5F, 40 cm, Cook, Bloomington, IN, United States; Figure 1) instead of a guidewire. A KMP catheter is short enough for easy manipulation and also reduces the risk of contamination during the procedure. The end of a KMP catheter is slightly angulated and turning the end is simple, which allows the KMP catheter to approximate the ERCP cannula, end-to-end. Herein, we evaluated the usefulness and safety of the new modified rendezvous technique using a KMP catheter to place inside stents into biliary strictures after LDLT and compared it with the rendezvous technique performed using a guidewire.

MATERIALS AND METHODS

Patients

Between November 2006 and June 2011, patients undergoing the rendezvous technique performed using a KMP catheter ($n = 19$) were compared retrospectively with those undergoing the rendezvous technique performed using a guidewire ($n = 19$) at a single institution. Abdominal computed tomography and magnetic resonance chol-

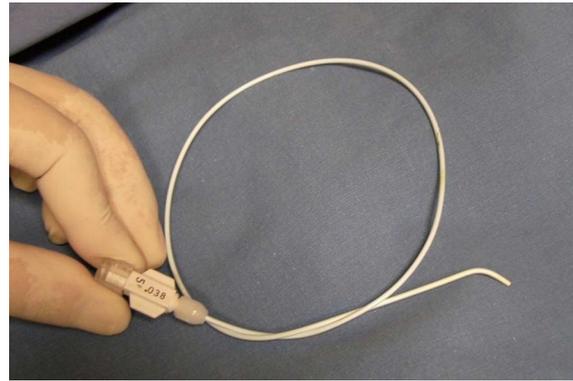


Figure 1 Kumpe catheter (5F, 40 cm).

angiography revealed that patients had biliary strictures at the anastomotic site after LDLT. They had PTBD catheters that were intended to be exchanged with inside stents. The rendezvous procedure was performed, because their anastomotic strictures were too angulated or twisted to place inside stents by ERCP. The rendezvous technique was performed using a guidewire before 2010. We invented the rendezvous technique using a KMP catheter in 2010, and have subsequently performed it ever since. No patient was treated using both techniques. All patients undergoing the rendezvous technique were consecutively enrolled. Patient anonymity was preserved and the Institutional Review Board of Seoul St. Mary's Hospital approved the study (KC11RISI0845). This study protocol was in complete compliance with the Declaration of Helsinki for medical research involving human subjects, as revised in Seoul in 2008.

Guidewire technique

One or two PTBD catheters were passed over the stricture in all patients. After an overnight fast, patients were sedated using midazolam and pethidine in the supine position. PTC was performed by injecting contrast medium through the PTBD catheter (Figure 2A). A guidewire (0.035 inch Jagwire; Boston Scientific, Natick, MA, United States) was introduced along the PTBD catheter until it advanced over the major ampulla into the duodenum, which was followed by removing the PTBD catheter (Figure 2B). After the patients were moved into a prone position, ERCP was performed using a video duodenoscope (ED-450XT5; Fujinon, Saitama City, Saitama, Japan). The guidewire exited the papilla and was identified inside the duodenal lumen using a duodenoscope. A minor sphincterotomy was performed alongside the guidewire in cases where an endoscopic sphincterotomy had not been performed. A bottle-top metal-tip ERCP cannula (MTW Endoscopie, Wesel, Germany) was introduced through the accessory channel of the duodenoscope and placed in front of the end of the guidewire (Figure 2C). The bottle-top metal-tip ERCP cannula and the guidewire were manipulated cautiously to insert the end of the guidewire into the ERCP cannula, which

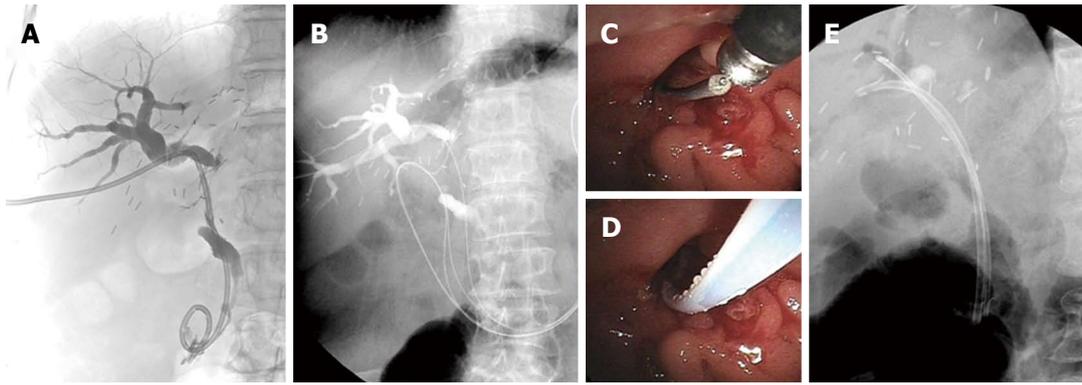


Figure 2 Guidewire technique. A: The percutaneous transhepatic biliary drainage (PTBD) catheter was located over the anastomotic stricture into the duodenum. The angle between the right hepatic duct and the common bile duct was steep (100°); B: The 0.035 inch guidewire was inserted through the PTBD catheter, and then the PTBD catheter was removed; C: The end of the guidewire was placed outside the papilla; D: The guidewire was inserted through a bottle-top metal-tip endoscopic retrograde cholangiopancreatography (ERCP) cannula, and then the ERCP cannula was advanced into the intrahepatic bile duct; E: Two inside stents were placed over the stricture in the anterior and posterior branches of the right hepatic duct in the recipient liver.

minimized damage to the guidewire (Figure 2D). The guidewire was pushed through the ERCP cannula and the ERCP cannula was advanced along the percutaneously inserted guidewire over the biliary stricture. Then, the percutaneously inserted guidewire was progressively withdrawn while another endoscopically inserted guidewire was pushed through the ERCP cannula. If the guidewire was not pushed easily through the ERCP cannula, the guidewire passing through the ampulla was captured by a basket and then withdrawn through the endoscopic working channel^[15]. After pulling the guidewire was completely out of the scope, the soft or floppy end of the guidewire was placed back into the ERCP cannula and advanced into the biliary tree. When a remaining stricture was suspected at the anastomotic site, balloon dilation of the anastomotic strictures was performed using a balloon catheter (6 or 8 mm in diameter; Hurricane RX; Boston Scientific, Natick, MA, United States). The inside stents were placed over the guidewire (Amsterdam-type biliary stents; 7F-11.5 F in diameter, 10-16 cm in length; Wilson-Cook Medical Winston-Salem, NC, United States, or Medi-Globe, Achenmuhle, Germany). The proximal side of the stent was located to cover the stricture, and the distal side of the stent passed 1-2 cm outside of the major papilla (Figure 2E). We intended to place the proximal end of the inside stent in the bile duct, not in the liver parenchyma, with assistance from fluoroscopic imaging. If we needed to insert another inside stent over the stricture, another guidewire was inserted retrogradely over the stricture site, and a second inside stent was placed along the second guidewire (Figure 2E). In cases where two guidewires had been inserted at different branches of the bile ducts along two PTBD tracts during PTC, two inside stents were placed along these guidewires.

The anastomotic angle between the common hepatic duct of the recipient and the right hepatic duct of the donor (confluence of the anterior and posterior branches) were measured. If a confluent duct was not obvious, we chose the intrahepatic duct (IHD) in which the PTBD catheter had been placed. After successful insertion of

the stents, a follow-up ERCP was performed within 3-6 mo. During the follow-up ERCP, the stents placed previously were removed, and the degree of improvement in biliary stricture and IHD or common bile duct (CBD) stones was evaluated. Restenting was performed if the stricture remained.

Kumpe catheter technique

The basic PTC and ERCP techniques were the same as the guidewire technique. During PTC, a guidewire was introduced along the PTBD catheter until it advanced over the major ampulla into the duodenum, which was followed by removal of the PTBD catheter. The KMP catheter (5F, 40 cm) was placed along the guidewire, and then the guidewire was removed (Figure 3A). In cases where two PTBD catheters had been inserted at different branches of the bile ducts, two KMP catheters were placed along two PTBD tracts. ERCP was performed after the patients were moved into the prone position. The KMP catheter was pulled back until the end of the catheter was located near the major ampulla in the duodenum (Figure 3B, C). The KMP catheter was rotated to approximate the short angulated tip of the KMP catheter and the end of the ERCP cannula, and then the preloaded guidewire in the ERCP cannula was advanced through the KMP catheter (Figure 3D, E). The KMP catheter was pulled back proximal to the stricture for placement of inside stents. Inside stents were placed over the stricture by endoscopy as described in the guidewire technique (Figure 3F). When additional information about the recipient's bile duct was required, a cholangiogram was performed by injecting contrast via the KMP catheter. The KMP catheter was removed after insertion of the inside stents.

Statistical analysis

Procedure time was defined as the time after positioning the end of the duodenoscope in front of the major ampulla until placement of the inside stents. A Pearson's χ^2 test or Fisher's exact test was used to compare categorical data and Student's *t*-test or the Mann-Whitney *U*-test was

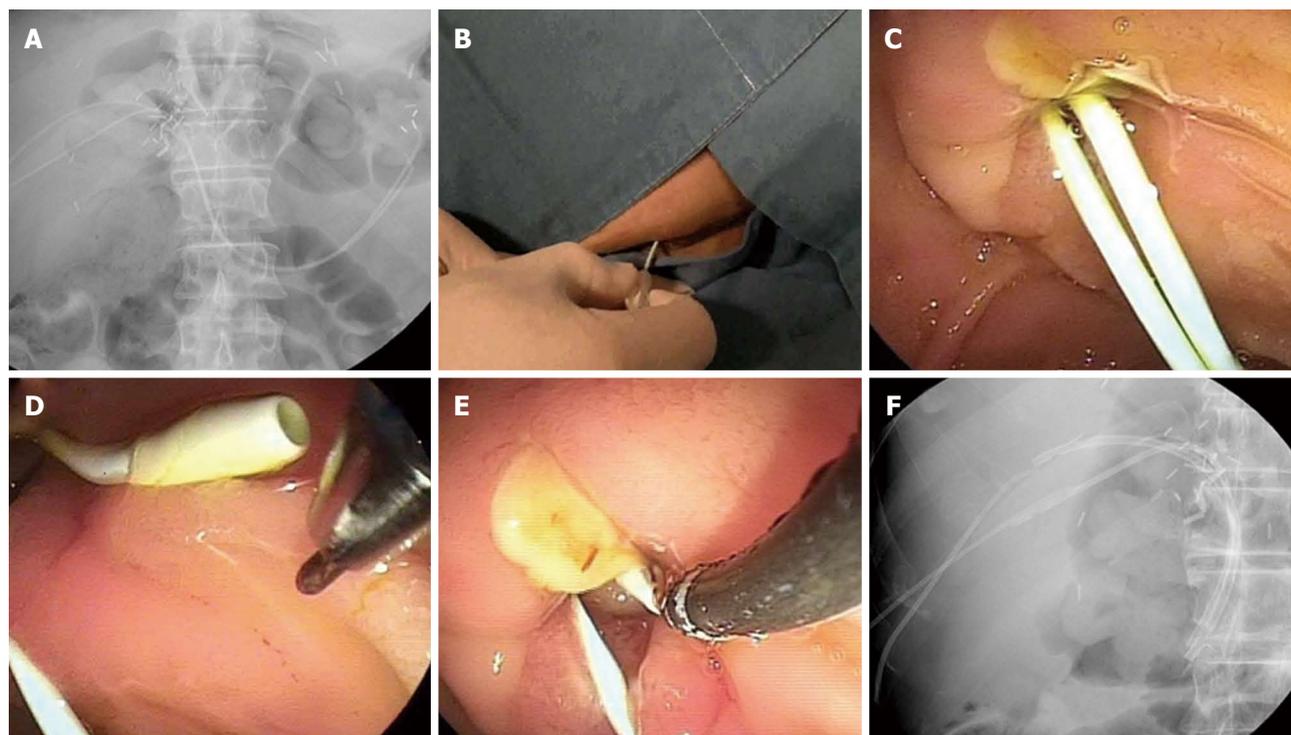


Figure 3 Kumpe catheter technique. A: Two Kumpe (KMP) catheters were placed along the previous percutaneous transhepatic biliary drainage tracts; B, C: The KMP catheters were located out of the major ampulla in the duodenum; D, E: The KMP catheter was pulled back and rotated to approximate the slightly angulated end of the KMP catheter and the end of the endoscopic retrograde cholangiopancreatography (ERCP) cannula. Then, a preloaded guidewire in the ERCP cannula was advanced through the KMP catheter; F: Two inside stents were placed over the stricture in the anterior and posterior branches of the right hepatic duct of the recipient liver.

used for comparisons of continuous data to analyze differences between the groups. The cumulative probability curves corresponding to the procedure time for each rendezvous technique were determined using the Kaplan-Meier method, and these were compared using the log rank test. A multivariate analysis was performed with the significant factors identified from the univariate analysis using the Cox proportional hazard regression model (forward: conditional method). Odds ratios and 95% confidence intervals were calculated. Statistical analyses were performed with SPSS software, version 14 (SPSS, Inc., Chicago, IL, United States). P -values < 0.05 were considered significant.

RESULTS

Patients

The baseline characteristics of the patients are described in Table 1. No significant differences were observed between the guidewire and KMP catheter groups. The mean duration between LDLT and the rendezvous procedure was 388 d (range: 31–2116 d), and the mean duration between PTBD and the rendezvous procedure was 154 d (range: 4–1526 d). Twenty-seven patients received one PTBD catheter, and 11 patients received two PTBD catheters. Laboratory findings showed normal or mildly elevated serum liver function tests, but no evidence of cholangitis.

Rendezvous procedure outcomes

Inside stents were successfully placed in all patients. Thus, the technical success rate in both groups was 100% (Table 1). No patient was treated with the KMP catheter technique after failure of the guidewire technique. In the guidewire group, the guidewire was pushed through the ERCP cannula and the ERCP cannula was advanced along the percutaneously inserted guidewire over the biliary stricture in 12 patients. In the remaining seven patients, the guidewire passing through the ampulla was captured by a basket and then withdrawn through the endoscopic working channel. IHD or CBD stones were identified in two patients in the KMP catheter group, and the stones were removed during the procedure. Dilation of the anastomotic stricture was performed in four patients in the guidewire group because of a tight stricture. We used the ERCP cannula preloaded with a guidewire in the KMP catheter group, and thus, it was not necessary to pull the guidewire back and reinsert it. It was easier to manipulate the KMP catheter than a guidewire. The procedure time was significantly shorter in the KMP group than in the guidewire group; the mean procedure time was 1012 s vs 2037 s, respectively ($P = 0.022$). In the cumulative probability curve corresponding to procedure time, the probability curves of the two rendezvous groups differed significantly according to the log rank test ($P = 0.008$, Figure 4), suggesting that the use of a KMP catheter was associated with a significantly shorter procedure time. Serum levels

Table 1 Patient characteristics and procedural outcomes

	Guidewire group (n = 19)	KMP catheter group (n = 19)	P-value
Mean age, yr (SD)	51.3 (9.3)	52.5 (10.2)	0.619
Male sex (%)	13 (68)	15 (79)	0.714
Pretransplantation liver disease (%)			0.524
End-stage liver cirrhosis	5 (26)	8 (42)	
Hepatitis B	2	6	
Hepatitis B and alcohol	2	1	
Cryptogenic	1	1	
Hepatocellular carcinoma	9 (47)	6 (32)	
Hepatitis B	9	5	
Hepatitis C	0	1	
Fulminant hepatitis	5 (26)	5 (26)	
Hepatitis A	1	2	
Hepatitis B	3	3	
Unknown origin	1	0	
Mean duration between LDLT and rendezvous procedure, d (SD)	338 (197)	438 (516)	0.704
Mean duration between PTBD and rendezvous procedure, d (SD)	91 (52)	217 (351)	0.511
Mean anastomotic angle ¹ , ° (SD)	118 (15)	125 (17)	0.148
Mean no. of PTBD catheters, (SD)	1.4 (0.5)	1.2 (0.4)	0.517
Mean diameter of PTBD catheter, F (SD)	10.7 (2.8)	9.1 (2.2)	0.365
Pre-laboratory findings ² , mean (SD)			
WBC (× 10 ⁹ /L)	4.61 (1.80)	4.87 (1.98)	0.569
Total bilirubin (mg/dL)	1.37 (0.90)	1.60 (1.51)	0.988
Alanine aminotransaminase (IU/L)	60.6 (41.6)	90.2 (128)	0.748
Alkaline phosphatase (IU/L)	332 (173)	289 (175)	0.649
γ-glutamyl transferase (IU/L)	248 (179)	278 (203)	0.800
Rendezvous success rate (%)	19 (100)	19 (100)	1.000
No. of stents inserted, mean (SD)	1.5 (0.5)	1.5 (0.5)	0.749
Inside stent diameter (F), mean (SD)	10.4 (2.7)	9.8 (0.7)	0.308
CBD or IHD stones (%)	0 (0)	2 (11)	0.152
Stricture dilation (%)	4 (21)	0 (0)	0.037
Mean procedure time ³ , s (range)	2037 (251-6758)	1012 (301-2006)	0.022
Post-laboratory findings ⁴ , mean (SD)			
White blood cell (× 10 ⁹ /L)	5.19 (1.88)	6.18 (2.34)	0.215
Total bilirubin (mg/dL)	2.15 (1.61)	2.11 (1.51)	0.942
Alanine aminotransaminase (IU/L)	85.1 (67.3)	115 (136)	0.953
Alkaline phosphatase (IU/L)	341 (167)	312 (198)	0.419
γ-glutamyl transferase (IU/L)	279 (189)	368 (319)	0.531
Amylase (U/L)	284 (585)	207 (241)	0.737
Complications			0.740
Acute cholangitis	1	0	
Hyperamylasemia	5	6	
Acute pancreatitis	1	2	
Migration of stents	1	0	

¹Between the right hepatic duct of the donor and the common bile duct of the recipient; ²One day before the rendezvous technique; ³Time after positioning the end of the duodenoscope in front of the major ampulla until placement of the inside stents; ⁴One day after the rendezvous procedure. LDLT: Living donor liver transplantation; PTBD: Percutaneous transhepatic biliary drainage; KMP: Kumpe; CBD: Common bile duct; IHD: Intrahepatic duct.

of liver enzymes were slightly elevated after the rendezvous procedure, but this was not clinically significant, and no differences were observed between the groups.

The factors related to procedure time were analyzed (Table 2). The method used for the rendezvous technique, the number of inside stents, the operator, and

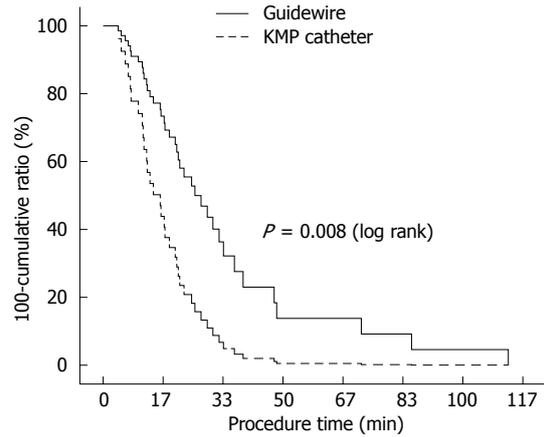


Figure 4 Cumulative probability of rendezvous procedures corresponding to procedure time. Kumpe catheter group vs guidewire group.

Table 2 Univariate analysis of factors related to procedure time

Factors	n	Procedure time, s, mean (range)	P-value
Rendezvous Technique	Guidewire 19	2037 (251-6758)	0.022
	KMP catheter 19	1012 (301-2006)	
IHD or CBD	Present 2	1517 (1202-1831)	0.513
	Absent 36	1525 (251-6758)	
Stone	One 19	1198 (251-5144)	0.04
	Two 19	1851 (666-6758)	
No. of inside stents	Lee IS 31	1292 (251-6758)	0.024
	Chang JH 7	2555 (735-5144)	
Operator	Yes 4	2568 (2189-2895)	0.008
	No 34	1402 (251-6758)	
Balloon dilation of the stricture	< 60 29	1571 (251-6758)	0.904
	> 60 9	1373 (301-2895)	
Age	First half ² 15	1009 (368-1934)	0.635
	Second half 16	1557 (251-6758)	

¹Performed by Lee IS; ²The rendezvous procedures were performed for the first half of each rendezvous group. KMP: Kumpe; CBD: Common bile duct; IHD: Intrahepatic duct.

balloon dilation of the stricture were significant factors related to procedure time. The procedure times did not differ between the first and second half in each rendezvous group.

Four significant factors in univariate analysis were evaluated for multivariate analysis. The number of inserted stents, operator, and balloon dilatation of the biliary stricture were not significant in the Cox proportional hazard regression model ($P > 0.05$). The rendezvous technique method was the only significant factor related to procedure time ($P = 0.010$, odds ratio 2.663, 95%CI 1.258-5.637, Table 3). Therefore, the rendezvous technique was an independent factor related to procedure time.

Complications and follow-up after the rendezvous procedure

Acute complications occurred in two patients after rendezvous procedures (10.5%) in each group. In the guide-

Table 3 Multivariate analysis of factors related to procedure time

Factors	P-value	Odds ratio (95%CI)
Rendezvous technique (guidewire <i>vs</i> rendezvous)	0.010	2.663 (1.258-5.637)
No. of inside stents (1 <i>vs</i> 2)	0.067	
Operator (Lee IS <i>vs</i> Chang JH)	0.195	
Balloon dilation (yes <i>vs</i> no)	0.289	

CI: Confidence interval.

wire group, one case of acute cholangitis and one mild case of acute pancreatitis developed after the procedures. Acute cholangitis with fever and epigastric pain occurred due to the migration of an inside stent proximally, resulting in obstruction of the distal end of the stent. We removed the original inside stent and put a new one in its place. The patient ultimately recovered. The patient with acute pancreatitis had the longest procedure time in the guidewire group (112 min) and a sustained elevation into total serum bilirubin for 4 d after the procedure. During the procedure, guidewire manipulation was difficult because the two guidewires were tangled. Two mild cases of acute pancreatitis developed after the procedure in the KMP catheter group. Peak serum amylase levels were 873 U/L and 847 U/L, respectively. Their abdominal pain lasted for 2 d.

We followed the patients in the guidewire and KMP catheter groups for average of 40 mo (range: 12-57 mo) and 8 mo (range: 2-14 mo), respectively. Inside stents were exchanged a mean of 0.8 times (range: 0-4 times) until they were free of biliary stricture. Finally, 19 patients reached stent-free status (seven in the KMP catheter group and 12 in the guidewire group), and 14 patients (nine in the KMP catheter group and five in the guidewire group) still had inside stents. Four patients had plastic inside stents that had been replaced with covered metal stents to treat a biliary stricture. The attempt to change an inside stent by ERCP failed in one patient, and the patient required to undergo PTBD again. IHD or CBD stones developed in seven patients and a biliary cast developed in one patient; these were removed by ERCP. Two patients died during follow-up. One patient in the guidewire group died from recurrent hepatocellular carcinoma 14 mo after the rendezvous procedure, and another patient in the KMP catheter group died from a hepatic artery occlusion and hepatic failure 12 mo later.

DISCUSSION

The present study demonstrated that the rendezvous technique using a KMP catheter is easy, fast, and safe when used to place inside stents for a biliary stricture after LDLT and represents a viable alternative to the rendezvous technique performed using a guidewire. No significant complications were observed.

Patients in whom ERCP stent placement failed needed to undergo PTBD or surgical treatment^[3,16,17]. Al-

though maintaining a PTBD catheter for a long period is beneficial for treating biliary strictures^[18-20], it may be difficult for patients due to the development of PTBD catheter-related complications, such as leakage, pain, infection, and accidental removal of the PTBD catheter^[21,22]. The discomfort caused by carrying a PTBD catheter also reduces the patient's quality of life and disturbs his or her daily routine. Hence, replacing PTBD catheters with inside stents is recommended. However, stenting using ERCP in patients with angulated or twisted biliary strictures is difficult and sometimes fails. Our previous study showed that the rendezvous technique is a useful alternative method for successful placement of inside stents in these patients^[13]. A few cases of biliary complication after liver transplantation have supported the usefulness of the rendezvous technique for biliary strictures and stones or biliary leakage from bile duct anastomosis^[23-25].

Although the rendezvous technique is useful and facilitates cannulation of the bile duct in cases where previous endoscopic attempts have failed^[6-9], there were some drawbacks to the conventional version of the rendezvous technique. In the classic rendezvous technique, grasping the guidewire outside of the ampulla with a forcep or snare is occasionally difficult due to its slippery surface. Kinking or breakage of the guidewire can also occur while grabbing and pulling guidewires through the accessory channel of the duodenoscope. A long guidewire outside the skin of the PTBD tract is difficult to manipulate and increases the risk of contamination. Additionally, it is inconvenient to pull the guidewire back and place the soft or floppy end of the wire back into the ERCP cannula, and then advance it into the biliary tree to reduce liver damage from the stiff end of the guidewire. To reduce these shortcomings, a modified rendezvous technique was introduced so that the end of the guidewire is pushed inside the lumen of an ERCP cannula and the ERCP cannula is advanced along the wire into the CBD^[14]. However, this technique also has its disadvantage. The guidewire is not easily pushed inside the ERCP cannula lumen, and this procedure is frequently time-consuming. A parallel cannulation technique using a sphincterotome in a retrograde fashion, alongside a biliary drainage catheter, can be useful for selective CBD cannulation^[26]. However, parallel cannulation is not suitable for selective IHD cannulation.

A KMP catheter is useful for overcoming these drawbacks. The KMP catheter was introduced as a vascular catheter and has been widely used in the interventional radiology. A KMP catheter is as short as 40 cm, so the portion outside the skin from the PTBD tract is short enough for easy manipulation including to-and-fro motion and turning, which are used to move the curved distal end of the KMP catheter up-and-down and right-to-left and reduces the risk of contamination. Because the end of a KMP catheter is slightly angulated and turning the end is simple, end-to-end contact between the ends of an ERCP cannula and a KMP catheter is easy to achieve without the use of a sphincterotome. It is pos-

sible to insert a preloaded guidewire within the ERCP cannula into the KMP catheter retrogradely. Therefore, it is unnecessary to pull the guidewire back out of the duodenoscope and to reinsert the soft or floppy end of the wire first. When ERCP is delayed after placing a KMP catheter, the KMP catheter can be kept in place for a few hours until ERCP is performed, and it is impossible when using the guidewire technique. Even if two KMP catheters are placed along two previous PTBD tracts, the degree of discomfort is reduced due to the thin caliber of the KMP catheter, and two KMP catheters are not likely to tangle, in contrast to guidewires. Additionally, a cholangiogram can be performed by injecting contrast via the KMP catheter during ERCP, which provides additional information about the recipient's bile duct. If the rendezvous procedure fails, reinserting a PTBD catheter is easy when a KMP catheter is in place. Recently a case report on the rendezvous technique with a C2 catheter which is similar to a KMP catheter, was introduced in a patient with gallbladder carcinoma and a metastatic right intrahepatic bile duct obstruction^[27].

Because this study was retrospective and not randomized, there were some limitations. First, the time to perform the procedures differed between the two groups. The guidewire group procedures preceded those of the KMP catheter group. It is possible that our familiarity with each of the rendezvous procedures differed somewhat. However, the procedure times in chronological order in each rendezvous group did not differ significantly; moreover, those of the first and second half in each group were not different in the factor analysis. Second, we performed an analysis of the factors related to procedure time, but other factors affecting procedure time that were not analyzed in our study may have played a role. For example, the severity and condition of stricture differed somewhat among the patients. However, we supposed that these factors were minor and not significantly related to procedure time. The rendezvous technique itself can overcome the state of the stricture in difficult situation. Third, although we connected the 5F KMP catheter with the tip of the ERCP cannula by a guidewire without great difficulty, the wider diameter of the catheter might make it easier to connect the catheter and ERCP cannula. If a straight catheter is used, a sphincterotome will facilitate the insertion of a guidewire through the catheter.

In conclusion, the rendezvous technique performed with a KMP catheter is a fast and safe method for placing inside stents in biliary strictures in LDLT patients who need to exchange the PTBD catheter for inside stents and represents a viable alternative to the guidewire technique. The KMP catheter rendezvous technique is recommended for LDLT patients who have angulated or twisted anastomotic biliary strictures. We expect further comparative prospective studies with a larger cohort of patients to demonstrate the benefits of the KMP catheter technique in the future.

COMMENTS

Background

The rendezvous technique allows for the successful placement of inside stents in angulated or twisted biliary strictures after liver transplantation. In the classic rendezvous technique, a guidewire is used for the endoscopic approach to the bile duct. However, manipulation of the guidewire is difficult and somewhat cumbersome, and kinking or breakage of the guidewire can occur.

Research frontiers

A Kumpe (KMP) catheter (5F, 40 cm) is useful for overcoming the drawbacks associated with the classic rendezvous technique. The KMP catheter is short enough for easy manipulation and it also reduces the risk of contamination during the procedure.

Innovations and breakthroughs

Because the end of a KMP catheter is shortly angulated and turning the end is simple, end-to-end contact between the ends of an endoscopic retrograde cholangiopancreatography (ERCP) cannula and a KMP catheter is easily achieved even without the use of a sphincterotome. It is possible to insert a preloaded guidewire within the ERCP cannula into the KMP catheter in retrograde fashion. The rendezvous technique involving use of the KMP catheter was a fast and safe method for placing inside stents in living donor liver transplantation (LDLT) biliary strictures and represents a viable alternative to use of the guidewire rendezvous technique.

Applications

The KMP catheter rendezvous technique is recommended for LDLT patients who have angulated or twisted anastomotic biliary strictures.

Peer review

The authors demonstrated the usefulness of a new rendezvous technique for placing stents using a KMP catheter in angulated or twisted biliary strictures. The results are interesting and suggest that rendezvous technique involving use of the KMP catheter was a fast and safe method for placing inside stents in patients with LDLT biliary stricture that represents a viable alternative to the guidewire rendezvous technique.

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Correlation between mitochondrial TRAP-1 expression and lymph node metastasis in colorectal cancer

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Abstract

AIM: To evaluate the effect of mitochondrial tumor necrosis factor receptor-associated protein-1 (TRAP-1) on the lymph node metastasis (LNM) in Chinese colorectal cancer (CRC) patients, and develop potential LNM-associated biomarkers for CRC using quantitative real-time polymerase chain reaction (RT-PCR) analysis.

METHODS: Differences in mitochondrial TRAP-1 gene expression between primary CRC with LNM (LNM CRC) and without LNM (non-LNM CRC) were assessed in 96 Chinese colorectal carcinoma samples using quantitative RT-PCR analysis, Western blotting, and confirmed with immunohistochemical assay. The relationship between clinicopathological parameters and potential diagnostic biomarkers was also examined.

RESULTS: TRAP-1 was significantly upregulated in LNM CRC compared with non-LNM CRC, which was confirmed by RT-PCR, Western blotting and immunohistochemical assay. The expression of TRAP-1 in two different metastatic potential human colorectal cancer cell lines, LoVo and HT29, was analyzed with Western blotting. The expression level of TRAP-1 was dramatically higher in LoVo than in HT29. Overexpression of TRAP-1 was significantly associated with LNM (90.2% in LNM group vs 22% in non-LNM group, $P < 0.001$), the advanced tumor node metastasis stage (89.1% in LNM group vs 26.9% in non-LNM group, $P < 0.001$), the increased 5-year recurrence rate (82.7% in LNM group vs 22.6% in non-LNM group, $P < 0.001$) and the decreased 5-year overall survival rate (48.4% in LNM vs 83.2% in non-LNM group, $P < 0.001$). Univariate and multivariate analyses indicated that TRAP-1 expression was an independent prognostic factor for recurrence and survival of CRC patients (Hazard ratio of 2.445 in recurrence, $P = 0.017$; 2.867 in survival, $P = 0.028$).

CONCLUSION: Mitochondria TRAP-1 affects the lymph node metastasis in CRC, and may be a potential biomarker for LNM and a prognostic factor in CRC. Overexpression of TRAP-1 is a predictive factor for the poor outcome of colorectal cancer patients.

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Key words: Colorectal cancer; Lymph node metastasis; Prognosis; Quantitative real-time polymerase chain reaction analysis; Hsp90 family; Mitochondria tumor necrosis factor receptor-associated protein-1

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INTRODUCTION

Invasion into adjacent tissues and metastasis to distant sites are major features of malignant cancer cells, which are complex processes that require a coordinated action of a large assortment of growth factors and their receptors, as well as downstream signaling intermediates^[1]. Colorectal cancer (CRC) is the third most common cancer in both men and women and the second most common cause of cancer-related death^[2]. CRC frequently migrates through the lymphatic route, depositing tumor cells into local lymph nodes, namely lymph node metastasis (LNM). The status of the local lymph nodes delivers crucial information concerning cancer staging, prognosis, and clinical decision making. The existence of LNM notably reduces the chance of CRC survivals^[3].

Unfortunately, the mechanisms related to LNM remain poorly understood at present because LNM is a complicated process that involves cancer cell detachment from the primary tumor, migration, invasion, adhesion and implantation in the new environment. A variety of dysregulated molecules play a significant role in this highly sophisticated process^[4,5]. Therefore, LNM has become a focus of cancer studies.

Clinicopathological features such as poor differentiation, depth of wall penetration, lymphovascular invasion, and tumor size are considered to be associated with CRC with LNM (LNM CRC)^[6,7].

Proper staging is important for choosing the right treatment for a patient: the most useful staging system is the tumor node metastasis (TNM) system established collaboratively by the American Joint Committee on Cancer and the International Union for Cancer Control^[8].

However, these characteristics are still insufficient to predict the existence of LNM. In order to improve the diagnosis and prognosis of CRC, there is an urgent need to identify specific tumor molecular markers to recognize patients with LNM, which can define a subset of CRC patients who could benefit from rational management.

Heat shock protein 90 (Hsp90) is an abundant molecular chaperone that is further overexpressed or activated in cancer cells, suggesting that it could be a crucial regulator of growth and/or survival of tumor cells^[9,10]. Recent data have shown that Hsp90 family may function as novel regulators of mitochondrial permeability transition, especially in tumor cells^[11-13]. Inhibitors of Hsp90 have been studied for the treatment of cancer, and a small molecule Hsp90 antagonist derived from geldanamycin (GA), i.e., 17-allylamino-demethoxygeldanamycin (17-AAG), has entered clinical testing in cancer patients, but progress has been questionable, and the response to this agent proved difficult to interpret^[14,15]. This reflected

a modest anticancer activity that was inconsistent with a predicted essential role of Hsp90 in tumor maintenance, paradoxical activation of oncogenic kinases, induction of anti-apoptotic mechanisms, and increased metastatic dissemination^[16].

Accordingly, Hsp90 and its ortholog, and tumor necrosis factor receptor-associated protein-1 (TRAP-1) are abundantly localized to the mitochondria of tumor, but not present in most of the normal cells. Mitochondria play a critical role in cell survival and cell death^[17]. Consistent with a general role of Hsp90 as a drug target in colorectal cancer, the mitochondria-compartmentalized cytoprotective pathway could provide a novel therapeutic target to enhance tumor cell apoptosis^[13].

TRAP-1 associated with cancer has been reported recently in several studies, especially in metastasis of cancer cells^[18-20], but there were a limited number of studies on the association of TRAP-1 with metastasis in Chinese CRC patients. In this study, we employed quantitative real-time polymerase chain reaction (RT-PCR) to analyze the expression of mitochondrial TRAP-1 in the groups of LNM CRC and non-LNM CRC. We studied the correlation between expression of TRAP-1 in LNM CRC and non-LNM CRC with quantitative RT-PCR and Western blotting. Confirmed with immunohistochemical (IHC) study, we also investigated the relationship between TRAP-1 expression and lymph node metastatic phenotype of CRC, and determined the prognostic value on the metastasis in Chinese CRC cases.

MATERIALS AND METHODS

Patient population and characteristics of tissue samples

A total of 96 Chinese colorectal carcinoma samples were collected in our hospital (Fudan University Shanghai Cancer Center, Shanghai, China) after informed consent was obtained from the patients. None of the patients received chemotherapy or radiotherapy before surgery. Resected specimens were reviewed by two senior pathologists according to the criteria described in the American Joint Committee on Cancer: Cancer Staging Manual (7th edition, 2009)^[8]. The number of lymph nodes retrieved was not less than 12 in the non-LNM CRC. None of them had distant metastasis. The fresh colorectal tumor tissues were obtained immediately after surgery, washed twice with chilled phosphate buffered saline, immediately stored in liquid nitrogen at -80 °C in our tissue bank until further use. This study was approved by the Cancer Center Research Ethics Committee of Fudan University.

Cell lines and culture

For detection of the expression of TRAP-1 in different metastatic potential human colorectal cancer cells, we chose two human colorectal cancer cell lines, LoVo and HT29 in the experiment, to make sure if the TRAP-1 expressed differently in different metastatic potential human colorectal cancer cell lines. The cell line HT29 was cultured in McCoy 5a medium containing 5% fetal bovine

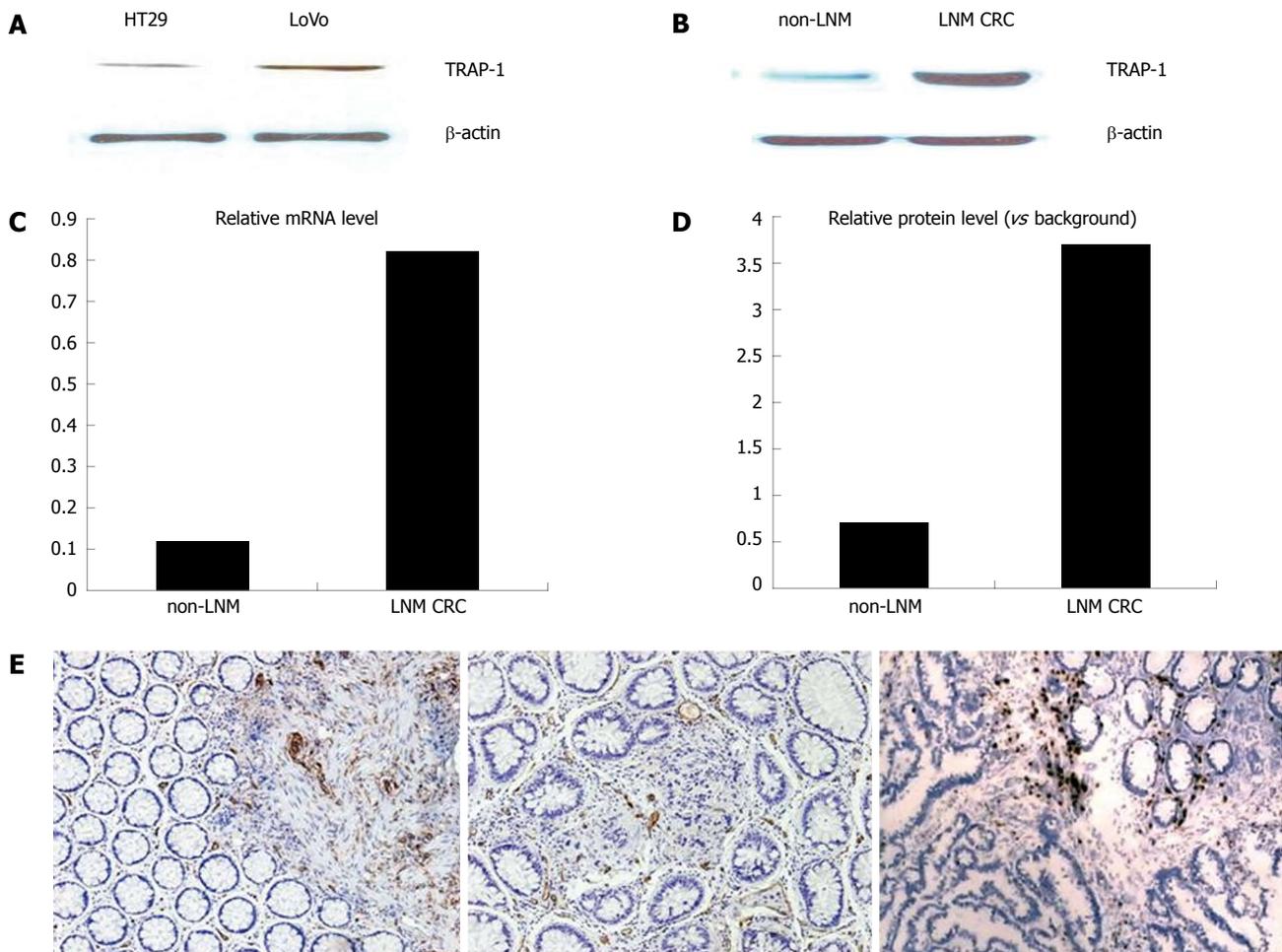


Figure 1 Confirmation of the overexpression of tumor necrosis factor receptor-associated protein-1 in colorectal cancer. A: Western blotting analysis for tumor necrosis factor receptor-associated protein-1 (TRAP-1) expression in different metastatic potential LoVo cell and HT29 cell. β -actin was used as the internal loading control. The histogram shows the relative expression levels of TRAP-1 in LoVo and HT29 cell; B: Western blotting analysis for TRAP-1 expression in non-lymph node metastasis (LNM) and LNM groups. Data represent the mean \pm SE ($P < 0.001$, Student *t* test); C, D: mRNA level of TRAP-1 via quantitative real-time polymerase chain reaction. TRAP-1 was consistently increased in the LNM group compared with non-LNM group. The mRNA level was normalized to that of β -actin. Data represent the mean \pm SE ($P < 0.001$, Student *t* test); E: Immunohistochemical labeling for the TRAP-1 in the CRC sample. TRAP-1 was identified in non-LNM cancer tissues (weak in middle) and strong staining in LNM cancer group (right), but was rare in normal mucosa (left).

serum (FBS). LoVo cells were cultured in F-12K medium containing 10% FBS. All the cell lines were maintained at 37°C in 95% air and 5% CO₂.

RNA isolation and reverse transcription

Total RNA was isolated from the human tissue or cultured colon cancer cell lines using the TRizol according to manufacturer's instructions (Invitrogen). After the RNA concentration measurement and the integrity of the isolated RNA analysis, 1 μ g of RNA was reverse-transcribed into cDNA according to the manufacturer's protocol (Promega).

Quantitative RT-PCR

Quantitative RT-PCR was analyzed using SYBR Green Supermix (Promega). For PCR, 10 ng of the RT reaction was used for a 25- μ L reaction using the ABI Prism 7700 sequence detector system (Applied Biosystems, Branchburg, NJ, United States). Target genes were normalized to β -actin and quantified using the comparative

Ct method^[21]. TRAP-1 expression levels were measured in triplicate, with a good reproducibility, and the average was calculated.

β -actin was applied as an internal control. The primers for β -actin (205 bp) were 5'-TGACGTGGACATCC-GCAAAG-3' (sense) and 5'-CTGGAAGGTGGACAGCGAGG-3' (antisense). The primers for TRAP-1 (185 bp) were 5'-ATGGTGG CTGACAGAGTGGAGG-3' (sense) and 5'-GCAGTCGGATTTCAGGTGGA TG-3' (antisense).

Western blotting

Briefly, 30- μ g protein samples from each case were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently transferred to polyvinylidene fluoride membranes. The membranes were incubated with rabbit polyclonal antibody against TRAP-1 (1:1000 dilution; Abcam, Cambridge, United Kingdom) and then incubated with a horseradish-peroxidase-conjugated secondary antibody (1:100 dilution; Proteintech,

Table 1 Relationship between tumor necrosis factor receptor-associated protein-1 expression and clinicopathological factors in colorectal cancer

Clinicopathological factors	n	TRAP-1 expression		P value ¹
		Negative	Positive	
Sex				
Male	46	16	30	0.225
Female	50	23	30	
Age (yr)				
≤ 60	66	30	36	0.486
> 60	30	10	20	
Tumor size (cm)				
≤ 5	70	30	40	0.065
> 5	26	11	15	
Tumor location				
Colon	31	11	20	0.915
Rectum	65	32	33	
Tumor differentiation ²				
I-II	80	45	35	0.212
III-IV	16	6	10	
Tumor status ²				
T1-2	34	14	20	0.512
T3-4	62	31	31	
Lymph node metastasis ²				
N0	45	35	10	< 0.001
N1-2	51	5	46	
TNM stage ²				
I-II	41	30	11	< 0.001
III-IV	55	6	49	

¹Statistical analysis was performed with χ^2 test; ²Grading of differentiation status and tumor node metastasis (TNM) classification for colorectal cancer were based on the American Joint Committee on Cancer: Cancer Staging Manual (7th edition, 2009). The tumors were classified into two groups: Well differentiated (grades I and II) and poorly differentiated (grades III and IV).

Chicago, IL, United States). β -actin was detected simultaneously as a loading control (anti- β -actin, 1:1000 dilution; Kangchen, Beijing, China). All blots were visualized using an ECL detection system (Amersham, Arlington Heights, IL, United States) and quantitated by densitometry using an LAS-3000 imager.

Immunohistochemistry

TRAP-1 expression was examined immunohistochemically using paraffin-embedded tissues. In brief, 4- μ m-thick tissue sections were heated in 6.5 mmol/L citrate buffer (pH 6.0) at 100 °C for 28 min, and incubated with antibody against TRAP-1 (1:200 dilution). Immunostaining was performed using the DAKO EnVision System (Dako Diagnostics, Zug, Switzerland). In the negative control group, PBS was used instead of primary antibody. TRAP-1 expression was scored by two independent experienced pathologists. Each sample was graded according to the intensity and extent of staining. The intensity of staining was scored as 0 (no staining), 1 (weak staining), and 2 (strong staining). The extent of staining was based on the percentage of positive tumor cells: 0 (no staining), 1 (1%-25%), 2 (26%-50%), 3 (51%-75%), and 4 (76%-100%). The final score was assessed by summarizing the results of intensity and extent of staining. The

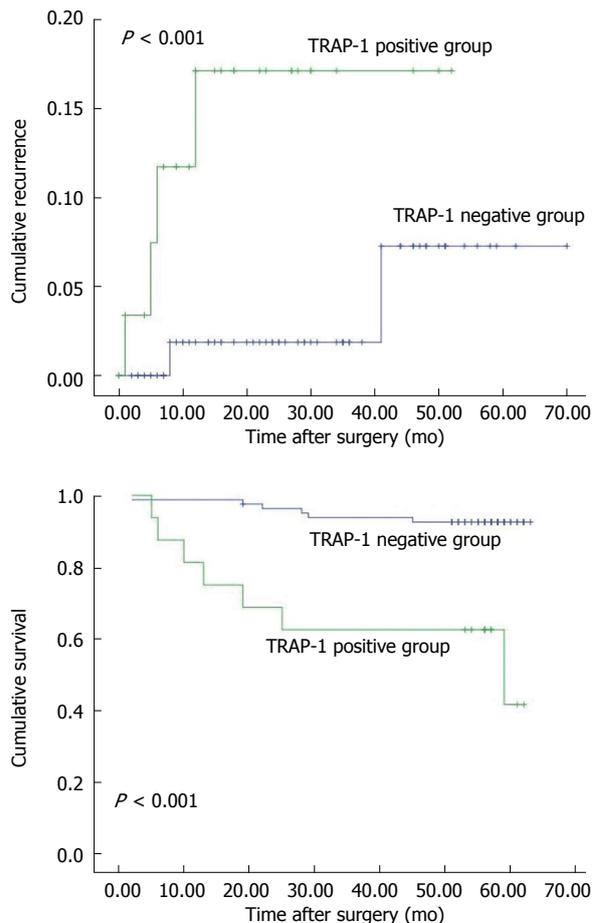


Figure 2 Overexpression of tumor necrosis factor receptor-associated protein-1 correlated with poor prognosis in 96 colorectal cancer patients. A: Cumulative recurrence; B: Cumulative survival. The tumor necrosis factor receptor-associated protein-1 (TRAP-1)-positive vs TRAP-1-negative groups (P < 0.001, log-rank test).

case was considered negative if the final score was 0 or 1 (-) or 2 or 3 (\pm), and positive if the score was 4 or 5 (+) or 6 or 7 (++) . In most cases, the two reviewers provided consistent results. Any inconsistencies were resolved through discussion to achieve a consensus score.

Statistical analysis

The Student *t* test was used to evaluate the differences in TRAP-1 expression between LNM CRC and non-LNM CRC. The χ^2 test was used to assess the relationship between TRAP-1 expression and clinicopathological factors. The cumulative recurrence and survival probability were estimated using the Kaplan-Meier method, and differences were calculated by log-rank test. Prognostic factors were determined using Cox regression analysis. The recurrence-free and overall survival duration were calculated from the first resection of the primary tumor to first evidence of recurrence or to death from any cause, respectively. The diagnosis of recurrence was based on the typical features presented on computed tomography/magnetic resonance imaging and elevated serum carcinoembryonic antigen. All P values were two-sided, and P < 0.05 was considered to be significant. Statistical analyses

Table 2 Univariate and multivariate analyses of recurrence and survival (Cox regression)

Variables	Recurrence		Survival	
	HR (95%CI)	P value	HR (95%CI)	P value
Univariate analysis				
Sex				
Male/female	0.813 (0.479-1.518)	0.604	0.829 (0.424-1.618)	0.662
Age (yr)				
≤ 60/> 60	1.506 (0.804-2.712)	0.163	1.822 (0.947-3.528)	0.065
Tumor size (cm)				
≤ 5/> 5	0.876 (0.163-0.665)	0.687	0.880 (0.438-1.723)	0.704
Tumor location				
Colon/rectum	0.812 (0.445-1.423)	0.518	0.904 (0.476-1.734)	0.778
Tumor differentiation				
I - II / III-IV	1.212 (0.654-2.308)	0.501	1.151 (0.576-2.358)	0.65
Tumor status				
T1-2/T3-4	0.866 (0.475-1.618)	0.687	1.020 (0.504-2.028)	0.904
Lymph node metastasis				
N0/N1-2	2.707 (1.502-4.912)	0.001	2.812 (1.413-5.509)	0.002
TNM stage				
I - II / III-IV	3.554 (1.932-6.526)	< 0.001	3.385 (1.677-6.843)	< 0.001
TRAP-1 expression				
Negative/positive	3.657 (1.919-6.957)	< 0.001	4.145 (1.913-8.712)	< 0.001
Multivariate analysis				
LNM				
N0/N1-2	0.210 (0.051-0.758)	0.018	0.196 (0.041-0.852)	0.028
TNM stage				
I - II / III-IV	8.905 (2.072-38.190)	0.003	9.037 (1.703-48.105)	0.010
TRAP-1 expression				
Negative/positive	2.445 (1.065-5.712)	0.017	2.867 (1.113-7.36)	0.028

HR: Hazard ratio; CI: Confidence interval; LNM: Lymph node metastasis; TNM: Tumor node metastasis; TRAP-1: Tumor necrosis factor receptor-associated protein-1.

were performed using SPSS 13.0 software.

RESULTS

TRAP-1 expression in CRC specimens and different metastatic potentials of two human colorectal cancer cell lines, LoVo and HT29, were determined by Western blotting and quantitative RT-PCR. The expression of TRAP-1 in LoVo cells and HT29 cells was analyzed with Western blotting. The expression level of TRAP-1 was dramatically higher in LoVo than in HT29 cells. Representative Western blotting results are presented in Figure 1A.

Quantitative RT-PCR and Western blotting were used to analyze the TRAP-1 expression in different groups of CRC. Relative gene and protein expression quantifications were calculated by the comparative Ct method using β -actin as an endogenous control. The results revealed that TRAP-1 mRNA and protein levels were higher in LNM CRC than in non-LNM CRC ($P < 0.001$, Figure 1B and C, D), which is consistent with the results by the Western blotting.

Immunohistochemistry was applied to confirm the upregulation of TRAP-1 in different groups (Figure 1E). In non-LNM CRC, there was weak staining in cancer cells compared to the strong staining in both primary and matched metastatic lymph node cancer cells in LNM CRC samples.

Association between TRAP-1 expression and clinicopathological features and postoperative prognosis of CRC patients

To detect the relationship between TRAP-1 expression and clinicopathological features and whether TRAP-1 could be a prognostic factor in predicting clinical outcomes of CRC patients, we evaluated TRAP-1 expression with the same samples. In the LNM CRC samples, 87% were positive for TRAP-1 expression, whereas 10.2% of the non-LNM CRC samples had positive expression.

Statistical analysis revealed that positive expression of TRAP-1 was significantly associated with LNM, and advanced TNM stage ($P < 0.001$). However, no significant correlations were observed between TRAP-1 expression and other clinicopathological parameters of sex, age, tumor size, tumor differentiation and tumor location (Table 1).

Furthermore, we found that patients with TRAP-1-positive CRC had significantly poorer prognosis than those with TRAP-1-negative CRC. The 5-year cumulative recurrence rate was significantly higher in patients with TRAP-1-positive CRC than in the TRAP-1-negative group ($P < 0.001$, Figure 2A). The 5-year cumulative survival rate in patients with TRAP-1-positive CRC was much lower than in those with TRAP-1-negative CRC ($P < 0.001$, Figure 2B). Univariate analyses revealed that LNM, TNM stage and TRAP-1 expression were associ-

ated with recurrence and overall survival. In multivariate analysis, LNM, TNM stage and TRAP-1 expression were also independent prognostic factors for recurrence and overall survival ($P < 0.05$, Table 2).

DISCUSSION

Metastasis remains one of the major challenges in management of CRC patients. LNM is the most common form of metastasis in CRC. To study the correlation between expression of TRAP-1 and LNM metastasis in East Asian CRC patients, develop potential LNM-associated biomarkers for CRC, we used the quantitative RT-PCR to determine the expression of TRAP-1 in clinical LNM CRC and non-LNM CRC patients, and employed immunohistochemical assay in the same samples to confirm the outcome of PCR and Western blotting.

Recently, several studies have shown that TRAP-1 is an important factor relevant to progression and prognosis in various human cancers, such as glioblastoma^[22], ovarian^[23], prostate^[24], colorectal^[25], and bladder^[26] cancer. In particular, some studies have revealed that overexpression of TRAP-1 strongly indicates the presence of LNM^[22-24], which accords with the objectives of our study. However, similar investigations have been limited to the relationship between TRAP-1 expression and LNM in CRC. Therefore, it is of interest to notice that TRAP-1, one of the significantly upregulated proteins identified in LNM CRC compared with non-LNM CRC, has been confirmed at the protein and mRNA levels.

TRAP-1 is abundantly and differentially expressed in metastatic CRC in humans, but in normal colon cells, TRAP-1 was undetectable or minimally expressed. The different distribution of TRAP-1 in colorectal cancer cells *vs* normal cells is in agreement with another survey of different tumor types, where TRAP-1 was also differentially expressed in tumors of breast, lung, prostate and pancreas as compared with normal matched tissues^[13]. The up-regulation of TRAP-1 in LNM CRC played a major role in crucial biological functions that influence various aspects of cell physiology, including proliferation and apoptosis, and differentiation and morphogenesis. It is also significantly involved in cell adhesion and motility, and cancer invasion and metastasis^[27,28].

The relationship between TRAP-1 expression and the LNM phenotype of CRC was also studied in the experiment with the same CRC samples. We found that the increase in TRAP-1 expression level was significantly correlated with LNM and advanced TNM stage, which suggests that TRAP-1 plays an important part in the progression of CRC from a localized to lymph node metastatic disease. In addition, patients with TRAP-1-positive CRC had an increasing risk of recurrence and significantly reduced overall survival. Univariate and multivariate analyses indicated that TRAP-1 expression is an independent prognostic factor for recurrence and overall survival in CRC, which indicates the considerable prognostic value

of TRAP-1 expression.

In conclusion, the present study provided evidences in the association between differently expressed TRAP-1 and LNM in CRC based on a quantitative mRNA expression analysis. TRAP-1 was identified and confirmed to be significantly overexpressed in LNM CRC. Further evaluation by Western blotting and IHC assay using the same sample sets suggested that TRAP-1 acts as a potential biomarker for LNM and prognosis in CRC.

Most epithelial malignancies, including colorectal cancer exhibit a higher anti-apoptotic threshold, which contributes to disease progression^[29-31]. However, many questions remain to be answered^[32-34] with respect to the cellular function of TRAP-1 and how it exerts its influence on metastatic progression, and the molecular basis for the different localization of Hsp90 and TRAP-1 to the tumor *vs* normal mitochondria awaits further studies.

COMMENTS

Background

Colorectal cancer (CRC) is the third most common cancer in both men and women and the second most common cause of cancer-related death. CRC frequently migrates through lymph node metastasis (LNM), but the variables used to predict the existence of LNM are not available. In order to improve the diagnosis and prognosis of CRC, there is an urgent need to identify specific tumor molecular markers to recognize patients with LNM, which can define a subset of CRC patients who could benefit from rational management.

Research frontiers

Accordingly, Hsp90, and its ortholog, tumor necrosis factor receptor-associated protein-1 (TRAP-1) are abundantly localized to mitochondria of tumor, but not to the normal cells. Mitochondria play a critical role in cell survival and cell death. Consistent with a general role of Hsp90 as a drug target in colorectal cancer, the mitochondria-compartmentalized cytoprotective pathway could provide a novel therapeutic target to enhance tumor cell apoptosis.

Innovations and breakthroughs

The association between TRAP-1 and cancer has been reported in several studies recently, especially the relationship of TRAP-1 with metastasis of cancer cells. There was a limited number of studies on the association of TRAP-1 with metastasis in Chinese CRC patients. In this study, the authors analyzed the expression of mitochondrial in different groups of LNM CRC and non-LNM CRC using quantitative real-time polymerase chain reaction (RT-PCR), and investigated the correlation between expression of TRAP-1 in LNM CRC and non-LNM CRC with quantitative RT-PCR and Western blotting. Confirmed with immunohistochemical assay in the same samples, the authors also investigated the relationship between TRAP-1 expression and lymph node metastatic phenotype of CRC, and determined the prognostic value on the metastasis in Chinese CRC cases.

Applications

This study suggests that the mitochondria TRAP-1 affect the lymph node metastasis in CRC, and it may be a potential biomarker for LNM and a prognostic factor in CRC. Over-expression of TRAP-1 is a predictive factor for the poor outcome of CRC patients.

Terminology

Hsp90 ortholog, TRAP-1 are abundantly localized to mitochondria of tumor, but not to normal cells. TRAP-1 is a 75 kDa heat shock protein that is encoded in humans by the TRAP-1 gene.

Peer review

The study is well-designed, although there are some technical points that need rethinking during the interpretation of the results. The topic is of great clinical importance as colorectal carcinoma represents a worldwide health problem, and finding possible biomarkers that can be prognostic factors may help the clinicians in their daily routine.

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Drug-induced liver injury in hospitalized patients with notably elevated alanine aminotransferase

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Abstract

AIM: To identify the proportion, causes and the nature of drug-induced liver injury (DILI) in patients with notably elevated alanine aminotransferase (ALT).

METHODS: All the inpatients with ALT levels above 10 times upper limit of normal range (ULN) were retrospectively identified from a computerized clinical laboratory database at our hospital covering a 12-mo period. Relevant clinical information was obtained from medical records. Alternative causes of ALT elevations were examined for each patient, including biliary abnormality, viral hepatitis, hemodynamic injury, malignancy, DILI or undetermined and other causes. All suspected DILI cases were causality assessed using

the Council for International Organizations of Medical Sciences scale, and only the cases classified as highly probable, probable, or possible were diagnosed as DILI. Comments related to the diagnosis of DILI in the medical record and in the discharge letter for each case were also examined to evaluate DILI detection by the treating doctors.

RESULTS: A total of 129 cases with ALT > 10 ULN were identified. Hemodynamic injury ($n = 46$, 35.7%), DILI ($n = 25$, 19.4%) and malignancy ($n = 21$, 16.3%) were the top three causes of liver injury. Peak ALT values were lower in DILI patients than in patients with hemodynamic injury (14.5 ± 5.6 ULN vs 32.5 ± 30.7 ULN, $P = 0.001$). Among DILI patients, one (4%) case was classified as definite, 19 (76%) cases were classified as probable and 5 (20%) as possible according to the CIOMS scale. A hepatocellular pattern was observed in 23 (92%) cases and mixed in 2 (8%). The extent of severity of liver injury was mild in 21 (84%) patients and moderate in 4 (16%). Before discharge, 10 (40%) patients were recovered and the other 15 (60%) were improved. The improved patients tended to have a higher peak ALT (808 ± 348 U/L vs 623 ± 118 U/L, $P = 0.016$) and shorter treatment duration before discharge (8 ± 6 d vs 28 ± 12 d, $P = 0.008$) compared with the recovered patients. Twenty-two drugs and 6 herbs were found associated with DILI. Antibacterials were the most common agents causing DILI in 8 (32%) cases, followed by glucocorticoids in 6 (24%) cases. Twenty-four (96%) cases received treatment of DILI with at least one adjunctive drug. Agents for treatment of DILI included anti-inflammatory drugs (e.g., glycyrrhizinate), antioxidants (e.g., glutathione, ademetionine 1,4-butanedisulfonate and tiopronin), polyene phosphatidyl choline and herbal extracts (e.g., protoporphyrin disodium and silymarin). Diagnosis of DILI was not mentioned in the discharge letter in 60% of the cases. Relative to prevalent cases and cases from wards of internal medicine, incident cases and cases from surgical wards had a higher risk of missed diagnosis in discharge letter [odds ratio (OR) 32.7, 95%CI (2.8-374.1),

and OR 58.5, 95%CI (4.6-746.6), respectively].

CONCLUSION: DILI is mostly caused by use of antibacterials and glucocorticoids, and constitutes about one fifth of hospitalized patients with ALT > 10 ULN. DILI is underdiagnosed frequently.

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Key words: Drug-induced liver injury; Abnormal liver enzyme; Alanine aminotransferase; Underdiagnosis; Adjunctive drugs

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INTRODUCTION

Hepatic injury is encountered frequently in clinical practice. Acute hepatic injury can be recognized by the increased activities of aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT) and can be diagnosed by the presence of ALT levels above 10 times upper limit of normal range (ULN). Viral hepatitis and toxic and ischemic hepatic injury are the most common causes of acute hepatic injury. Rarely, Wilson disease and autoimmune hepatitis can also present as acute hepatic injury^[1]. The frequency of different causes of acute hepatic injury remains unknown and varies worldwide.

Among these causes of acute hepatic injury, drug-induced liver injury (DILI) represents an important challenge for physicians^[2]. DILI is the leading cause of death from acute liver failure and accounts for approximately 13% of cases of acute liver failure in the United States^[3,4]. Furthermore, drug-induced hepatotoxicity is one of the main reasons for postmarketing regulatory decisions, including drug withdrawal^[5]. However, because there are no specific markers coupled with the highly variable clinical presentations of DILI, the recognition and diagnosis of DILI are often difficult and delayed due to the need to exclude more common competing causes of liver injury^[6,7]. The real proportion and seriousness of DILI in patients with acute hepatic injury remain unknown. A previous study showed that DILI was observed in 18%-22% of medical inpatients fulfilling Council for International Organizations of Medical Sciences (CIOMS) laboratory criteria^[8]. Since hepatocellular DILI is the most common type of DILI, the proportion of DILI in patients with ALT > 10 ULN might be higher than in medical inpatients.

The aim of this study was to determine the propor-

tion of DILI in patients with ALT > 10 ULN and identify the causes and the nature of DILI. And we also wanted to know the applicability of ALT > 10 ULN as a criterion for screening DILI patients.

MATERIALS AND METHODS

Case identification

This study was carried out in a 2300-bed teaching hospital of Zhejiang University School of Medicine, China. All hospitalized patients with ALT > 10 ULN (reference value 0-50 U/L) were retrospectively identified from a computerized clinical laboratory database at our hospital covering a 12-mo period (January 2010-December 2010). The medical records for all these patients were independently reviewed by two clinical pharmacists. Alternative causes of ALT elevations were examined for each patient, including biliary abnormality, viral hepatitis, hemodynamic injury, malignancy, DILI or undetermined and other causes (such as liver surgery or trauma, and autoimmune disease). Causes of liver injury were determined based on clinical data and results of investigations such as virology detection, ultrasonography, computed tomographic scanning or magnetic resonance imaging of the liver and biliary tree, antinuclear antibody, smooth-muscle antibody, and gamma globulins detection. A history of alcohol consumption or hypotension which might cause ischemic hepatitis was also needed for diagnosis. As chronic hepatitis B is prevalent in China, presence of hepatitis B surface antigen could not exclude diagnosis of DILI, unless it was accompanied by elevated HBV DNA titer > 1 × 10³^[9].

DILI was suspected based on the following criteria: (1) an appropriate temporal relationship between the intake of the drug and the onset of liver injury, and between the withdrawal of the drug and the course of the reaction; and (2) exclusion of other causes of liver disease^[10]. The CIOMS scale was used to assess the suspected DILI cases^[11], and only the cases classified as highly probable, probable, or possible were diagnosed as DILI. Immune-mediated DILI is an important component of idiosyncratic DILI, which is characterized by presence of fever, rash, eosinophilia and autoantibodies. Extrahepatic manifestations of immune-mediated DILI such as rash, fever, arthralgia, eosinophilia and cytopenia were also reviewed. Prevalent cases referred to the DILI cases with no documented normal baseline values of aminotransferase during hospitalization. In contrast, incident cases were defined as the patients having at least one normal liver parameter prior to DILI development during the hospitalization.

Type of DILI

According to the CIOMS criteria, cases with ALT > 2 ULN alone or an ALT/alkaline phosphatase (AP) ratio ≥ 5 (ALT and AP expressed as multiples of their upper normal limit) were classified as hepatocellular, cases with ALT/AP ratio ≤ 2 as cholestatic and cases with an

ALT/AP ratio between 2 and 5 as mixed liver injuries.

Severity of DILI

The severity of DILI was categorized according to the previous report with minor modifications^[12]: mild [ALT was elevated, but total serum bilirubin was < 2.5 mg/dL and International Normalized Ratio (INR) was < 1.5]; moderate (ALT was elevated and serum bilirubin was ≥ 2.5 mg/dL or INR was ≥ 1.5); and severe [ALT was elevated, and total serum bilirubin ≥ 2.5 mg/dL and with at least one of the following: (1) hepatic failure (INR ≥ 1.5, ascites or encephalopathy); (2) other organ failure believed to be caused by DILI event; and (3) death or liver transplantation because of DILI event].

Drug causality assessment

Drug causality for each case of DILI was assessed according to the WHO definitions for adverse drug reactions: The drug causality was classified as probable if only one drug could be identified as causative for the DILI; it was labeled as certain if a rechallenge was positive; and labeled as possible if more than one drug could have caused the DILI. Drugs were sorted and grouped according to the Anatomical Therapeutic Chemical classification.

Outcome

Cases with a documented decline of all liver parameters < 3 ULN before discharge were labeled as recovery, whereas cases with a documented decline but with liver parameters remaining > 3 ULN were labeled as improvement. Cases without a documented decline of liver parameters before discharge were labeled aggravation.

Detection and evaluation of DILI by treating doctors

In order to evaluate DILI detection by the treating doctors, any comments related to the diagnosis of DILI in the medical record and in the discharge letter for each case were examined. With respect to cases in which DILI was mentioned, we checked whether the causative drugs were specified in these documents.

Statistical analysis

Data were expressed in mean ± SD or mean (range) unless otherwise stated, and were analyzed using the SPSS version 13.0 (Chicago, IL, United States). Continuous variables were compared using independent-sample *t* test between two groups and one-way analysis of variance and *Post hoc* tests among more than two groups. Categorical variables were compared by χ^2 (Fisher's exact) test and the likelihood ratio test. Differences were reported as statistically significant if *P* < 0.05.

RESULTS

Causes of increased ALT > 10 ULN

A total of 129 patients with ALT > 10 ULN were identified in this study. Their mean age was 51 years (range, 6-83

Table 1 Characteristics of patients with alanine aminotransferase > 10 upper limit of normal range (mean ± SD)

Causes	<i>n</i> (%)	Age (yr)	Female <i>n</i> (%)	Peak ALT values (× ULN)
Hemodynamic injury	46 (25.7)	54.2 ± 17.2 ^a	14 (30.4)	32.5 ± 30.7 ^b
Drug-induced liver injury	25 (19.4)	45.4 ± 16.8	9 (36.0)	14.5 ± 5.6
Malignancy	21 (16.3)	55.8 ± 14.6 ^a	2 (9.5)	17.5 ± 10.3
Biliary abnormality	15 (11.6)	52.3 ± 14.4	7 (46.7)	14.3 ± 7.7
Viral hepatitis	12 (9.3)	47.8 ± 16.8	4 (33.3)	18.6 ± 10.0
Undetermined and others	10 (7.8)	42.9 ± 12.0	5 (50)	30.9 ± 33.5 ^a
Total	129 (100)	51.1 ± 16.4	41 (31.8)	23.0 ± 22.7
<i>P</i> (between different causes)	-	0.085	0.15	0.004

Post hoc tests ^a*P* < 0.05, ^b*P* < 0.01 vs drug-induced liver injury group. ALT: Alanine aminotransferase; ULN: Upper limit of normal range.

years). Table 1 shows the number of patients with ALT > 10 ULN, age, female percentage as well as peak ALT values derived from all causes. Hemodynamic injury (*n* = 46, 35.7%), DILI (*n* = 25, 19.4%) and malignancy (*n* = 21, 16.3%) were the top three causes of acute liver injury. The other causes included biliary abnormality (11.6%), viral hepatitis (9.3%) and undetermined and other causes (7.8%). There was no difference in gender distribution among different cause groups, but patients were older (54.2 ± 17.2 years vs 45.4 ± 16.8 years, *P* = 0.029) and the magnitude of ALT alteration was higher (32.5 ± 30.7 ULN vs 14.5 ± 5.6 ULN, *P* = 0.001) in the group caused by hemodynamic injury than in the group caused by DILI.

Features of DILI

Among the DILI patients, the median age was 47 years (range, 20-83 years) and 9 (36%) were female. A total of 10 (40%) patients were 50 years or older and 3 (12%) had a history of alcohol consumption. Sixty-eight percent of patients developed DILI during hospitalization. The median duration between first exposure to the implicated agent and DILI recognition was 6 d (range, 1-40 d). The peak values for serum biochemistries (mean ± SD) were as follows: ALT, 733.7 ± 290.3 U/L; AP, 167.8 ± 120.0 U/L; and total bilirubin, 1.2 ± 1.3 mg/dL. A hepatocellular pattern was observed in 23 (92%) cases and mixed in 2 (8%) cases, and 4 (16%) cases had jaundice. Two (8%) cases had rash, 12 (48%) had fever and 6 (24%) had cytopenia. Neither arthralgia nor eosinophilia was observed. The degree of severity of the liver injury was judged to be mild in 84% and moderate in 16%. The median time to biochemical resolution ≥ 50% from the peak values following discontinuation of the offending drug was 6 d (range, 2-12 d). A definite causal relationship was found in one (4%) case, probable in 19 (76%) and possible in 5 (20%) cases. Ten (40%) cases were recovered and the other 15 (60%) were improved before discharge. Clinical features of 25 DILI patients are listed in Table 2. The improved patients tended to have a

Table 2 Demographic, clinical and laboratory parameters of 25 cases of drug-induced liver injury

Variables	Outcome	
	Recovery	Improvement
Age, yr	44.6 ± 18.4	45.9 ± 16.4
Female	3 (30)	6 (40)
Body mass index, kg/m ²	24.3 ± 1.6	21.6 ± 2.8
Alcohol use	2 (20)	1 (6.7)
Jaundice	2 (20)	2 (13.3)
Extrahepatic manifestations		
Rash	1 (10)	1 (6.7)
Fever	6 (60)	6 (40)
Cytopenia	2 (20)	4 (26.7)
Incident cases	6 (60)	11 (73.3)
Surgical wards	5 (50)	9 (60)
Laboratory parameters, peak values		
Alanine aminotransferase (U/L)	623 ± 118	808 ± 348 ^a
Aspartate aminotransferase (U/L)	338 ± 187	636 ± 651
Alkaline phosphatase (U/L)	169 ± 172	167 ± 75
Total bilirubin (mg/dL)	1.4 ± 1.6	1.1 ± 1.0
γ-glutamyl transpeptidase (U/L)	190 ± 224	213 ± 149
Hepatocellular type	9 (90)	14 (93.3)
Causality assessment		
Highly probable	0	1 (6.7)
Probable	9 (90)	10 (66.7)
Possible	1 (10)	4 (26.7)
Time to onset, d	5.3 ± 3.8	8.9 ± 10.2
Time to enzymes resolution, d	6.7 ± 2.4	6.0 ± 3.0
Number of adjunctive drugs	2.6 ± 1.6	2.5 ± 0.8
Adjunctive drugs for treatment		
Anti-inflammation	7 (70)	14 (93.3)
Antioxidants	7 (70)	11 (61.1)
Phospholipids	6 (60)	7 (46.7)
Herbs	0	3 (20.0)
Time from treatment of liver injury to discharge, d	28 ± 12	8 ± 6 ^a

^a*P* < 0.05 *vs* recovery group. In parentheses: Standard deviation; In brackets: Percentage.

higher peak ALT and shorter treatment duration before discharge compared with the recovered patients (mean peak ALT, 808 ± 348 U/L *vs* 623 ± 118 U/L, *P* = 0.016; and mean treatment duration before discharge, 8 ± 6 d *vs* 28 ± 12 d, *P* = 0.008).

Causative agents

DILI was caused by a single prescription medication in 16% of the cases, by multiple agents in 40% and by herbs in 16%. Twenty-two chemical agents and 6 herbs were associated with DILI. Antibacterials were the most common class of agents associated with DILI in 8 (32%) cases. The antibacterials most often encountered were furbenicillin (*n* = 2), cefminox (*n* = 2), ornidazole (*n* = 2), panipenem and betamipron (*n* = 2), meropenem (*n* = 1), piperacillin and sulbactam (*n* = 1) and ceftiofloxacin (*n* = 1). DILI was caused by glucocorticoids in 6 cases (24%), including 5 cases treated with methylprednisolone, which was the leading individual drug and one case treated with dexamethasone. Implicated causative chemical drugs in 25 subjects with DILI are shown in Table 3. Characteristics of the 6 DILI patients caused by glucocorticoids are presented in Table 4.

Table 3 Implicated causative chemical drugs in 25 subjects with drug-induced liver injury

Classification (<i>n</i>)	Specific drugs (<i>n</i>)
Proton pump inhibitors (2)	Omeprazole (2)
Cardiovascular system (3)	Amiodarone (1), atorvastatin (1), cinepazide (1), telmisartan (1)
Glucocorticoids (6)	Methylprednisolone (5), dexamethasone (1)
Antibacterials for systemic use (8)	Furbenicillin (2), cefminox (2), ornidazole (2), panipenem and betamipron (2), meropenem (1), piperacillin and sulbactam (1), ceftiofloxacin (1)
Monoclonal antibodies (1)	Bevacizumab (1)
Musculo-skeletal system (4)	Diclofenac (1), celecoxib (1), parecoxib (1), baclofen (1)
Nervous system (2)	Olanzapine (2), fluoxetine (1)

Underdiagnosis rate of DILI

DILI was mentioned in medical records of 19 (76%) cases and in the discharge letter of 10 (40%) cases. Those who had missed diagnosis of DILI in medical records also had a higher risk of missed diagnosis of DILI in discharge letters [odds ratio (OR) 1.7, 95%CI (1.1-2.5), *P* = 0.022]. Among the 19 cases of DILI, there were 9 (47.4%) cases without information of specified causative drugs. As shown in the discharge letter, patients from surgical wards had a higher risk of missed diagnosis of DILI compared with those from internal medicine wards [OR 58.5, 95%CI (4.6-746.6), *P* < 0.001]. Similarly, incident cases had a higher risk of underdiagnosis of DILI in their discharge letter compared with the prevalent cases [OR 32.7, 95%CI (2.8-374.1), *P* = 0.001].

Adjunctive drugs for treatment of DILI

Only one case was not treated by any hepatic disease adjunctive drugs. DILI was treated by a single medication in 12% of the cases, and by at least two agents in 84% cases (Table 5). Ten agents were used for treatment of DILI. Anti-inflammatory drugs such as glycyrrhizinate were the most common agents used for treatment of DILI, with a total of 21 (84%) cases. The antioxidants were prescribed for 18 (72%) cases, including glutathione (*n* = 17), ademetionine 1, 4-butanedisulfonate (*n* = 6) and tiopronin (*n* = 1). Polyene phosphatidyl choline was used in 13 (52%) cases.

DISCUSSION

Among the hospitalized patients with ALT > 10 ULN, DILI was the second common cause of liver injury, accounting for 19.4% of cases. Antibacterials and glucocorticoids were the most frequently causative agents for DILI. The degree of severity of DILI was mild or moderate. Ninety-six percent of DILI cases received at least one adjunctive drug for treatment of DILI. All DILI patients were either recovered or improved when they discharged from the hospital. The underdiagnosis rate of DILI in discharge letters was 60%.

ALT and AST are enzymes highly concentrated in the

Table 4 Characteristics of 6 cases of drug-induced liver injury caused by glucocorticoids

Age/sex	Principal disease	Drug	Dose (mg/d)	Treatment duration (d)	Peak values					Follow-up
					ALT (U/L)	AST (U/L)	AP (U/L)	TB (mg/dL)	GGT (U/L)	
20/F	SLE	MP	20-160	84	600	138	83	2.31	183	Recovered
36/F	Chronic glomerulonephritis	MP	32-40	17	1085	439	162	0.34	41	Improved
25/M	Brain trauma	MP	20	10	515	257	148	0.61	446	Improved
49/M	Brain trauma	MP	40	10	516	363	108	1.19	190	Improved
42/F	Drug eruption	MP	30-160	24	668	34	110	0.61	146	Improved
49/M	Brain tumor	DXM	5-15	12	602	282	231	0.52	424	Improved

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AP: Alkaline phosphatase; TB: Total bilirubin; GGT: γ -glutamyl transpeptidase; F: Female; SLE: Systemic lupus erythematosus; MP: Methylprednisolone; DXM: Dexamethasone.

Table 5 Adjunctive drugs used for treatment of drug-induced liver injury

Classification (n)	Specific drugs (n)
Anti-inflammation (21)	Diammonium glycyrrhizinate (9), magnesium isoglycyrrhizinate (8), compound monoammonium glycyrrhetate S (4), compound glycyrrhizin (3)
Antioxidants (18)	Glutathione (17), ademetonine 1,4-butanedisulfonate (6), tiopronin (1)
Phospholipids (13)	Polyene phosphatidyl choline (13)
Herbal extrats (3)	Protoporphyrin disodium (2), silymarin (1)

liver and are sensitive indicators of hepatocyte damage. Patients with a marked increase in aminotransferase levels (> 10 ULN) typically have acute hepatic injury. The study of the causes of notably elevated AST of liver origin in the United Kingdom^[13] showed that hepatic hypoxia was the most common cause (50%), followed by pancreato-biliary disease. Since viral hepatitis is prevalent in many Asian countries and it is the primary cause in 95%-100% of patients with acute hepatic failure in India^[14], viral hepatitis was supposed to be the most common cause of elevated ALT > 10 ULN in China. However, our results showed that hemodynamic injury is the most common cause of notably elevated ALT (36%) similar to the results from the AST study. Viral hepatitis accounts for 9.3% patients with ALT > 10 ULN, which is more than two times higher than the proportion (3.6%) in the AST study. From the results of present study, we should take a new look at causes of acute liver injury in China. Unlike the results from the study of AST, DILI accounts for 19.4% of inpatients with ALT > 10 ULN, which is more than two times higher than the proportion of 8.8% in patients with AST > 10 ULN, indicating that ALT is a more suitable indicator for diagnosis of drug-induced hepatocellular injury than AST.

Although there can be grey areas in which a range of causes overlap, the magnitude and rate of ALT change may provide initial insight into a different diagnosis. A very high ALT (> 75 ULN) are more likely caused by ischemic or toxic liver injury^[6]. In our study, we found that the average peak ALT value in patients with hemodynamic liver injury was more than two times higher than the value in patients with DILI and ALT > 50 ULN was

less likely caused by DILI. However, the average peak ALT values were similar among patients with DILI, liver malignancy, biliary abnormality and viral hepatitis. Therefore, it was hard to identify these etiologies of liver injury merely according to the ALT levels.

DILI is usually recorded by spontaneous reporting in postmarketing surveillance. However, spontaneous reporting frequencies of DILI to the pharmacovigilance authorities are always low in most countries^[15]. The CIOMS laboratory criteria are widely used in studies of DILI. The CIOMS laboratory criteria require at least two determinations of ALT > 2 ULN, conjugated bilirubin > 2 ULN, or combined increases of AST, AP and total bilirubin with one value > 2 ULN, which are too complicated as criteria for screening DILI in real practice. Proportion of DILI in different populations varies depending on different screening laboratory criteria. In our study, among 129 inpatients with ALT > 10 ULN, 25 patients had DILI resulting in a positive predictive value of 19.4% for a case detection based on ALT > 10 ULN. This is similar to the values of 18%-22% in medical inpatients fulfilling CIOMS laboratory criteria^[8]. Because of the hepatocellular predominance of ALT, 92% of DILI cases in this study were of hepatocellular injury. Thus, positive predictive rate of drug-induced hepatocellular injury was 18% in cases with ALT > 10 ULN, which was more than two times higher than the values of 8% in patients fulfilling CIOMS laboratory criteria^[16]. However, it must be noted that the criteria used in our study increased the positive predictive value for diagnosis of drug-induced hepatocellular injury at a cost of missing drug-induced cholestatic injury.

The underdiagnosis of DILI is well known^[8]. In the present study, among 25 cases of DILI, comments about the diagnoses of DILI were given in only 10 (40%) cases in their discharge letters. Nineteen (76%) cases had a diagnosis of DILI during hospitalization, but causative drugs were specified in only less than half of the cases. Underdiagnosis of DILI occurred more often in incident cases and cases from surgery wards. Although the diagnosis rate in our study was not optimistic according to the patients discharge letters, the rate of 40% was more than five times higher than the rate of 7% in a previous study by Meier *et al.*^[8]. This may be due to different detection criteria used, e.g., CIOMS laboratory criteria were

used in that study, but ALT > 10 ULN was used in our study. Doctors might pay more attention to ALT >10 ULN than to ALT > 2 ULN, which is one of the CIOMS laboratory criteria. Underdiagnosis of DILI may lead to a severe underestimation of the prevalence and incidence of DILI, especially for the studies based on a computerized diagnosis database. It is still a worldwide problem with respect to the methods for improving the reporting rate of DILI. One of the aims of our study is to identify whether ALT >10 ULN is a good cut-off point for screening DILI patients. From the results of our study, we can conclude that a cut-off in all patients with ALT >10 ULN for screening DILI has yielded at least the same positive predictive value as the CIOMS laboratory criteria which might be more easily accepted by doctors.

Because inpatients rarely received only a single drug for treatment, which makes it difficult to determine which drug is the most responsible agent inducing liver injury even using the CIOMS criteria, we labeled possible for all suspected drugs. The drugs causing DILI vary among countries. In European countries and the United States, antibiotics, cardiovascular and central nervous system drugs are the most frequent causes of DILI^[3,5,10,17]. In Asian countries, herbal and dietary supplements are often the most common causes of DILI^[9,18]. The current study revealed that the most common type of drugs was antibacterials, accounting for 32% of all DILI cases. The ratio of DILI caused by antibacterials in our study is very similar to the data in outpatients of a Swedish university hospital and inpatients in Switzerland hospitals^[8,15]. Discrepancies in the published data on the category of antibacterials related with DILI may attribute to the different local therapeutic strategies and prescribing behaviors in different countries^[15,19]. Similar to previous studies, hepatotoxicity induced by NSAIDs, omeprazole, amiodarone, statins and olanzapine was recognized. Unexpectedly, we found that 6 cases of DILI might be caused by glucocorticoids. Glucocorticoid is a relatively uncommon cause of liver injury; moreover, it is the choice of treatment for severe hepatitis. However, some cases of corticosteroid-induced hepatotoxicity, mostly induced by the high-dose methylprednisolone have been reported^[20]. In our study, liver injuries were all induced by low-doses of glucocorticoids. The mechanisms of corticosteroid-induced liver injury still remains unclear. Reactivation of HBV infections, excipient of the methylprednisolone preparation and/or idiosyncrasy reactions might all be associated with the liver injury. The significance and mechanism of liver injury caused by glucocorticoids deserve more attention and need more researches. Herbal medicines seem to be the major causes of DILI in Asian countries^[9]. In our study, we found that herbal medicines were responsible for 16% of DILI cases which is higher than the proportion rate in Japan^[18], but much lower than the rate in Singapore^[9]. This may be explained by different herbs consumptions among countries.

Up till now, therapeutic interventions for DILI remain principally limited to the cessation of the causative drugs, supportive therapy and monitoring for acute he-

patic failure^[21]. However, in our study, adjunctive drugs were used in 96% of DILI patients after cessation of the inducing drugs. Glycyrrhizinate, antioxidants and polyene phosphatidyl choline were the most common agents used for treatment of DILI. Data from some clinical studies showed that these drugs are beneficial to patients with DILI^[22-25]. In the current study, none of the patients died from DILI and all patients were either recovered or improved when they were discharged, in spite of the fact that 4 (16%) patients had jaundice. If both drug-induced hepatocellular injury and jaundice occur at the same time, a mortality of at least 10% can be expected according to the Hy's rule^[26]. The reason why we did not detect any fatal cases may somewhat attribute to the use of adjunctive drugs. We did not find any significant difference between recovered patients and improved patients in how many adjunctive drugs were used and which drug was chosen for treating DILI. Although our sample size was small and the follow-up of the patients was done only till discharge, but not till the time of complete recovery, our findings suggest that adjunctive drugs may be effective for treatment of DILI, however, further studies are needed to determine their efficacy and safety in subjects with DILI.

Our study is limited by its retrospective nature. A detailed medical history of the DILI patients before hospitalization could not be obtained if the doctors had not described it in the medical records. For prevalent cases, our data were mainly based on the doctors' judgments. Some patients had not undergone a complete investigation to exclude other possible causes of liver disease. We found that even among cases of DILI diagnosed by treating doctors, many critical elements needed for a diagnosis of DILI were not available. A checklist of minimum elements required for diagnosis of DILI^[19] would be helpful for improving the DILI diagnosis rates and future DILI research.

In conclusion, our study demonstrates that DILI was most frequently caused by use of antibacterials and glucocorticoids and constituted about one fifth of hospitalized patients with ALT > 10 ULN. Underdiagnosis of DILI is common, especially in patients from surgical wards and incident cases. An online warning for diagnosis of DILI when a patient' ALT was > 10 ULN and a checklist of minimum elements required for diagnosis of DILI may both be helpful for improving DILI diagnosis rates and future DILI research.

COMMENTS

Background

Drug-induced liver injury (DILI) is being increasingly recognized as an important cause of acute hepatic injury. It is the leading cause of acute liver failure in several Western countries and the most common reason for postmarketing regulatory decisions. The recognition and diagnosis of DILI are often difficult and delayed because of the lack of specific diagnostic markers coupled with the highly variable clinical presentations of DILI.

Research frontiers

The real incidence of DILI remains unknown because of the difficulty in diagnosis and the low spontaneous reporting frequency to the pharmacovigilance

authorities. The focus of DILI research is how to improve the detection of DILI to better understand the causes, risk factors and nature of DILI. Screening laboratory results of patients and using diagnostic scales, such as the Council for International Organizations of Medical Sciences scale for patients with suspected DILI, may help improve the detection of DILI.

Innovations and breakthroughs

In this study, the authors identified that DILI was the second common cause of alanine aminotransferase (ALT) > 10 times upper limit of normal range (ULN) and a positive predictive value of 19.4% for DILI detection based on ALT > 10 ULN. Antibacterials were the most common causative agents for DILI. Unexpectedly, glucocorticoids, which are commonly used for severe hepatitis treatment, were found to be the second common causative agents. A high rate (60%) of underdiagnosis of DILI was found in patients with ALT > 10 ULN, especially in incident cases and cases from surgical wards.

Applications

The results suggest that underdiagnosis of DILI is frequent and ALT > 10 ULN can be used as a laboratory screening criterion to improve the detection of DILI. The significance and mechanism of causative drugs of DILI, such as glucocorticoids, await further researches.

Terminology

CIOMS scale: This scale is determined by a score based on 7 criteria, including temporal relationship, clinical course (response after withdrawal of drug), risk factors, concomitant drugs, exclusion of other non-drug etiologies, likelihood of a reaction based on package labeling, and rechallenge. The categories of suspicion are definite or highly probable (score > 8), probable (score 6-8), possible (score 3-5), unlikely (score 1-2) and excluded (score ≤ 0).

Peer review

The authors retrospectively investigated the proportion and causality assessment of possible DILI cases in hospitalized patients in a university hospital in China. The research has novelty in identifying and classifying DILI cases. The causality assessment of the DILI cases in this research also provides meaningful information about unique drugs implicated in non-fulminant DILI in the studied patients.

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Dedifferentiated liposarcoma of the rectum: A case report

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Abstract

Liposarcoma is one of the most common soft tissue sarcomas found in adults, and it usually occurs in the retroperitoneum and the extremities. Here, we describe a case of dedifferentiated liposarcoma originating from a well-differentiated liposarcoma of the mesorectum that presented as a protruding mass in the rectal lumen. Hartmann's operation with total mesorectal excision was performed and the tumor was removed radically. No management guidelines are currently available for liposarcoma of the rectum. We propose that complete surgical resection be required for the treatment of rectal liposarcoma and that a long-term detailed follow up is necessary.

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Key words: Dedifferentiated liposarcoma; Soft tissue sarcoma; Rectum; Management; Surgery

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INTRODUCTION

Liposarcoma is one of the most common malignant soft tissue tumors found in adults. Most liposarcoma patients are between 40 and 60 years of age, and the incidence in men and women with this disease is approximately equal. Currently, classification of liposarcoma is divided into five subtypes: myxoid, pleomorphic, dedifferentiated, round cell, and well-differentiated liposarcoma (WDLPS)^[1]. WDLPS is the most common histological subgroup, whereas dedifferentiated liposarcoma (DDLPS) has a comparatively worse prognosis. In this report we describe a WDLPS arising from the mesorectum and presenting as an endoluminal mass. Additionally, this tumor had histological characteristics consistent with DDLPS and had transmurally invaded into the rectum.

CASE REPORT

A 77-year-old Japanese male patient complaining of constipation and urinary retention, was admitted to Kurashiki Central Hospital and examined by a gastroenterologist. The patient had diabetes mellitus that was under control with anti-diabetic drugs. Physical examination revealed a large mass in the lower abdomen, and an abdominal computed tomography (CT) scan showed a large tumor mass in the rectum accompanied by prostatomegaly. Pelvic magnetic resonance imaging revealed a 9 cm × 7 cm × 5 cm sized polypoid tumor in the rectal cavity, with the dorsal side of the mesorectum appearing to protrude into the tumor (Figure 1). A colonoscopy revealed a smooth-

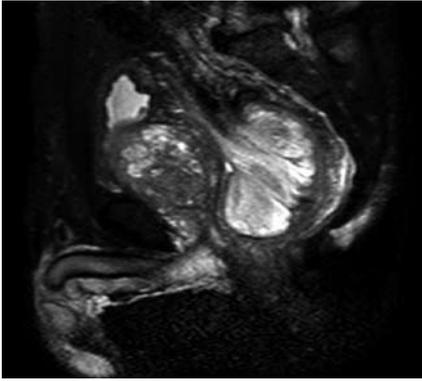


Figure 1 T2-weighted magnetic resonance image demonstrating a high-intensity mass in rectum with prostatomegaly.

surfaced, oval-shaped, large tumor in the rectum, with the lower end located about 10 cm from the anal verge. The first preoperative colonoscopic biopsy retrieved some necrotic but no malignant tissue. The immunohistochemical analysis of the second biopsied specimen demonstrated positive staining for CD34, CD31 and Ki67, and negative staining for c-kit, AE1/AE3 and epithelial membrane antigen, with the MIB-1 index at 30%. Preoperative diagnosis was not definitive but indicated a possible high-grade sarcoma. The tumor marker carbohydrate antigen 19-9 was detected at an abnormally high level, while other tumor markers were within normal ranges. The gastroenterologist suspected that the preoperative diagnosis was angiosarcoma, malignant solitary fibrous tumor, or c-kit negative gastrointestinal stromal tumor and introduced the patient to the surgical department for surgical resection. A preoperative digital examination confirmed that the distance between the lower end of the tumor and the anal verge was 10 cm and that the tumor was mobile.

The patient underwent a laparotomy, which revealed a large palpable tumor in the rectum. Since the preoperative diagnosis indicated a possible high-grade sarcoma, the local recurrence rate after the operation was not expected to be low, even if the tumor was resected radically. Therefore, total mesorectal excision was performed in addition to Hartmann's operation to help decrease the risk of pelvic recurrence. A part of the mesorectum was slightly hard and the tumor was resected radically. Gross examination revealed a large submucosal tumor with invagination of the surrounding large intestine. Following surgery, the patient was discharged without any postoperative complications.

The resected solid tumor was 85 mm × 67 mm × 32 mm in size and pedunculated (Figure 2A). Histological examination revealed a high-grade sarcoma consisting of spindle-shaped tumor cells with hyperchromatic oval nuclei and eosinophilic cytoplasm (Figure 2B, C). The differentiation status of the tumor was determined morphologically as well as immunohistochemically. The tumor was positive for Bcl-2, vimentin, desmin, CD34, CD56, CD10, and CD99. However, the mass was found to be present transmurally in the rectum, and was con-

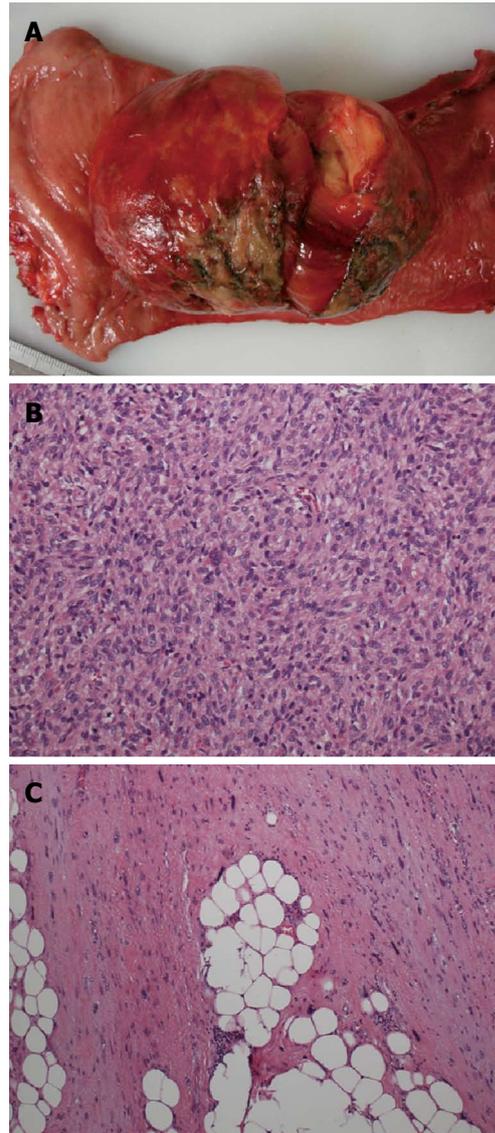


Figure 2 Dedifferentiated liposarcoma in rectum. A: Gross picture showing a huge dedifferentiated liposarcoma in rectum; B: Dedifferentiated component identified in polypoid lesion at rectum [hematoxylin and eosin (HE), × 400]; C: Well-differentiated liposarcoma at mesorectum (HE, × 400).

tinuous to WDLPS, consisting of mature adipose tissue, intervening fibrous tissue, and scattered atypical cells with large, unusual nuclei in the mesorectum. Thus, a diagnosis of DDLPS arising from WDLPS was rendered.

Three months postoperatively, no recurrence or metastasis was identified *via* CT scan.

DISCUSSION

Retroperitoneal malignant tumors are rare; however, liposarcoma is the most common type^[2-6]. Liposarcoma tends to occur in the fourth to sixth decades of life, with no difference in frequency among the sexes. Liposarcomas have been divided into five subtypes by the World Health Organization (well differentiated, dedifferentiated, myxoid, pleomorphic, and mixed type)^[1]. In 1979, Evans^[7] was the first to characterize a liposarcoma. He

described a combination of WDLPS and a non-lipogenic dedifferentiated sarcoma-like component. In 1971 Dahlin *et al*^[8] described the dedifferentiated chondrosarcoma as a morphologically biomorphic neoplasm showing areas of well-differentiated, low-grade tumors juxtaposed with high-grade non-chondroblastic tumors without obvious areas of gradual transition. Dedifferentiation can take place through a *de novo* mechanism or through the recurrence of WDLPS in which additional changes have occurred^[9]. The original definition of DDLPS has been modified over time. Dedifferentiation into exclusively low-grade areas or into a combination of low and high-grade areas has been included in this subtype. DDLPS has a less aggressive clinical course than other types of high grade sarcoma, although the underlying mechanism remains unclear. Approximately 40% of DDLPSs will recur locally and 17% will metastasize and 28% of patients will ultimately die as a result of the tumor^[1].

In our case, the mesorectum spindle cells in the pleomorphic lipoma were positive for CD34, indicating a well-differentiated liposarcoma. Preoperative immunohistochemical staining with CD117 was negative, suggesting that the tumor was not a typical GIST. Postoperative histological findings revealed a transition from WDLPS to a non-lipogenic sarcoma with a variable grade at the polypoid lesion indicating a DDLPS.

The most common sites of DDLPS are the retroperitoneum and extremities, with other anatomic locations occasionally reported. Excluding case of DDLPS in the retroperitoneum, we found nine cases of localized DDLPS. Six of the DDLPS cases occurred in the small bowel mesentery^[10,11] and two cases of primary advanced DDLPS occurred in the colon^[12,13]. The final case of DDLPS occurred in the sigmoid mesocolon^[14]. The case we present here is an example of primary retroperitoneal WDLPS with secondary involvement of the rectum. An unusual feature of this case was that the DDLPS was detected in a polypoid lesion in the rectum.

The appropriate diagnostic and therapeutic approach to treat DDLPS has not yet been determined; although, it is generally accepted that complete surgical resection of the tumor should be performed to increase the cure rate of this disease. In our case, total mesorectal excision of the rectum was performed and was expected to have removed the tumor radically. The patient has been followed-up and no signs have been detected to suggest further need for therapy. However, prognosis of DDLPS mainly depends on local recurrence and almost all retroperitoneal cases recur locally in 10-20 years following

treatment^[15]. Therefore, we recommend that complete surgical resection and long-term follow up after surgery be required for the treatment of DDLPS due to the risk of recurrence.

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Synchronous double cancers of the common bile duct

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Abstract

We report an extremely rare case of synchronous double cancers of the common bile duct without pancreaticobiliary maljunction. Only two similar cases have been reported in the English literature. Endoscopic retrograde cholangiopancreatography showed a tubercous filling defect in the middle and superior parts of the common bile duct, and mild stenosis in the inferior duct. Computed tomography (CT) showed a well enhanced mass in the middle and superior parts of the common bile duct. A single cancer of the middle and superior bile duct was suspected and extra-hepatic bile duct resection was performed. CT eleven months after surgery revealed enhanced inferior bile duct wall and a slightly enhanced tumor within it. Retrospective review of the CT images taken before first surgery showed enhanced inferior bile duct wall without intrabiliary tumor only on the delayed phase. The inferior bile duct tumor was suspected to have originally co-existed with the middle and superior bile duct tumor. Pancreaticoduodenectomy was performed subsequently. Histopathological examination revealed that the middle and superior bile duct tumor was a moderately differentiated tubular adenocarcinoma while the inferior bile duct tumor was a papillary adenocarcinoma. The two tumors were separated and had different histological findings and growth patterns, further suggesting that they were two primary cancers.

INTRODUCTION

Synchronous double cancers in the biliary system are rare^[1]. Most such tumors are double cancers in the common bile duct (CBD) and the gallbladder associated with pancreaticobiliary maljunction (PBM)^[1-3]. We report here an extremely rare surgical case of synchronous double cancers of the CBD without PBM. To our knowledge, only two similar cases have been reported in the English literature^[4,5].

CASE REPORT

A 78-year-old male presented with abdominal pain and jaundice. Computed tomography (CT) showed dilatation of the intrahepatic bile duct and a well-enhanced mass in the middle and upper parts of the CBD (Figure 1A). No mass was detected in the inferior part of the CBD (Figure 1B). The results of laboratory tests showed elevated serum levels of several liver enzymes (total bilirubin 19.6 mg/dL, glutamic oxaloacetic transaminase 54 mg/dL, glutamic pyruvic transaminase 103 mg/dL), and high levels of carcinoembryonic antigen (5.4 ng/dL) and carbohydrate antigen 19-9 (375 U/mL). Endoscopic ret-



Figure 1 Imaging findings before the first surgery. A: Computed tomography showed a well enhanced mass in the middle and superior parts of the bile duct (black arrow); B: No mass was detected in the inferior part of the bile duct (white arrow); C: Endoscopic retrograde cholangiopancreatography revealed a tuberos filling defect in the middle and superior parts of the bile duct (black arrows) and mild stenosis in the inferior part (white arrows).

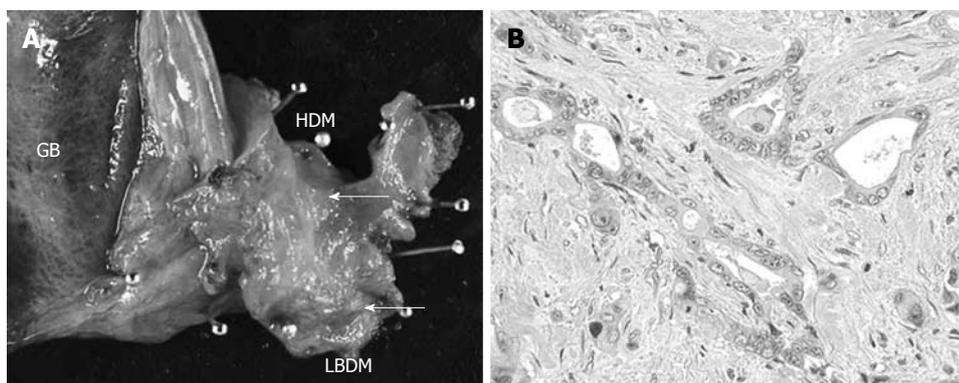


Figure 2 Macroscopic and microscopic findings of resected specimen at the first surgery. A: Resected specimen of the extra-hepatic bile duct showed whitish tuberos tumor in the middle and inferior parts of the bile duct (cancer marked by the white arrows); B: Microscopically, the tumor was moderately differentiated tubular adenocarcinoma with invasive growth (hematoxylin-eosin; magnification; $\times 100$). GB: Gallbladder; HDM: Hepatic duct margin; LBDM: Lower bile duct margin.

rograde cholangiopancreatography (ERCP) showed the absence of PBM and the presence of a tuberos filling defect in the middle and superior parts of the CBD and mild stenosis in the inferior bile duct (Figure 1C). Endoscopy showed no abnormal finding in the papilla of Vater. Although a mild stenosis of the inferior part of the CBD was detected on ERCP, no tumor could be detected in that part of the duct on CT, suggesting that the stenosis was not caused by a malignant tumor. The provisional diagnosis was a solitary tumor in the middle and superior parts of the bile duct. Extra-hepatic bile duct resection with regional lymph node dissection was performed. Histopathological examination showed a moderately differentiated tubular adenocarcinoma with invasive growth (Figure 2). Both the proximal and distal ductal margins

were negative. The postoperative course was uneventful. A repeat CT taken 11 mo after surgery showed enhanced inferior bile duct wall and a slightly enhanced tumor measuring 1.6 cm in diameter within the duct (Figure 3A). Cholangioscopy revealed a papillary tumor in the remaining inferior bile duct (Figure 3B). Retrospective review of the CT images before the first surgery confirmed the lack of abnormal findings in the inferior bile duct on the arterial and portal venous phases. However, the enhanced inferior bile duct wall was detected only on the delayed phase (Figure 3C). Based on the clinical course and CT findings, the inferior bile duct tumor was suspected to have originally co-existed with the middle and superior bile duct tumor although it was not detected before the first surgery. Pancreaticoduodenectomy was performed

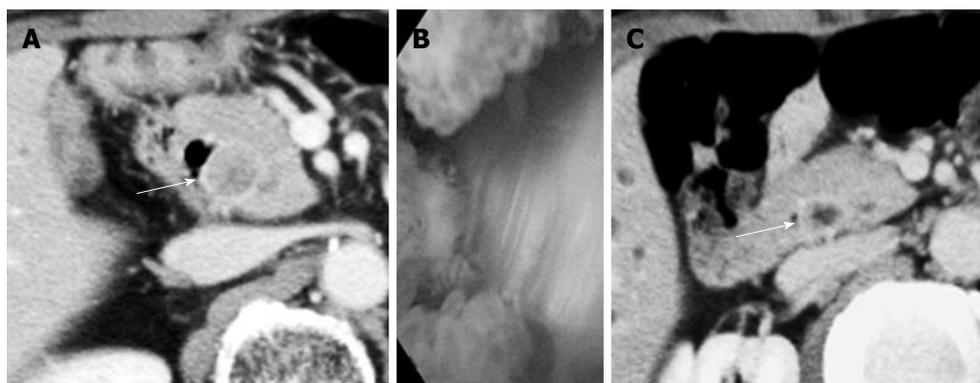


Figure 3 Imaging findings before the second surgery and retrospectively reviewed computed tomography finding before the first surgery. A: Computed tomography (CT) taken 11 mo after the first surgery showed enhanced inferior bile duct wall (white arrow) and slightly enhanced tumor within the duct; B: Cholangioscopy revealed a papillary tumor in the remaining inferior bile duct; C: Retrospective review of the CT images before the first surgery revealed enhanced inferior bile duct wall (white arrow) only on the delayed phase.

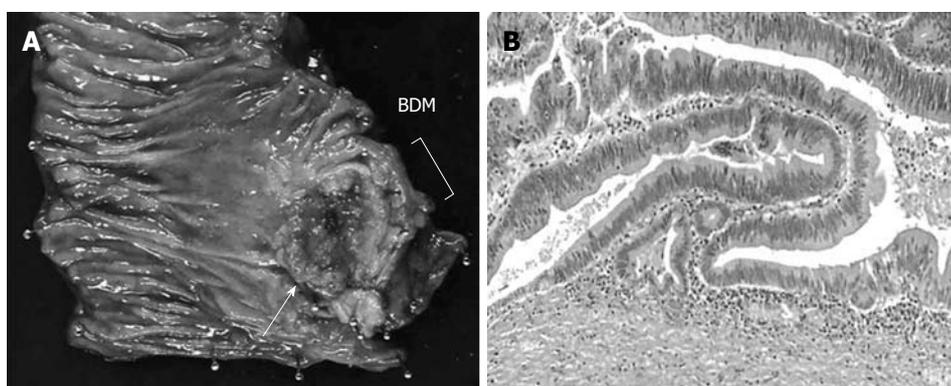


Figure 4 Macroscopic and microscopic findings of resected specimen at the second surgery. A: Specimen resected during pancreaticoduodenectomy. Note the papillary tumor in the inferior bile duct (white arrow); B: Microscopic examination showed papillary adenocarcinoma with expansive growth (hematoxylin-eosin, magnification; $\times 100$). Tumor cells were confined within the fibromuscular coat. BDM: Bile duct margin.

as the second surgery. Histopathological examination showed a papillary adenocarcinoma with expansive growth (Figure 4), and a negative distal ductal margin. Postoperatively, the patient received adjuvant chemotherapy with S-1. However, multiple liver metastases were detected 10 mo after the second surgery. The patient received additional chemotherapy with gemcitabine for the recurrent metastases, but the metastatic foci showed aggressive growth, resulting in death at 31 mo after the first surgery (18 mo after the second surgery).

DISCUSSION

Synchronous double cancers in the biliary system are rare. Most of the reported cases are double cancers of the CBD and the gallbladder associated with PBM^[1-3]. Fujii *et al*^[6] reported that 62.5% of synchronous double cancers and 100% of metachronous double cancers of the biliary tract were associated with PBM. Biliary cancers with PBM are thought to develop multicentrally, due to the effect of pancreatic juice reflux on the mucosa of the biliary tract^[7]. Our patient had synchronous double cancers of the middle and superior bile ducts and the inferior bile duct. Although ERCP before the first surgery revealed a

mild stenosis in the inferior part of the bile duct, no mass was detected on CT, and the diagnosis was a single bile duct cancer. However, retrospective review of the first CT images identified enhanced inferior bile duct wall only on the delayed phase, and the site of the enhanced wall was identical to the site of the inferior bile duct tumor detected on the CT eleven months after the first surgery. These imaging findings and the clinical course indicate that the inferior bile duct tumor originally co-existed with the tumor identified in the middle and superior bile duct. We believe that other tests such as endoscopic ultrasonography or cholangioscopy should have been conducted before the first surgery, considering the finding of ERCP. As an extremely rare entity, synchronous double cancers of the CBD without PBM can exist. When a tumor is suspected in the biliary tract, careful and meticulous pre-operative assessment is necessary.

A search of the PubMed database identified only two reports of synchronous double cancers of the CBD without PBM in the English-language literature^[4,5]. With regard to cases of biliary cancer without PBM, the presence of synchronous double tumors poses the question of whether they are independent primary tumors or one tumor that have metastasized from a single tumor. Dif-

ferentiation between these events is important since these two origins imply different stage of the disease, as well as different subsequent treatment and prognosis. In their case report, Ogawa *et al*^[6] described synchronous double cancers of the superior and middle bile duct and inferior bile duct. The upper cancer was pathologically diagnosed as poorly differentiated adenocarcinoma, while the lower one was moderately differentiated adenocarcinoma. In their case, the upper cancer was considered a metastasis from the lower one, based on genetic analysis of loss of heterozygosity^[8,9]. Bedoui *et al*^[5] reported another case of synchronous double cancers of the middle bile duct and inferior bile duct, and both cancers were pathologically diagnosed as adenocarcinomas. That case was preoperatively diagnosed as a single bile duct cancer, similar to the present case. The diagnosis of another inferior bile duct tumor was made during surgery. In their case, there was no communication in either the mucosal layer or the subepithelial layer between the two cancers, and the two tumors were thought to be primary. In our case, the two cancers were separate entities with different histopathological diagnosis, suggesting that they were two primary tumors.

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Mucosal necrosis of the small intestine in myopathy, encephalopathy, lactic acidosis, and stroke-like episodes syndrome

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Abstract

This report presents a case of massive mucosal necrosis of the small intestine in a patient with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS), which particularly affects the brain, nervous system and muscles. A 45-year-old Japanese female, with an established diagnosis of MELAS, presented with vomiting. Computed tomography showed portomesenteric venous gas and pneumatosis intestinalis. She underwent a resection of the small intestine. A microscopic study showed necrosis of the mucosa and vacuolar degeneration of smooth muscle cells in the arterial wall. Immunohistochemistry showed anti-mitochondrial antibody to be highly expressed in the crypts adjacent the necrotic mucosa. The microscopic and immunohistochemical findings suggested the presence of a large number of abnormal mitochondria in MELAS to be closely linked to mucosal necrosis

of the small intestine.

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Key words: Myopathy, encephalopathy, lactic acidosis, and stroke-like episodes syndrome; Acute mesenteric ischemia; Immunohistochemistry; Anti-mitochondrial antibody; Pathology

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INTRODUCTION

Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) is one of a family of mitochondrial cytopathies^[1]. MELAS particularly affects the brain and nervous system and muscles. Gastrointestinal symptoms, such as nausea, vomiting and anorexia, are also common^[2]. Although scattered focal necrosis is sporadically seen^[3], massive intestinal necrosis in MELAS is rare. Only two cases of massive large intestinal necrosis in MELAS have been reported to date^[4,5]. This report presents the case of a MELAS patient with massive mucosal necrosis of the small intestine. This is the first case report of MELAS with massive mucosal necrosis of the

small intestine.

CASE REPORT

A 45-year-old Japanese female presented with vomiting. Abdominal computed tomography (CT) showed portomesenteric venous gas; therefore she was referred to this hospital for further investigation and treatment.

Her medical history revealed that she presented with progressive sensorineural hearing loss at 24 years of age. In addition, she experienced headaches, seizures, homonymous hemianopsia, right hemi paresis, limb weakness and a stroke-like episode at 43 years of age. Her lactic acid and pyruvate level were 112.4 mg/dL (normal < 17 mg/dL) and 2.16 mg/dL (normal < 0.94 mg/dL), respectively. She did not undergo a muscle biopsy. However, the mitochondrial mutation A3243G was detected in her son. A diagnosis of MELAS was therefore established because MELAS is transmitted by maternal inheritance^[2,6].

Her height and body weight were 143 cm, and 26.6 kg, respectively, at the time of admission. Physical examinations revealed abdominal distension. Guarding, rebound tenderness or rigidity could not be estimated properly due to the presence of generalized muscle atrophy and difficulties in communicating with the patient. She was in a state of septic shock. Her heart rate and blood pressure were 88 beats/min and 73 mmHg/31 mmHg, respectively. A semi-quantitative measurement showed her procalcitonin level to be ≥ 10 ng/mL.

CT showed a massive amount of portomesenteric venous gas and pneumatosis intestinalis involving the duodenum, jejunum and ileum (Figure 1). An emergency operation was indicated. Mucosal necrosis from the duodenum to the ileum was recognized transluminal from the serosal. The pulse of the superior mesenteric artery and the vasa recta was palpable. She underwent a massive resection of the small intestine from 10 cm distal to the Treitz ligament to 30 cm proximal to the ileocecal valve, followed by jejunostomy and ileostomy, as damage control surgery because her vital signs indicated the patient to be in a state of shock.

The specimen was 150 cm in length (Figure 2A). Macroscopic observation revealed diffuse mucosal necrosis in two thirds of oral-sided area, and mottled necrosis in the remaining area. The microscopic analysis revealed coagulation necrosis of the mucosa and the lamina propria (Figure 2B). The external longitudinal layer of muscularis propria was also degenerated and partially diminished (Figure 2B). Vacuolar degeneration of smooth muscle cells was observed in the arterial wall and the muscular layer of the intestinal wall (Figure 3).

Anti-mitochondrial antibody (AMA), ab92824, was highly expressed in the crypt and slightly in the remaining mucosa that was observed in the necrotic area (Figure 4). On the contrary, AMA expression in the crypts was moderate in the non-necrotic area. The resected strangulated ileum of a non-MELAS patient stored in the pathological department was used as a control. This sample showed



Figure 1 Preoperative computed tomography. There was a massive amount of portomesenteric venous gas (arrow head) and pneumatosis intestinalis in the small intestine (arrow).

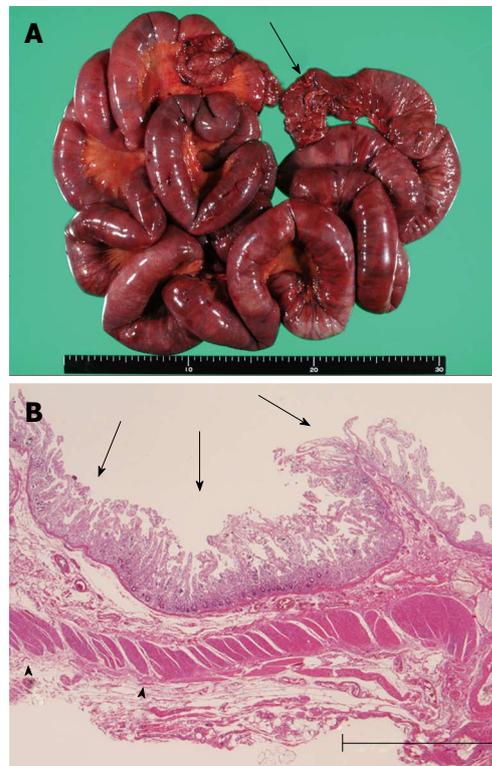


Figure 2 Macroscopic findings of resected tissue and specimen. A: Macroscopic findings of resected tissue. The specimen measured 150 cm in length. There was diffuse mucosal necrosis in two thirds of oral-sided area, and mottled necrosis in the remaining area. Arrow: Oral stump; B: Microscopic findings of the resected specimen. Coagulation necrosis of the mucosa and lamina propria was observed (arrows). The external longitudinal layer of the muscularis propria was partially diminished (arrow heads). Hematoxylin and eosin, Bar = 500 μ m.

moderate AMA expression in the crypt in comparison to that of the current patient (Figure 5; Table 1). Expression patterns of AMA in both specimens are shown in Table 1.

A series of antibiotics were administered based on culture evaluations in order to control infection. She required support for renal insufficiency with continuous hemodiafiltration for a 3-d period after surgery; blood pressure support with catecholamine administration, mechanical ventilation support for a 3-wk period after

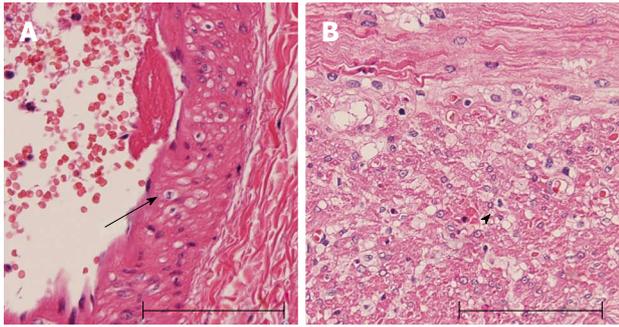


Figure 3 Vacuolar degeneration. A: Artery wall (arrow); B: Muscular layer (arrow head). Hematoxylin and eosin, Bar = 100 μ m.

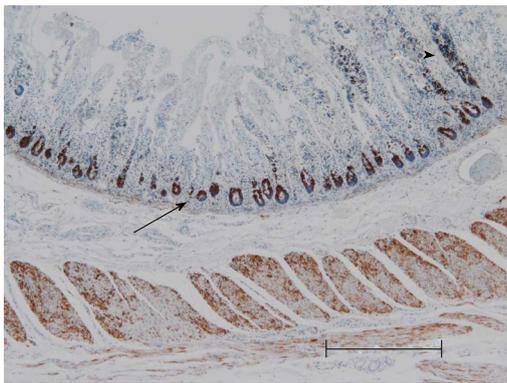


Figure 4 Immunohistochemical study. Anti-mitochondrial antibody is markedly expressed in the crypts (arrow) and preserved mucosa (arrow head). Bar = 500 μ m.

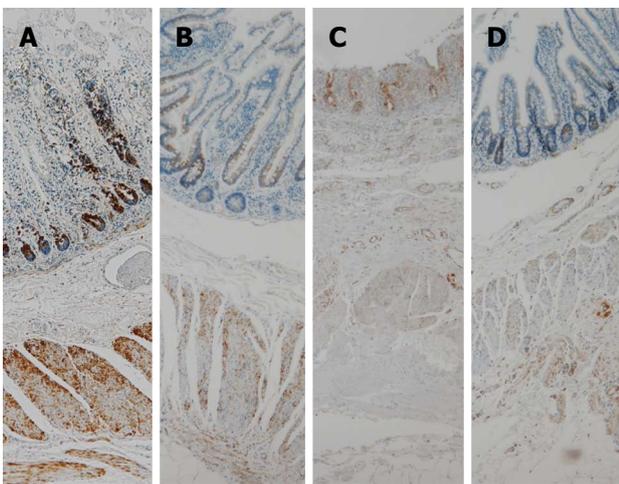


Figure 5 Expression of anti-mitochondrial antibody. A: Necrotic area of the present patient; B: Non-necrotic area of the present patient; C: Necrotic area of strangulated small intestine of a non-myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) patient; D: Non-necrotic c area in the strangulated small intestine of a non-MELAS patient. Bar = 500 μ m.

surgery. The patient's condition eventually recovered along with the restoration of the necrotic mucosa, based on observations of the oral stoma and thereafter she was discharged. Home parenteral nutrition was indicated because intestinal continuity had not been reestablished

Table 1 Summary of anti-mitochondrial antibody expression

	MELAS patient (in this case)		Non-MELAS patient (in our control)	
	Necrotic lesion	Non-necrotic lesion	Necrotic lesion	Non-necrotic lesion
Expression of AMA				
Mucosa				
Villi	Diminished/+++	+	Diminished	+
Crypt	+++	+/-	+	+
Lamina propria	+/-	+/-	+/-	+/-
Muscularis mucosa	+	+	+/-	+/-
Submucosa	+/-	+/-	+/-	+/-
Muscular layer				
Inner circular	++	++	+	+
External longitude	++	++	+	+

AMA: Anti-mitochondrial antibody; MELAS: Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes.

in consideration of her general condition. She died of severe metabolic disorder due to MELAS 3 mo after discharge.

DISCUSSION

MELAS is a rare type of metabolic disorder causing multi-organ disorders, such as brain ischemia and the degeneration of skeletal muscle. MELAS is caused by mutations in mitochondrial DNA encoding transfer RNA^{LEU(UUA/UUG)} and it is transmitted by maternal inheritance^[2,6]. Though MELAS particularly affects the brain, nervous system and muscles, the occurrence of massive intestinal necrosis due to MELAS is rare.

Many MELAS symptoms caused by mitochondrial A4243G mutation are thought to depend on the mutation load and the tissue distribution of abnormal mitochondria^[7]. Therefore, an immunohistochemical study was conducted using AMA, which is a highly sensitive and specific method for identifying the mitochondria^[8], to investigate the relationship between mucosal necrosis and the distribution of mitochondria. This method is applied when the distribution of mitochondria cannot be determined by electron microscopy because the tissue was fixed for light microscopy, as in the present case^[9]. Though an increase in the number of mitochondria does not always indicate the presence of abnormal mitochondria, an increase in the number of mitochondria is more likely to be a sign of mitochondrial disease and aggregation of large mitochondria a characteristic ultrastructural finding in mitochondrial disease^[10,11]. The current patient showed a marked expression of AMA in the crypts, where adjacent villi had almost completely disappeared (Figure 4; Table 1). A marked expression was also observed in the remaining mucosa in the necrotic area. No such marked expression was observed in the crypts of non-necrotic areas in the present case or a necrotic area in the non-MELAS specimen (Figure 5; Table 1). These findings suggest that an increase in the number of

mitochondria, which is likely abnormal, therefore, may contribute to mucosal necrosis. The mucosa containing a higher number of abnormal mitochondria may be vulnerable to ischemia.

Microscopic examination revealed vacuolation of the smooth muscle cells in the small arteries in the current case (Figure 3). However, there was no narrowing of the small arteries. An electron microscopic study of cerebral vessels of patients with MELAS showed a striking increase in the number of mitochondria in the smooth muscle and endothelial cells causing the vascular changes^[12]. The increase is most prominent in arterioles and small arteries. These MELAS-related vascular changes are likely to attribute to a decrease in blood flow^[5]. Therefore, the current patient probably experienced transient non-occlusive ischemia, such as a vasospasm that eventually triggered mucosal necrosis.

In addition to the mucosa, the muscularis mucosa, and inner circular and external longitudinal layer of the muscularis propria were also affected in the present case. Therefore, MELAS may affect any layer of the small intestine.

In conclusion, the presence of abundant abnormal mitochondria may be closely linked to necrosis in the intestinal mucosa, and MELAS-related vascular changes may therefore be a prerequisite for necrosis.

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Newly developed autoimmune cholangitis without relapse of autoimmune pancreatitis after discontinuing prednisolone

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Abstract

A 57-year-old man presented with a 2-wk history of painless jaundice and weight loss. He had a large ill-defined enhancing mass-like lesion in the uncinat process of the pancreas with stricture of the distal common bile duct. Aspiration cytology of the pancreatic mass demonstrated inflammatory cells without evidence of malignancy. Total serum immunoglobulin G level was slightly elevated, but IgG4 level was normal. After the 2-wk 40 mg prednisolone trial, the patient's symptoms and bilirubin level improved significantly. A follow-up computed tomography (CT) scan showed a dramatic resolution of the pancreatic lesion. A low dose steroid was continued. After six months he self-discontinued prednisolone for 3 wk, and was presented with jaundice again. A CT scan showed newly developed intrahepatic biliary dilatation and marked concentric wall thickening of the common hepatic duct and the proximal common

bile duct without pancreatic aggravation. The patient's IgG4 level was elevated to 2.51 g/L. Prednisolone was started again, after which his serum bilirubin level became normal and the thickening of the bile duct was resolved. This case suggests that autoimmune pancreatitis can progress to other organs that are not involved at the initial diagnosis, even with sustained pancreatic remission.

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Key words: Autoimmune disease; Pancreatitis; Cholangitis; Prednisolone

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INTRODUCTION

Autoimmune pancreatitis (AIP) is a type of chronic pancreatitis characterized by an autoimmune inflammatory process in which prominent lymphocyte infiltration with associated fibrosis of the pancreas leads to organ dysfunction. Steroids are the first choice of therapy in

patients with AIP and the response to steroid therapy is usually dramatic^[1]. However, 20% to 60% of patients with AIP are found to relapse after the initial course of corticosteroid therapy^[2]. The relapse can occur with or without the involvement of other organs such as the bile duct, retroperitoneum, kidneys, salivary or lacrimal gland^[3]. However, cholangitis in the proximal extrahepatic bile duct without pancreatic relapse has rarely been reported. Herein we report a case of newly developed autoimmune cholangitis in a patient with AIP with sustained pancreatic remission.

CASE REPORT

A 57-year-old man presenting with painless jaundice and 2 kg weight loss in 2 wk, was admitted to our hospital. He was an inactive carrier of hepatitis B and his liver function test was within the normal limits at a regular follow up 6 mo ago. He drank socially and had a 30-pack-year history of smoking. On physical examination, he was deeply jaundiced, but other clinical examinations were unremarkable. Laboratory tests revealed white blood cell count of 5850/mm³, aspartate aminotransferase 112 U/L, alanine aminotransferase 230 U/L, alkaline phosphatase (ALP) 326 IU/L, gamma-glutamyl transpeptidase (GGT) 584 U/L, total bilirubin 24.7 mg/dL, amylase 91 U/L and lipase level of 56 IU/L. Abdominal computerized tomography (CT) revealed an ill-defined enhancing mass-like lesion in the uncinate process of the pancreas measuring 5.7 cm × 3.2 cm with regional infiltrations and small amounts of fluid collections around the pancreas head (Figure 1). Magnetic resonance cholangiopancreatography showed moderate dilation of the intrahepatic duct and common bile duct (CBD) but the pancreatic duct was unremarkable (Figure 2A). Endoscopic retrograde cholangiopancreatography revealed a distal biliary stricture (Figure 2B), and brush cytology demonstrated no malignant cells. 7-Fr Amsterdam type inside stent was placed to decompress the biliary system. A subsequent endoscopic ultrasound (EUS) demonstrated a hypoechoic lesion located in the head of the pancreas with blurred delineated margins, which suggested inflammation rather than malignancy. EUS guided fine needle aspiration cytology revealed lymphocytes, neutrophils, and irregular sheets of bland ductal epithelial cells lacking atypia (Figure 3). Serum tumor markers were unremarkable; carbohydrate antigen 19.9 was 20.36 U/mL (normal < 37 U/mL), and carcino-embryonic antigen was 0.8 ng/mL (normal < 7.5 ng/mL). Serum immunoglobulin G level was slightly elevated to 1703 mg/dL (normal < 1600 mg/dL), but serum IgG4 level was 0.41 g/L (normal < 1.21 g/L). In consideration of autoimmune pancreatitis, we started with 40 mg/d prednisolone orally for 2 wk. After that, the patient's symptoms and bilirubin level improved significantly. A follow-up CT scan showed a dramatic resolution of the pancreatic lesion. The daily dose of prednisolone was gradually tapered and 2.5 mg of prednisolone per day was maintained. The stent in the CBD was removed two months later. He remained well

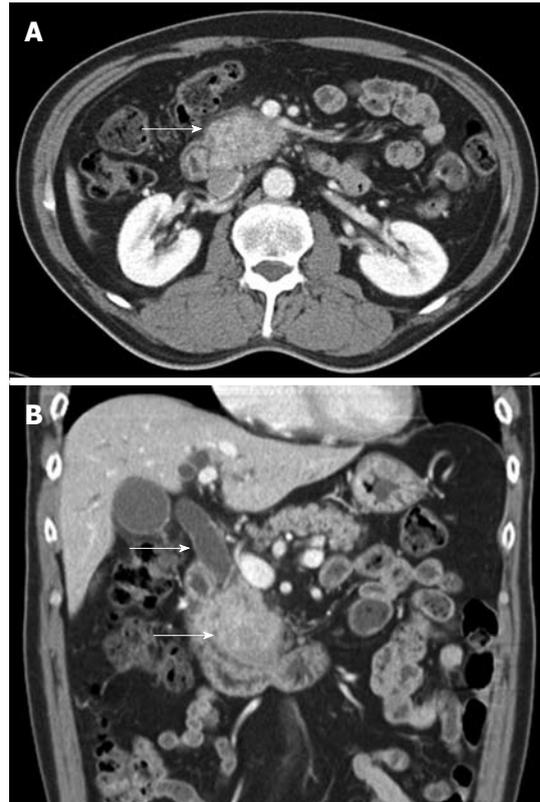


Figure 1 Initial abdominal computed tomography scan revealed an ill-defined enhancing mass-like lesion in the uncinate process of the pancreas with a dilatation of common bile duct. A: The uncinate process of the pancreas (white arrow) measuring 5.7 cm × 3.2 cm with regional infiltrations; B: In addition to this, there is small amounts of fluid collections around the pancreas head (white arrows).

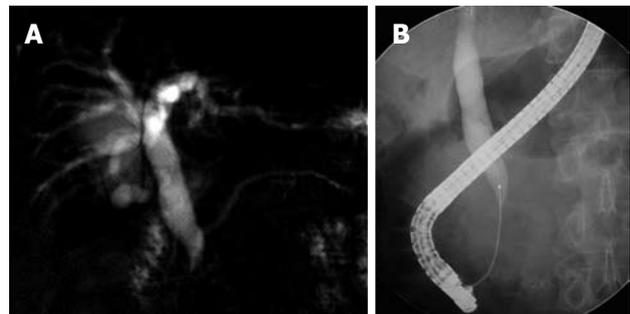


Figure 2 Magnetic resonance cholangiopancreatography and endoscopic retrograde cholangiography. A: Magnetic resonance cholangiopancreatography showed moderate dilatation of the intrahepatic and common bile duct. The distal common bile duct had an abrupt narrowing. The pancreatic duct was unremarkable; B: Endoscopic retrograde cholangiography revealed a beak shaped stricture of the distal common bile duct with biliary dilatation above it.

until six months later, when he was readmitted with acute jaundice after self-discontinuation of prednisolone for 3 wk. The total bilirubin level was elevated to 19.2 mg/dL. ALP and GGT were 222 IU/L and 447 IU/L, respectively. IgG4 level was also elevated to 2.51 g/L, which was two times above the upper limit of the normal range. The following CT scan showed a stricture at the proximal extrahepatic bile duct and dilation of the intrahepatic bile duct (Figure 4A). The common hepatic duct and proxi-

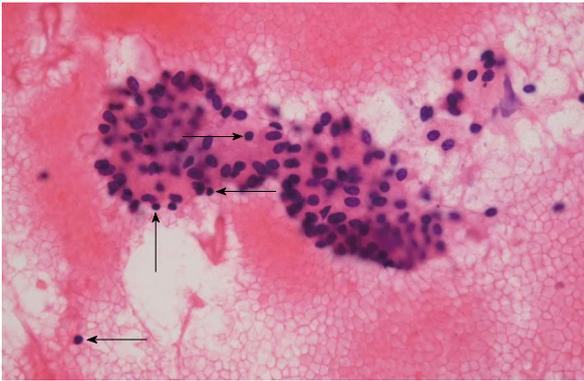


Figure 3 Photomicrograph of cytologic specimen obtained by endoscopic ultrasound-fine needle aspiration, showing lymphocytes (arrows) and irregular sheets of bland ductal epithelial cells on the bloody background (hematoxylin and eosin, $\times 400$).

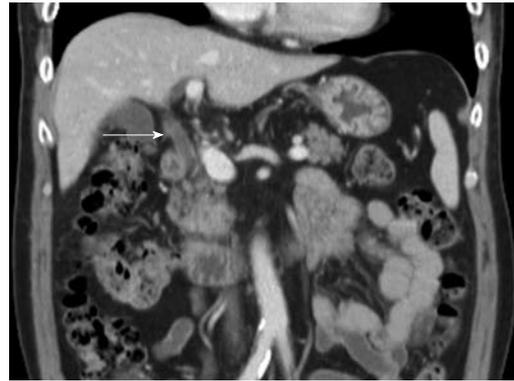


Figure 5 Abdominal computed tomography scan after retreatment with prednisolone showed resolution of the thickening of the bile duct (white arrow).

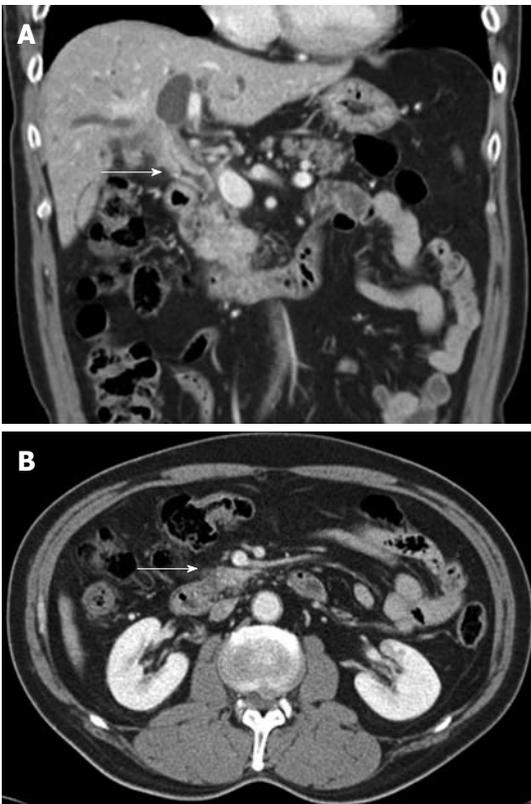


Figure 4 Follow up abdominal computed tomography scan. A: Coronal image revealed a stenosis of the common hepatic and the proximal common bile duct (white arrow) with significant thickening and inner wall enhancement of the bile duct; B: There was no pancreatic relapse (white arrow).

mal CBD showed marked concentric wall thickening and enhancement. However, there was no pancreatic aggravation (Figure 4B). Newly developed autoimmune cholangitis was strongly suspected. He was treated with 40 mg prednisolone/d for 2 wk, after which serum bilirubin, ALP, and GGT became normal and the following CT scan showed resolution of the thickening of the bile duct (Figure 5). After tapering of the prednisolone, he took 5 mg of prednisolone daily without aggravation of clinical findings.

DISCUSSION

AIP is a form of chronic pancreatitis of presumed autoimmune etiology^[1]. It is frequently presented with obstructive jaundice and pancreatic swelling. AIP presents with lympho-plasmacytic infiltration and fibrosis in the pancreas and shows a dramatic response to steroids^[4]. Lympho-plasmacytic infiltrate in AIP shows abundant IgG4-positive plasma cells on immunostaining^[5]. Elevation of serum IgG4 is the most remarkable characteristic of this disease. Since AIP can mimic pancreatic cancer, its diagnosis is important to avoid unnecessary surgery. The classic appearance of AIP on abdominal CT is sausage-shaped enlargement of the pancreas with a capsule-like rim. However, AIP can be presented as a pancreatic mass-like lesion, similar to the present case. In such case, pancreatic cancer should be discriminated from AIP. Serum autoimmune markers are helpful for the diagnosis of mass forming AIP, but histological diagnosis is necessary to confirm AIP in many cases^[1]. Because histological confirmation of AIP is difficult, “a 2-wk steroid trial and subsequent assessment of its response” is introduced as a diagnostic tool in patients whose clinical and laboratory findings are equivocal for AIP^[6]. Our patient showed rapid resolution of the pancreatic mass after treatment with steroid despite the normal IgG4 level. Since serum IgG4 level was elevated afterward, our case was diagnosed as type I AIP by International Consensus Diagnostic Criteria for AIP^[4].

AIP can be associated with sclerosing extrapancreatic lesions^[7]. Because all tissues involved have characteristic infiltration of IgG4-positive cells, the term “IgG4-associated systemic disease” has been proposed. The most common sites of extrapancreatic involvement in AIP are the bile duct, followed by salivary glands, retroperitoneal fibrosis, orbital pseudotumors, lymphadenopathy, and renal parenchyma^[5,7]. Although the stenosis of the CBD in the intrapancreatic area usually occurs with AIP, there are some debates about whether the involvement of only distal CBD should be included in the category of IgG4-associated cholangitis (IAC). Because the narrowing of the intrapancreatic CBD may merely be a secondary

phenomenon from an extrinsic compression owing to the pancreatic enlargement of AIP, intrapancreatic CBD involvement is considered as a part of AIP rather than as a IAC^[8]. Our patient had a beak shaped intrapancreatic CBD stenosis without proximal extrahepatic or intrahepatic biliary involvement at initial presentation. Therefore, IAC was not combined at first.

AIP responds well to steroids. The general initial recommended dose of oral prednisolone for the induction of remission is 0.6 mg/kg per day for 2-4 wk^[9]. Pancreatic size usually normalizes within a few weeks, and biliary drainage becomes unnecessary within about 1 mo. Rapid response to the steroid confirms the diagnosis of AIP^[10]. Remission of AIP is defined as the disappearance of clinical symptoms and the resolution of the pancreatic and/or extrapancreatic manifestations in the imaging studies. Our patient had a rapid response to the steroid treatment showing improvements in symptoms, hepatic biochemistry, biliary stricture, and pancreatic lesion.

Relapse of AIP is defined as reappearance of symptoms such as weight loss, jaundice, or abdominal discomfort and elevation of serum IgG4 concentrations with reappearance of pancreatic and/or extrapancreatic abnormalities in the bile duct, salivary gland, or retroperitoneum on imaging studies^[3]. The relapse rate after remission of AIP is variable between 20% and 60%^[2,10]. Relapse patterns regarding the pancreas or extrapancreatic lesions have not been established and it is not certain that the extrapancreatic involvements at the diagnosis is related to the relapse of AIP. Although initial extrapancreatic involvement was not defined, Kamisawa *et al.*^[9] reported that the relapse of AIP occurred in the pancreas (52%), bile duct (34%) and other lesions ($n = 19$). Moreover, Sandanayake *et al.*^[11] reported that all relapsed AIP patients have had extrapancreatic or proximal biliary strictures at the time of diagnosis. When defining relapse, authors generally do not distinguish between relapse of the pancreatic manifestation of IgG4-associated systemic disease, namely AIP, versus occurrence of the disease in another organ, either de novo or true relapse of a previously treated disease in that organ^[3]. Newly developed proximal extrahepatic biliary involvement without pancreatic relapse is very rare. One case of hilar and proximal extrahepatic bile duct involvements with sustained pancreatic remission in a diffuse type AIP patient was reported briefly^[12,13]. In our case, the focal enlargement type AIP relapsed as the proximal extrahepatic biliary stricture with marked wall thickening and no pancreatic aggravation. The feature of our case is that the serum IgG4 level was normal at initial diagnosis of AIP, but it was markedly elevated with the relapsed autoimmune cholangitis. Although it has been reported that the predictors for relapse of AIP is elevated serum IgG4 levels during remission^[10], this case showed that seronegative AIP could relapse as seropositive autoimmune cholangitis, which

means progression of the autoimmune disease.

In summary, autoimmune pancreatitis may relapse to other organs as IgG4-associated systemic disease without pancreatic aggravation, even if the organs were not involved and IgG4 level was normal at initial diagnosis. Therefore, clinicians should pay close attention to involvement of other organs during follow up of patients with AIP even with sustained pancreatic remission.

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S- Editor Gou SX L- Editor A E- Editor Zhang DN

Medical treatment for a fish bone-induced ileal micro-perforation: A case report

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Abstract

Ingested fish bone induced intestinal perforations are seldom diagnosed preoperatively due to incomplete patient history taking and difficulties in image evidence identification. Most literature suggests early surgical intervention to prevent sepsis and complications resulting from fish bone migrations. We report the case of a 44-year-old man suffered from acute abdomen induced by a fish bone micro-perforation. The diagnosis was supported by computed tomography (CT) imaging of fish bone lodged in distal ileum and a history of fish ingestion recalled by the patient. Medical treatment was elected to manage the patient's condition instead of surgical intervention. The treatment resulted in a complete resolution of abdominal pain on hospital day number 4 without complication. Factors affecting clinical treatment decisions include the nature of micro-perforation, the patient's good overall health condition,

and the early diagnosis before sepsis signs develop. Micro-perforation means the puncture of intestine wall without CT evidence of free air, purulent peritoneum or abscess. We subsequently reviewed the literature to support our decision to pursue medical instead of surgical intervention.

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Key words: Fish bone ingestion; Micro-perforation; Decision-making; Medical treatment; Small intestine

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INTRODUCTION

The ingestions of foreign bodies result in gastrointestinal (GI) perforations in less than 1% of patients. Fish bones are the most common objects leading to gastrointestinal perforations^[1]. Correct preoperative diagnoses are seldom made. Few patients can recall the instance of foreign body ingestions. The most common site of perforation by fish bones is the distal ileum^[2]. Abdominal computed tomography (CT) examination may provide preoperative diagnosis of foreign bodies^[1,3], but more often, they are confirmed after diagnostic laparoscopy^[4-6]. Most literature emphasizes early surgical intervention with resection of the partial bowel, because of high risk of intra-abdominal

abscess formation and the potential of delayed complications due to fish bone migration^[7,8]. We report a case in which medical treatment was selected, instead of surgical intervention, for a fish bone-induced micro-perforation of distal ileum.

CASE REPORT

A 44-year-old man with abdominal pain came to emergency room (ER) at 8 PM. He started to feel abdominal discomfort 6 h before. He began experiencing severe sharp abdominal pain with a sudden onset 3 h before. The pain persisted even after the patient took some anti-acid drug at home. He recalled that he had dinner with fish the previous night. He appeared acutely ill without any vomiting, shortness of breath, diarrhea or fever. At the time of his arrival in the ER, he was alert and oriented. His blood pressure was 144/86 mmHg, heart rate 72 beats/min, respiratory rate 18 breaths/min, and body temperature 37.1 °C. He reported no past history of hypertension, diabetes or abdominal surgery. The initial physical examination revealed normal breathing sounds and regular heart beat without murmur. He had normal active bowel sounds and diffuse abdominal tenderness particularly over the right lower quadrant abdomen, coupled with muscle guarding and rebounding pain. Focus echogram showed no ascites, distended gall bladder with Murphy's sign on sonography, or hydronephrosis. Radiography of the kidney-ureter-bladder revealed normal bowel gas without signs of intestinal obstruction or free air. Serum laboratory examinations showed white blood cell count of 10 100/ μ L, neutrophils of 82.2%, lymphocytes of 12.1%, hemoglobin of 14.8 g/dL, and platelet count of 210 000/ μ L. Serum biochemistry tests revealed a glucose level of 121 mg/dL, and aspartate aminotransferase of 27 U/L, cereal third transaminase of 43 U/L, total bilirubin of 0.5 mg/dL, direct bilirubin of 0.2 mg/dL, creatinine of 1.3 mg/dL, and Na^+/K^+ of 145/3.8 mEq/L. After primary ER medical treatment with intravenous tenoxicam 20 mg and buscopan 20 mg, his pain was localized to the right lower quadrant abdomen, but rebounding pain was still noted. Abdominal CT revealed a 26 mm radiopaque linear shadow transversely lodged in the distal ileum with thickened wall, which is consistent with signs of fish bone retention. Minimal peritoneal contamination without pneumoperitoneum or abscess formation was noted. A normal appendix was identified. A fish bone-induced micro-perforation in the distal ileum was highly suspected (Figure 1).

A general surgeon was consulted. The patient and his families were informed of the indication for surgical intervention and the option of a more conservative medical treatment. Given the nature of micro-perforation, the patient's good overall health condition, and the early diagnosis (6 h after symptom onset) before sepsis signs developed, initial medical treatment was elected to manage the patient's condition, instead of surgical intervention with the consent of the patient and his families.



Figure 1 An unenhanced abdominal computerized tomography image reveals a 26 mm in length radiopaque linear shadow in the distal ileum lodged into the thickened intestinal wall at both ends (black arrow). Minimal peritoneal contamination without pneumoperitoneum, or abscess formation is noted, which is consistent with signs of fish bone induced micro-perforation.

Intravenous saline hydration without oral intake, subacillin (ampicillin 2 g + sulbactam 1 g) and SABS (metronidazole) 500 mg were provided at ER. After admission to the ward, fever was noted up to 38 °C in the first 2 d. Subacillin 1.5 g *iv* every 8 h was prescribed for 5 d, followed by Soonmelt (amoxicillin 250 mg/clavulanic acid 125 mg) one tablet orally every 8 h for 7 d. During the first day of admission, the pain score went down from 10 to 3. Rebounding pain and muscle guarding also subsided. However, tenderness over the right lower quadrant abdomen and the periumbilical area was still noted.

On the fourth day, the patient felt hungry and experienced no more abdominal pain or tenderness. Laboratory examinations revealed white blood cell (WBC) count of 3700/ μ L, neutrophils of 49.7%, lymphocytes of 37.3%, hemoglobin of 14.1 g/dL, platelet count of 227 000/ μ L and a C-reactive protein level of 2.97 mg/dL (normal < 0.5 mg/dL). As a result of the improved clinical condition, an oral soft diet was initiated. The patient was tolerant of the soft diet without any deterioration of symptoms. A follow up abdominal CT without contrast, which revealed the radiopaque linear shadow still lodged in the same intestinal segment, was performed on the fourth day. The fish bone rotated and became parallel to the distal ileum lumen with one end penetrating out the intestinal wall into the mesenteric fat. Minimal local inflammatory infiltration was seen around the protruding part. No free air or abscess was noted (Figure 2). There was no surgical intervention because clinical symptoms had been completely resolved. The patient was discharged on the sixth day with normal oral intake and stool passage. At 3- and 6-mo follow-ups with the patient, no recurrent abdominal pain or complication was noted.

DISCUSSION

Unintentional, unconscious foreign body ingestions in adults are usually dietary. Nearly two-thirds of foreign

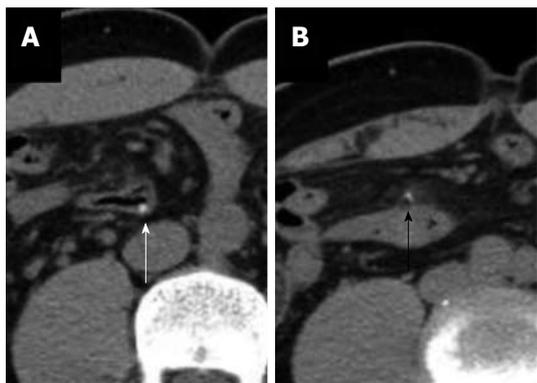


Figure 2 Two follow-up unenhanced abdominal computer tomography images, which reveal the radiopaque shadow still lodged in the intestinal segment. The fish bone rotates and becomes parallel to the distal ileum lumen. A: Most of the fish bone is still inside the intestinal lumen (white arrow). One end of the fish bone penetrates out the intestinal wall into the mesenteric fat; B: Minimal local inflammatory infiltration contains the protruding part. No free air or abscess is noted (black arrow). The distance between these two images is 18 mm.

bodies are fish bones^[2]. However, most digested foreign bodies pass through the GI tract within a week, and seldom cause major complications^[1,9-12]. The ingestion of foreign bodies results in gastrointestinal perforations in less than 1% of patients. Fish bones are the most common objects leading to gastrointestinal perforation^[1]. The most common perforation site is the distal ileum^[2]. Clinical presentations of GI tract perforation caused by digested foreign bodies vary from case to case, and can be acute, subtle, chronic or even asymptomatic^[2,13,14]. The clinical presentations include acute peritonitis, abdominal wall tumor or abscess^[2,15], intra-abdominal mass and abscess formation^[2,16]. Patients who experience gastric and duodenal perforation tend to present with highly acute pain due to a rapid chemical peritonitis, often followed by the systemic inflammatory response syndrome, which can lead to rapid clinical deterioration^[8,17,18]. Patients often recall the exact time of symptom onset. The perforation may progress to an infected peritonitis and sepsis in untreated patients or in patients who have late-stage presentations^[8]. Colon perforations may present without immediate perforation-associated pain and tend to have a slower clinical progression, with the development of a secondary bacterial peritonitis or localized abscess formation partly due to the relatively neutral and non-erosive nature of the chemical environment within the colon^[19-21]. Because of the variety of clinical manifestations, the correct preoperative diagnosis is seldom made. Goh *et al*^[2] reported that a correct preoperative diagnosis was made in only 10 (23%) of 44 patients. Furthermore, only a few patients can recall foreign body ingestion. In the report of Goh *et al*^[2], only one (2%) patient provided a definitive history of foreign body ingestion.

Plain radiographs are usually unhelpful with a sensitivity of 32% for fish bones, which varies according to species^[22,23]. CT scan is preferred and will usually demonstrate a linear calcified lesion, which if initially

missed, can be seen in retrospect. Goh *et al*^[1] reported that the sensitivity of a CT scan in the detection of intra-abdominal fish bones was 71.4% (5/7) in initial reports. Gastrointestinal perforation causes considerable mortality and usually requires emergency surgery. Mortality of secondary peritonitis is still 30% to 50% despite advances in antibiotics, surgical technique, radiographic imaging, and resuscitation therapy^[7,8]. The reported indications for surgical intervention are as follows: (1) bowel perforation; (2) peritonitis due to bowel perforation; (3) migration to other organs adjacent to the perforation site; (4) bleeding or severe inflammation in the abdominal cavity; (5) penetration of vessels; and (6) abscess formation^[24]. Nonsurgical management highly depends on the time of diagnosis, location and size of the perforation, degree of contamination, and condition of the patient. Nonsurgical management can be successful in stable patients who have minimal signs and symptoms of peritonitis and who have small injuries to the stomach, duodenum, and retroperitoneal portions of the colon^[25]. These locations offer possible anatomic containment of the perforation by the retroperitoneal space or omentum. Perforations of the intra-peritoneal small bowel and colon usually require surgery, except for micro-perforations. Micro-perforations often cause minimal peritoneal contamination and can seal spontaneously^[25,26]. Micro-perforation means puncture of intestine wall but no CT evidence of free air, purulent peritoneum or abscess. Selected colon perforations, such as certain iatrogenic injuries or perforation secondary to diverticulitis may also be managed non-operatively. Spontaneously sealed perforations and perforations that are contained with the development of an associated abscess cavity can often be successfully managed without surgery^[27]. An excellent and clinically useful classification system for diverticular perforations was developed by Hinchey and colleagues and modified by others^[19,27]. The treatment of gastrointestinal perforation includes fluid resuscitation, antibiotics, source control, organ system support, and nutrition. Antibiotics are standard treatment for gastrointestinal perforation^[7,8,18,28-30]. Many efficacious regimens have been described, and no single agent or combination of agents has been found to be superior to the others^[28-32]. We started Subacillin plus SABS initially at ER. Owing to a good response, we then shifted to Subacillin only in the ward.

The duration of antibiotic coverage is controversial^[28,33]. Some authors advocate a standard treatment of 7 to 14 d, whereas others recommend continuing antibiotics until the WBC count has normalized and the patient is afebrile^[28,30]. Current general consensus advocates antimicrobial therapy for 5 to 7 d if clinical signs of infection have resolved^[28,33]. If the patient fails to improve or worsens during this period, the adequacy of source control or the appropriateness of antibiotic coverage must be questioned^[28]. Our patient responded well to medical treatment. He was afebrile on the third day. WBC count had normalized on the fourth day. Clinical signs of infection were resolved after oral intake on the fourth day.

After 5 d of intravenous Subacillin, the patient received 7 additional days of the oral antibiotic Soonmelt.

We found one recent case report of fish bone induced distal ileum micro-perforation which was spontaneously relieved one day after admission while awaiting surgical intervention^[34]. There were two previous documented cases of hepatic abscess secondary to fish bone perforation that were successfully treated with medical therapy, because of contraindication for operation^[35,36]. The impacted fish bone remains unchanged in the pylorus. The patient remained asymptomatic during the 18 mo of follow-up.

The clinical improvement is not necessarily a result of fish bone pass out. Because the fish bone is sharp and linear, it could penetrate the small intestinal wall and migrate into the surrounding soft tissue. It may cause delayed complications. Reported complications of migrated fish bones include retropharyngeal abscesses^[37], gastric submucosal mass^[38], inflammatory omentum mass^[39], pancreatitis with intraluminal thrombosis of superior mesenteric vein due to penetrating into the superior mesenteric vein^[40], migration into the right renal vein^[41], and liver abscess^[35,36,42-45]. Most complications following foreign body impaction will require surgery at some stage, even many years after ingestion has occurred^[46]. Because there are vital organs nearby (such as the mediastinum, great vessel, liver and pancreas), fish bones migrated from the esophagus, stomach or duodenum may induce catastrophic complications. It is important to be mindful of the delayed complications of fish bones migration.

In conclusion, ingestion of foreign bodies is a common event. However, perforation of the GI tract by fish bones is not common. Key hints for the diagnosis of a fish bone induced GI tract perforation are the following: acute onset of peritonitis signs, patient's dietary history with an emphasis on fish, and image evidence of abdominal CT. Key factors affecting clinical treatment decisions include the nature of perforation, the patient's overall health condition, and the timing of diagnosis. Medical treatment may be one of the choices in micro-perforation of the distal ileum induced by fish bone in select patient.

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Focal peliosis hepatis in a colon cancer patient resembling metastatic liver tumor

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Abstract

Peliosis hepatis (PH) is a rare benign condition characterized by the presence of multiple, randomly distributed, blood filled cystic areas of variable size within the liver parenchyma. PH is difficult to recognize and may be mistaken for neoplasm, metastases or multiple abscesses. A 75-year-old female with a previous history of colon cancer was admitted when a liver mass in the right liver lobe was found 11 mo after surgery during the follow-up period. Computed tomography and magnetic resonance imaging scan of the abdomen were performed. The initial possible diagnosis was metastatic hepatocellular carcinoma. The patient underwent excision of the hepatic segment where the nodule was located. The pathological diagnosis of the surgical specimen was PH. PH should be considered in the differential diagnosis of new liver lesions in patients whose clinical settings do not clearly favor metastasization. Clinicians and radiologists must recognize these lesions to minimize the probability of misdiagnosis and inappropriate treatment.

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INTRODUCTION

Peliosis hepatis (PH) is a rare benign condition characterized by the presence of multiple, randomly distributed, blood filled cystic areas of variable size within the liver parenchyma^[1]. PH has been associated with malignancies, immunosuppression, infections and medications. PH is difficult to recognize, and the diagnosis is often missed or delayed because its imaging findings are often non-specific and the condition may be mistaken for neoplasm, metastases or multiple abscesses. Here, we present a case of focal PH in a colon cancer patient mistaken for liver metastases in the initial diagnosis. A review of the literature on PH in relation to etiology and imaging diagnosis was performed, and the presentation and management of this rare condition are discussed.

CASE REPORT

A 75-year-old female with a previous history of colon

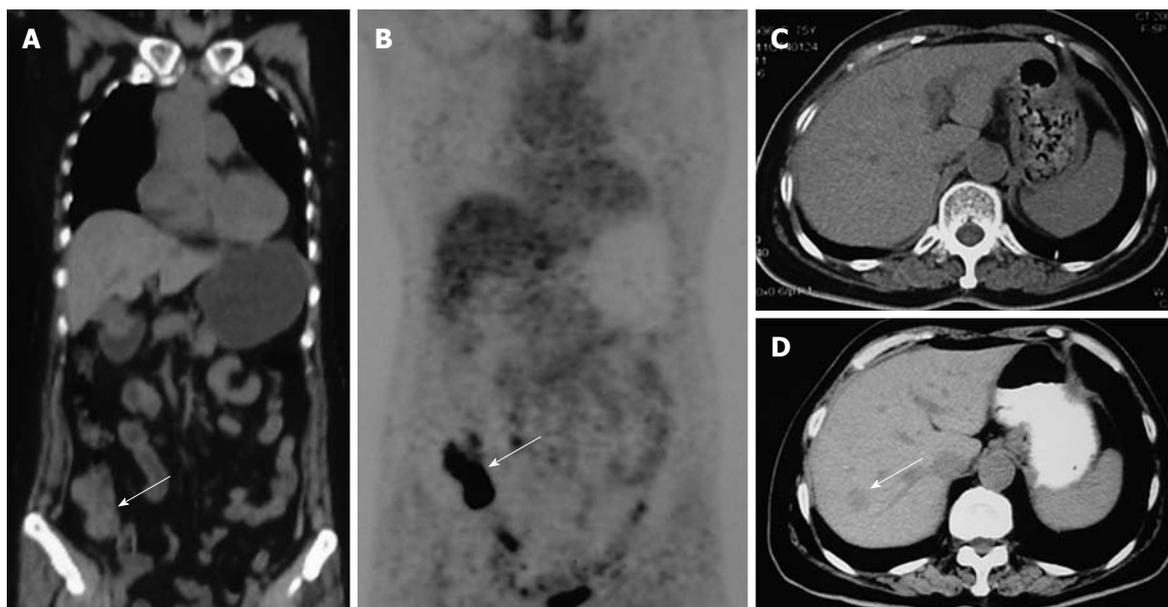


Figure 1 Positron emission tomography plus computed tomography and computed tomography scan findings in our patient. A: Positron emission tomography plus computed tomography (PET/CT) scan showed thickening of the ileocecal region (white arrow), and no liver lesions; B: PET/CT scan showed increased metabolic areas in the ileocecal region (white arrow), no increased metabolic areas in the liver; C: Six months later a follow-up CT scan showed no liver lesions; D: Eleven months later a follow-up CT scan showed one low-density lesion in the right liver lobe (white arrow).

cancer treated with radical resection, was admitted to Shanghai East Hospital when a liver mass in the right liver lobe was found 11 mo after surgery during the follow-up period. The pathological diagnosis of colon cancer was moderately differentiated adenocarcinoma. After colon cancer surgery, the patient received a number of chemotherapeutic protocols including capecitabine (Xeloda) and oxaliplatin. She had a history of type 2 diabetes for over 20 years. She took gliclazide for the treatment of diabetes, and glucose was well-controlled. She had no history of viral hepatitis or alcohol abuse.

Laboratory evaluations revealed hemoglobin of 120 g/L, and a normal white cell and platelet count. Prothrombin time, electrolytes, BUN and creatinine were normal. Liver chemistry revealed aspartate aminotransferase 24 IU/L, alanine aminotransaminase 16 IU/L, total bilirubin (TB) 10.6 $\mu\text{mol/L}$, direct bilirubin (DB) 4.5 $\mu\text{mol/L}$, alkaline phosphatase 97 IU/L, lactate dehydrogenase 184 IU/L and albumin 44 g/L. Hepatitis B virus examination showed: hepatitis B surface antigen, hepatitis B e antigen, hepatitis B e antibody negative, hepatitis B surface antibody, hepatitis B core antibody positive and HBV DNA < 500 copies/mL. α -fetoprotein was 2.78 ng/mL, carcinoembryonic antigen (CEA) 3.32 ng/mL, CA125 24.50 U/mL, CA153 12.29 U/mL, CA199 52.49 U/mL, and CA724 1.24 U/mL. Thyroid function tests were normal. Human immunodeficiency virus was negative.

CT and magnetic resonance imaging (MRI) scan of the abdomen were performed. Plain CT scan at the level of the upper abdomen (Figure 1D) showed the presence of one hypodense lesion in the right liver lobe, which was not present on a previous positron emission tomography and computed tomography (PET/CT) or CT scan per-

formed 11 mo previously (Figure 1A-C).

MRI T1-weighted images (Figure 2A) of the upper abdomen showed that the hepatic lesion demonstrated low signal intensity compared with adjacent liver tissue, while on T2-weighted images (Figure 2B) hyperintense signal intensity was observed. Following administration of gadolinium contrast medium (Figure 2C) the mass remained unenhanced. During the portal (Figure 2D), and delayed (Figure 2E) phases, the lesion displayed peripheral enhancement with a centripetal progression.

Because the patient had a history of colon cancer, the initial diagnosis was possible metastatic hepatocellular carcinoma. After informed consent was obtained, the patient underwent excision of hepatic segment VII, where the nodule was located. The pathological diagnosis of the surgical specimen was PH (Figure 3). The patient had an uneventful postoperative recovery without complications, and was discharged one week after surgery.

DISCUSSION

PH was first reported in the German literature in 1861 by Wagner, and named by Schoenlank in 1916. The first description in the English literature was in 1950 by Zak^[2]. Although the exact origins of this disorder are unknown, PH has been associated with the prolonged use of a number of drugs and infectious causes such as *Bartonella henselae*^[3], tuberculosis, acquired immunodeficiency syndrome^[4], gummatous syphilis^[5] and several debilitating illnesses such as hematological malignancies^[6]. In this case, PH was found during the follow-up period after capecitabine and oxaliplatin treatment which suggests that chemotherapy could be responsible for the development of PH. Drugs reported to be associated with PH

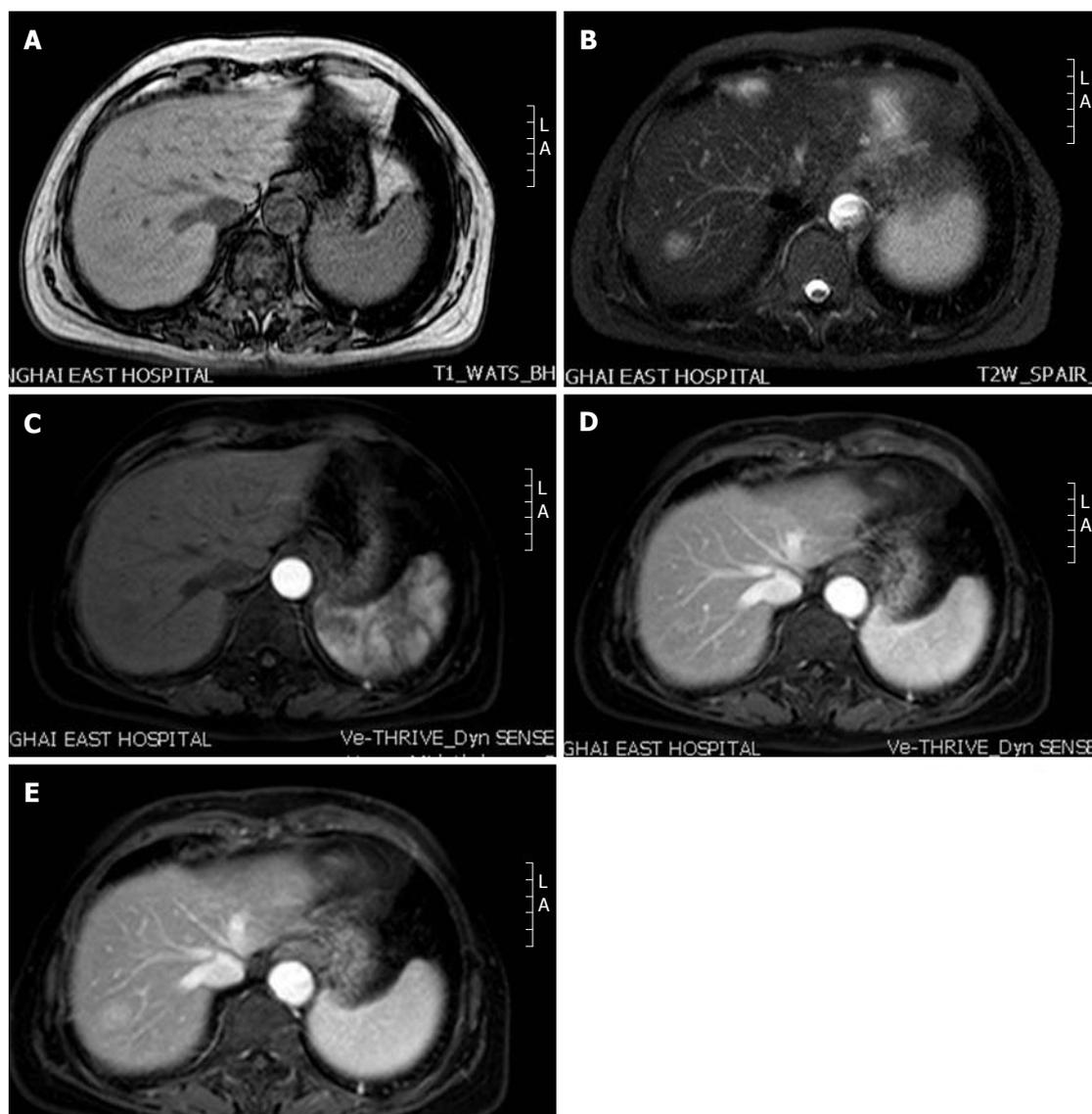


Figure 2 Magnetic resonance imaging scan findings in our patient. A: On T1-weighted images, the hepatic lesion shows low signal intensity; B: On T2-weighted images, the lesion shows hyperintense signal intensity; C: In the arterial phase, the lesion remains unenhanced; D, E: During the portal and delayed phases, the lesion displays peripheral enhancement with a centripetal progression. L: Left; A: Ahead.

include thiopurine^[7,8], azathioprine^[9], 6-thioguanine^[10], tamoxifen^[11], androgen^[12], oral contraceptives, diethylstilbestrol and toxin (e.g., polyvinyl chloride, arsenic, thorium oxide) exposure^[13]. To our knowledge, PH associated with capecitabine and oxaliplatin treatment has not been reported.

The clinical presentation and laboratory data in patients with PH are non-specific. Its clinical presentation is variable, ranging from asymptomatic cases discovered at autopsy to progressive cases with cholestasis, liver failure, portal hypertension or even spontaneous rupture^[14]. Death may result from hepatic failure or intraperitoneal hemorrhage. PH regresses after drug withdrawal, cessation of steroid therapy, or resolution of an associated infectious disease. Its variable clinical presentation makes the correct diagnosis of PH important, because misdiagnosis could lead to inappropriate treatment in asymptomatic cases, and in advanced cases could lead to progres-

sion and a fatal outcome if appropriate treatment is not given. Cohen *et al*^[15] reported a case of PH simulating a hepatic abscess both clinically and radiographically in a patient with sepsis. CT-guided drainage of the presumed liver abscess led to a fatal outcome.

The definitive diagnosis of PH is by histology. A percutaneous needle biopsy can also be used to confirm the diagnosis. However, even when ultrasound-guided, the procedure has a high risk of life-threatening hemorrhage^[16]. The imaging appearance of PH is difficult to differentiate from multiple abscesses, adenoma, focal nodular hyperplasia, hemangiomas, and metastases. CT findings of PH have been reported to display early globular enhancement centrally with centrifugal progression and eventual homogenous enhancement on the delayed phase^[17]. Wannesson *et al*^[18] reported CT findings in a case of PH which displayed peripheral enhancement with a centripetal progression between the arte-

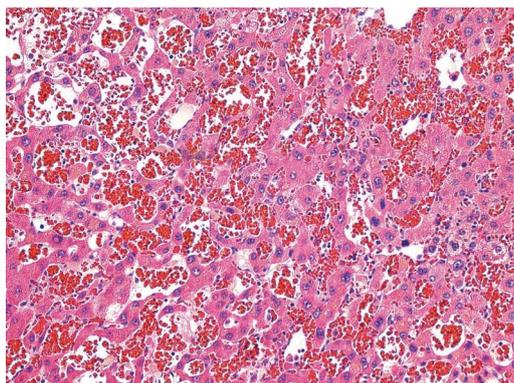


Figure 3 Photomicrograph of a liver section from our case showing variable-sized, blood-filled cystic spaces (hematoxylin and eosin, 200 ×).

rial and portal phases. Iannaccone *et al.*^[13] reported PH lesions with typical centrifugal progression of contrast enhancement, however, centripetal enhancement can also be observed. The enhancement pattern of PH varies depending on the freshness of the blood filling the peliotic cavities. Fresh circulating blood within the peliotic cavities is associated with marked enhancement, whereas retention of old blood is associated with mild or no enhancement^[19]. Characteristic MRI findings of PH include T1 hypointense and T2 hyperintense lesions, which show early peripheral and late diffuse contrast enhancement on dynamic imaging. Additionally, several T1 and T2 hyperintense hemorrhagic lesions may be detected due to hemorrhage^[20].

There is no specific treatment for PH. Treatment is primarily symptomatic and includes discontinuation of offending medications, partial hepatectomy or transarterial embolization^[21] due to liver rupture causing intraabdominal bleeding, or occasionally liver transplantation^[22] in patients with irreversible liver insufficiency.

In our case, points supporting metastatic liver cancer were: (1) a new liver lesion on follow-up; (2) a history of colon cancer; and (3) MRI imaging compatible with a metastasis. Points not supporting metastatic liver cancer were: (1) the presence of just one lesion; and (2) normal CEA level. An uncertain diagnosis in this situation led to antitumor therapies.

In conclusion, it is likely that PH is underdiagnosed in radiologic studies. PH should be considered in the differential diagnosis of new liver lesions in patients whose clinical settings do not clearly favor metastasization. Clinicians and radiologists must recognize these lesions to minimize the probability of misdiagnosis and inappropriate treatment.

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Upper oesophageal images and Z-line detection with 2 different small-bowel capsule systems

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Abstract

Transmission of oesophageal images may vary between different small-bowel capsule endoscopy models. A retrospective review of 100 examinations performed with 2 different Small-bowel capsule endoscopy (SBCE) systems (PillCam[®] and MiroCam[®]) was performed. The oral cavity/aero-digestive tract (i.e., tongue, uvula and/or epiglottis) was captured/identified in almost all (99%) of PillCam[®] videos but in none of MiroCam[®] cases, $P < 0.0001$. Furthermore, oesophageal images (i.e., from the upper oesophageal sphincter to the Z-line) were captured in 99% of PillCam[®] videos (mean \pm SD, 60.5 \pm 334.1 frames, range: 0-3329 frames) and in 66% of MiroCam[®] cases (mean \pm SD, 11.1 \pm 46.5 frames, range: 0-382 frames), $P < 0.0001$. The Z-line was identified in 42% of PillCam[®] videos and 17% of MiroCam[®], $P = 0.0002$. This information might be useful when performing SBCE in patients with high risks for aspiration.

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Key words: Capsule endoscopy; PillCam; MiroCam; Oesophagus; Aspiration; Detection; Z line; Transmission

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TO THE EDITOR

Small-bowel capsule endoscopy (SBCE) is likely one of the safest procedures in every day gastroenterology practice. Aside acute small-bowel obstruction from retained capsules^[1,2], aspiration of capsule endoscopes has also been reported^[3-5]. Albeit rare -and likely associated with spontaneous resolution-, capsule aspiration is a potentially life-threatening complication and a single fatality has been reported to date^[6].

We have previously noted that the MiroCam[®] SBCE system (IntroMedic[®] Co, Seoul, Korea) has a theoretical advantage, over other SBCE systems, of being smaller in size (11 mm \times 24 mm), as well as lighter (3.25 g)^[5,7]. The fact that to date there are no reported cases of tracheal aspiration involving MiroCam[®] capsules concurs to this. Conversely, PillCam[®] is 10.8 mm \times 26 mm and weighs 3.45 g. Furthermore, the 2 capsule systems have different centre of gravity and that may have some role in determining the direction of propagation in the small-bowel^[7]. The transmission of oesophageal images though may vary between different SBCE models and it is our experience that it fails more frequently with MiroCam[®]. In our hospital, a tertiary-care referral centre for capsule endoscopy for the East of Scotland, we have simultaneously operated two SBCE systems (MiroCam[®] and PillCam[®] SB, Given[®] Imaging Ltd, Yokneam, Israel) since May 2009^[8].

We retrospectively reviewed the last 100 examinations performed with each SBCE system in order to check the performance of the 2 systems in capturing images of

the upper most part of the gastrointestinal (GI) tract. Procedures involving endoscopic capsule placement ($n = 2$) and those where a capsule stayed for > 30 min in the oesophagus were excluded (one for each SBCE system). MiroCam[®] was used in 51 males/49 females (age: 55.3 ± 16.9 years) with the following indications: suspected/established Crohn's disease: 25; overt/occult GI bleeding: 64; other indication/s: 11. PillCam[®] was used in 34 M/66 F (age: 56.3 ± 14.9 years) of which, 32 had suspected/established Crohn's disease, 62 overt/occult GI bleeding and 6 other indication/s.

A single, experienced capsule endoscopist (> 1000 SBCE reviews) re-evaluated the SBCE videos for confirmation of capture of oropharyngeal images, number of oesophageal frames and detection of Z-line. Any frame that included a part of Z-line, was calculated as positive for the latter. The Fisher's exact test, mean \pm SD were used; P values of < 0.05 are considered statistically significant. All analyses were performed with GraphPad InStat (GraphPad Software, Inc, La Jolla, United States).

Interestingly, the oral cavity/aero-digestive tract (i.e., tongue, uvula and/or epiglottis) was captured/identified in almost all (99%) of PillCam[®] videos but in none of MiroCam[®] cases, $P < 0.0001$.

Furthermore, oesophageal images (i.e., from the upper oesophageal sphincter to the Z-line) were captured in 99% of PillCam[®] videos (mean \pm SD, 60.5 ± 334.1 frames, range: 0-3329 frames) and in 66% of MiroCam[®] cases (mean \pm SD, 11.1 ± 46.5 frames, range: 0-382 frames), $P < 0.0001$. The Z-line was identified in 42% of PillCam[®] videos and 17% of MiroCam[®], $P = 0.0002$. MiroCam[®] uses electric field propagation rather than radiofrequency, with a single skin electrode, two external contact plates on the capsule and the body as a conductor for signal transmission^[9]. This effectively means that a water interface would offer a better conductive surface area and it might explain the scarcity of images from the higher part of the GI tract and -potentially- the lack of documentation of a capsule aspiration.

In a recent retrospective study, Hong *et al*^[10] compared the diagnostic yield of MiroCam[®] and PillCam[®] SB capsules using the detection rates of the Z-line and the duodenal ampulla in a cohort of 141 individuals who under-

went SBCE for various clinical indications. In this cohort, the Z-line was detected in 36.9% of PillCam[®] videos and 47.7% of MiroCam[®] examinations ($P = 0.227$).

It is useful to remember that the advent of real-time viewers allows us to follow the procedure from the very first steps of capsule ingestion, providing of course that images are captured during this phase. This may have clinical implications, especially when the procedure is performed in unfit, elderly patients or individuals with known swallowing difficulties, since a number of capsule aspiration cases are relatively asymptomatic^[4,5].

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January 13-15, 2012
 Asian Pacific *Helicobacter pylori*
 Meeting 2012
 Kuala Lumpur, Malaysia

January 19-21, 2012
 American Society of Clinical
 Oncology 2012 Gastrointestinal
 Cancers Symposium
 San Francisco, CA 3000,
 United States

January 19-21, 2012
 2012 Gastrointestinal Cancers
 Symposium
 San Francisco, CA 94103,
 United States

January 20-21, 2012
 American Gastroenterological
 Association Clinical Congress of
 Gastroenterology and Hepatology
 Miami Beach, FL 33141,
 United States

February 3, 2012
 The Future of Obesity Treatment
 London, United Kingdom

February 16-17, 2012
 4th United Kingdom Swallowing
 Research Group Conference
 London, United Kingdom

February 23, 2012
 Management of Barretts
 Oesophagus: Everything you need
 to know
 Cambridge, United Kingdom

February 24-27, 2012
 Canadian Digestive Diseases Week
 2012
 Montreal, Canada

March 1-3, 2012
 International Conference on
 Nutrition and Growth 2012
 Paris, France

March 7-10, 2012
 Society of American Gastrointestinal
 and Endoscopic Surgeons Annual
 Meeting
 San Diego, CA 92121, United States

March 12-14, 2012
 World Congress on
 Gastroenterology and Urology
 Omaha, NE 68197, United States

March 17-20, 2012
 Mayo Clinic Gastroenterology and
 Hepatology
 Orlando, FL 32808, United States

March 26-27, 2012
 26th Annual New Treatments in
 Chronic Liver Disease
 San Diego, CA 92121, United States

March 30-April 2, 2012
 Mayo Clinic Gastroenterology and
 Hepatology
 San Antonio, TX 78249,
 United States

March 31-April 1, 2012
 27th Annual New Treatments in
 Chronic Liver Disease
 San Diego, CA 92121, United States

April 8-10, 2012
 9th International Symposium on
 Functional GI Disorders
 Milwaukee, WI 53202, United States

April 13-15, 2012
 Asian Oncology Summit 2012
 Singapore, Singapore

April 15-17, 2012
 European Multidisciplinary
 Colorectal Cancer Congress 2012
 Prague, Czech

April 18-20, 2012
 The International Liver Congress
 2012
 Barcelona, Spain

April 19-21, 2012
 Internal Medicine 2012
 New Orleans, LA 70166,
 United States

April 20-22, 2012
 Diffuse Small Bowel and Liver
 Diseases
 Melbourne, Australia

April 22-24, 2012
 EUROSON 2012 EFSUMB Annual

Meeting
 Madrid, Spain

April 28, 2012
 Issues in Pediatric Oncology
 Kiev, Ukraine

May 3-5, 2012
 9th Congress of The Jordanian
 Society of Gastroenterology
 Amman, Jordan

May 7-10, 2012
 Digestive Diseases Week
 Chicago, IL 60601, United States

May 17-21, 2012
 2012 ASCRS Annual Meeting-
 American Society of Colon and
 Rectal Surgeons
 Hollywood, FL 1300, United States

May 18-19, 2012
 Pancreas Club Meeting
 San Diego, CA 92101, United States

May 18-23, 2012
 SGNA: Society of Gastroenterology
 Nurses and Associates Annual
 Course
 Phoenix, AZ 85001, United States

May 19-22, 2012
 2012-Digestive Disease Week
 San Diego, CA 92121, United States

June 2-6, 2012
 American Society of Colon and
 Rectal Surgeons Annual Meeting
 San Antonio, TX 78249,
 United States

June 18-21, 2012
 Pancreatic Cancer: Progress and
 Challenges
 Lake Tahoe, NV 89101, United States

July 25-26, 2012
 PancreasFest 2012
 Pittsburgh, PA 15260, United States

September 1-4, 2012
 OESO 11th World Conference
 Como, Italy

September 6-8, 2012
 2012 Joint International

Neurogastroenterology and Motility
 Meeting
 Bologna, Italy

September 7-9, 2012
 The Viral Hepatitis Congress
 Frankfurt, Germany

September 8-9, 2012
 New Advances in Inflammatory
 Bowel Disease
 La Jolla, CA 92093, United States

September 8-9, 2012
 Florida Gastroenterologic Society
 2012 Annual Meeting
 Boca Raton, FL 33498, United States

September 15-16, 2012
 Current Problems of
 Gastroenterology and Abdominal
 Surgery
 Kiev, Ukraine

September 20-22, 2012
 1st World Congress on Controversies
 in the Management of Viral Hepatitis
 Prague, Czech

October 19-24, 2012
 American College of
 Gastroenterology 77th Annual
 Scientific Meeting and Postgraduate
 Course
 Las Vegas, NV 89085, United States

November 3-4, 2012
 Modern Technologies in
 Diagnosis and Treatment of
 Gastroenterological Patients
 Dnepropetrovsk, Ukraine

November 4-8, 2012
 The Liver Meeting
 San Francisco, CA 94101,
 United States

November 9-13, 2012
 American Association for the Study
 of Liver Diseases
 Boston, MA 02298, United States

December 1-4, 2012
 Advances in Inflammatory Bowel
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 Hollywood, FL 33028, United States

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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 PMCID: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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- 9 Outreach: Bringing HIV-positive individuals into care. *HRS A Careaction* 2002; 1-6 [PMID: 12154804]

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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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- 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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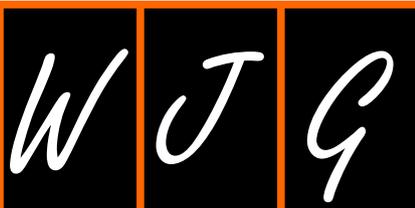
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Signaling pathway/molecular targets and new targeted agents under development in hepatocellular carcinoma

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Abstract

Advances in molecular cell biology over the last decade have clarified the mechanisms involved in cancer growth, invasion, and metastasis, and enabled the development of molecular-targeted agents. To date, sorafenib is the only molecular-targeted agent whose survival benefit has been demonstrated in two global phase III randomized controlled trials, and has been approved worldwide. Phase III clinical trials of other molecular targeted agents comparing them with sorafenib as first-line treatment agents are ongoing. Those agents target the vascular endothelial growth factor, platelet-derived growth factor receptors, as well as target the epidermal growth factor receptor, insulin-like growth factor receptor and mammalian target of rapamycin, in addition to other molecules targeting other components of the signal transduction pathways. In addition, the combination of sorafenib with standard treatment, such as resection, ablation, transarterial embolization, and hepatic arterial infusion chemotherapy are ongoing. This review outlines the main pathways involved in the development and progression of hepatocellular carcinoma and the new agents that target these pathways. Finally, the current statuses of clinical trials of new agents or combination therapy with sorafenib and standard treatment will also be discussed.

INTRODUCTION

Advances in molecular cell biology over the last decade have clarified the mechanisms involved in cancer growth, invasion and metastasis, and enabled the development of molecular-targeted agents, best represented by trastuzumab for breast cancer, imatinib and rituximab for hematopoietic tumors, and gefitinib and erlotinib for lung cancer. These molecular-targeted agents are broadly classified into two categories: drugs targeting cancer cell-specific molecules, and nonspecific molecular-targeted drugs for molecular biological abnormalities induced in the host stroma or blood vessels by the presence of cancer. Examples of the former approach include trastuzumab, which targets human epidermal growth factor receptor 2 (HER2), the expression of which is a poor prognostic factor for breast cancer; rituximab, which is used to treat B-cell lymphoma, targets CD20 expressed on normal and neoplastic mature B cells; while imatinib binds to the ATP-binding site of Bcr-abl, a protein that causes chronic myelogenous leukemia. However, no critical target molecules responsible for treatment response have been identified in hepatocellular carcinoma (HCC).

In recent years, clinical trials have been conducted for many agents that act on growth factor receptors, such as epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR), and intracellular signaling pathways. In addition, multi-kinase inhibitors, including sorafenib, have emerged and been evaluated. Clinical trials are ongoing to compare drugs with the same mechanism of action and to test the combined efficacy and relative merits of these drugs with existing drugs for many cancers. Since the main treatment option for metastatic, advanced stage cancers, such as breast and colorectal cancer, is systemic chemotherapy, clinical trials are ongoing to investigate how to combine molecular-targeted agents with standard therapies based on the results of long-term, large-scale clinical trials, and to identify which molecular-targeted agents should be used as initial or second-line therapy.

However, for HCC, background liver damage limits the indication for systemic chemotherapy and no anti-cancer drugs were found to be effective in large-scale randomized controlled trials except sorafenib. Now that the usefulness of sorafenib has been demonstrated in two large scale randomized clinical trials, the development of new drugs that are effective for poor-prognosis advanced HCC, who are resistant to a standard of care agent, sorafenib.

In this review, the clinical impact of sorafenib and ongoing trials of new agents or combination trials with sorafenib will be described.

SIGNALING PATHWAYS AND MOLECULAR-TARGETED AGENTS IN HCC

As in other cancers, the molecular mechanisms involved in the development and progression of HCC are complex. After hepatitis B virus and hepatitis C virus infection and alcohol or aflatoxin B1 exposure, genetic and epigenetic changes occur, including oncogene activation and tumor-suppressor gene inactivation due to inflammation-induced increase in hepatocyte turnover and oxidative stress-induced DNA damage. Through apoptosis and cell proliferation, these changes lead to the multistep development and progression of a hyperplastic to dysplastic nodule, early HCC, and advanced HCC. A number of studies have reported changes in gene expression, chromosomal amplification, mutations, deletions and copy number alterations (gain/loss), somatic mutations, CpG hypermethylation, and DNA hypomethylation, as well as molecular abnormalities, which can constitute therapeutic targets^[1-5].

The binding of growth factors to their receptor proteins activates protein-phosphorylating enzymes, thus activating a cascade of proliferative signaling pathways to transmit proliferative signals into the nucleus. Growth factors, such as EGF, transforming growth factor (TGF)- α / $-\beta$, insulin-like growth factor (IGF), and VEGF, also function in liver regeneration after injury, while fibroblast

growth factor (FGF) and the platelet-derived growth factor (PDGF) family are involved in liver fibrosis and HCC growth^[6-8]. The receptors for these growth factors are broadly classified into G protein-coupled receptors and protein kinases. On ligand binding, these receptors activate their downstream intracellular molecules in a cascade fashion. Many of the growth factor receptors and oncogenes have tyrosine kinase activity, and the tyrosine kinases are classified into transmembrane receptor tyrosine kinases, such as the EGFR and VEGFR, and cytoplasmic non-receptor tyrosine kinases, such as Abl and Src. On the other hand, Raf, mitogen-activated protein kinase (MAPK)/extracellular signaling-regulated kinase (ERK) kinase (MEK), and mammalian target of rapamycin (mTOR) are serine/threonine kinases.

In general, the MAPK, phosphoinositide 3-kinase (PI3K)/Akt/mTOR, c-MET, IGF, Wnt- β -catenin and Hedgehog signaling pathways, and the VEGFR and PDGF receptor (PDGFR) signaling cascades show altered activity in HCC, and agents targeting these pathways are under development (Figures 1-3; Table 1)^[9-12]. Many molecular-targeted agents are now under development and the target signaling pathways and growth factors are outlined below.

MAPK pathway (Ras/Raf/MEK/ERK)

The MAPK intracellular signaling pathway, which is mainly involved in cell growth and survival, and regulates cell differentiation, is upregulated in cancer cells. Therefore, this pathway has been extensively studied as a therapeutic target. The MAPK pathway is a common downstream pathway for the EGFR, PDGFR and VEGFR, and is universally used for signal transduction downstream of cytokine receptors, integrin complexes, and G-protein receptors to Ras. The MAPK pathway also plays an important role in HCC, in that its activation is reportedly involved in HCC growth and survival. The downstream ERK is activated by two upstream protein kinases, which are coupled to growth factor receptors by Ras proteins. Ras, which is activated by ligand binding, activates Raf serine/threonine kinases and MEK (MAP kinase/ERK kinase), while MEK phosphorylates and activates ERK, which phosphorylates proteins involved in cell growth, apoptosis resistance, extracellular matrix production and angiogenesis^[13-15].

Raf and Ras inhibitors: Raf and Ras are proto-oncogenes. In particular, K-Ras mutations are commonly observed in many cancers, including pancreatic and colorectal cancers. One study reported that 30% of HCCs have Ras mutations^[16]. To our knowledge, no agents targeting Ras are planned to enter clinical trials at the present. However, because the binding of Ras protein to the cell membrane and its functional activation require farnesylation, several farnesyltransferase inhibitors are being tested for Ras-related tumors. In addition, vaccine therapy for mutant Ras proteins is currently being tested for solid cancers, including HCC.

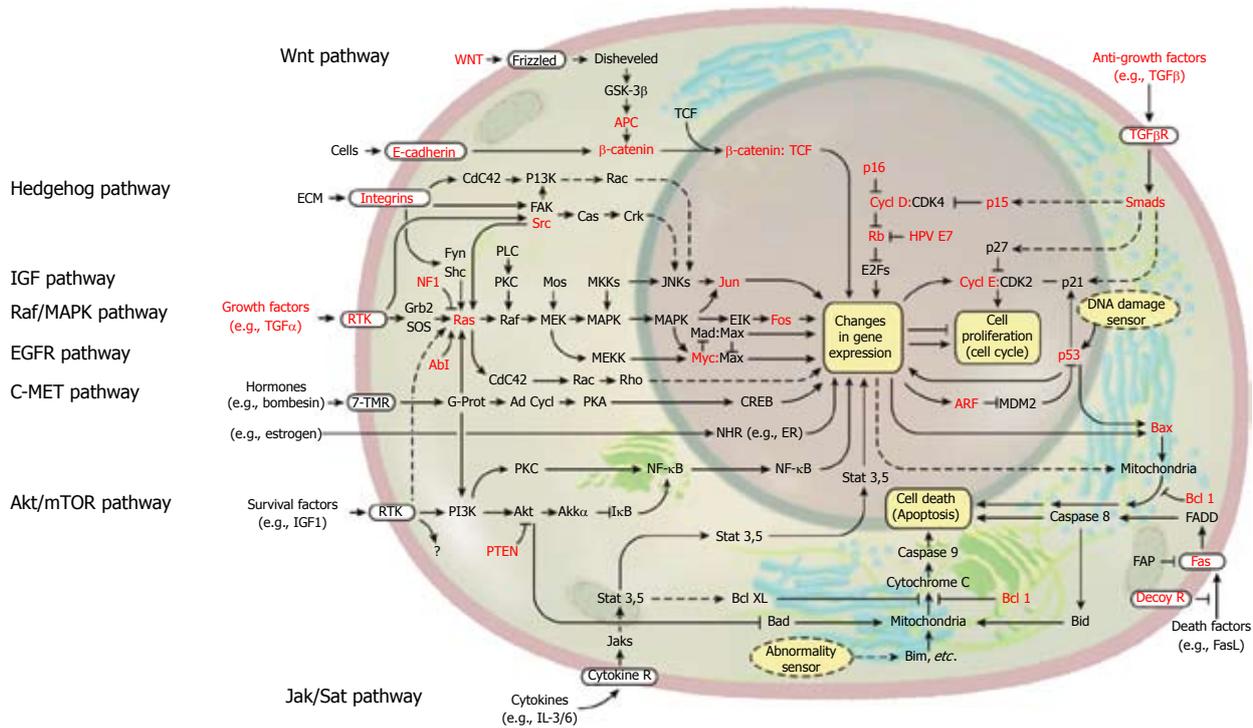


Figure 1 Signal transduction in solid cancer cells including hepatocellular carcinoma. Some of the genes known to be functionally altered are highlighted in red. These signaling pathways, including growth factor pathway, Wnt pathway, Hedgehog pathway, Akt/mammalian target of rapamycin (mTOR) pathway, and Jak/Stat pathway, can be a molecular targets for treatment of hepatocellular carcinoma. (Cited and modified from Hanahan *et al*^[10] with permission.) EGFR: Epidermal growth factor receptor; TGF: Transforming growth factor; IGF: Insulin-like growth factor; MAPK: Mitogen-activated protein kinase; PI3K: Phosphoinositide 3-kinase; ERK: Extracellular signaling-regulated kinase; NF-κB: Nuclear factor-kappa B; IL: Interleukin.

Table 1 Molecular targeted agents for hepatocellular carcinoma: Targets and development status

Agents	Targets (angiogenesis)				Targets (proliferation)			Positioning	Development status
	VEGFR	PDGFR	FGF	EGFR	Raf	mTOR	TRAIL-R2		
Sorafenib	•	•			•			1st line	Approved
Sunitinib	•	•						1st line	P III terminated
E7080	•	•	•					1st/2nd line	P II ongoing
Tigatuzumab (CS1008)							•	1st line (sorafenib combination)	P I / II ongoing
Linifanib (ABT-869)	•	•						1st line	P III ongoing
Brivanib		•			•			1st line, 2nd line, TACE adjuvant	P III ongoing
TSU-68		•	•					TACE combination	P III ongoing
Ramucirumab		•						2nd line	P III ongoing
Everolimus (RAD001)						•		2nd line	P III ongoing
Axitinib		•	•					2nd line	P III ongoing

VEGFR: Vascular endothelial growth factor receptor; PDGFR: Platelet-derived growth factor receptor; FGF: Fibroblast growth factor; EGFR: Epidermal growth factor receptor; mTOR: Mammalian target of rapamycin; TACE: Transarterial chemoembolization.

The Raf family consists of three isoforms, A-Raf, B-Raf and C-Raf/Raf-1. Genetic abnormalities, such as point mutations and gene rearrangements, have been reported in various cancers^[17]; however, in HCC, *ras/raf* mutations are rare, and no *k-ras* or *b-raf* mutations have been detected^[18]. On the other hand, wild-type Raf-1 was reported to be hyperactivated in many cancers, including HCC^[19-21]. Sorafenib inhibits Raf, and has multiple characteristics in that it exhibits strong inhibitory activity against Raf-1 (C-Raf) kinase, B-Raf (wild-type B-Raf and mutant V600E B-Raf) serine/threonine kinase, the pro-angiogenic receptor tyrosine kinases VEGFR,

PDGFR and FGFR1, and tyrosine kinases, such as c-kit, Flt-3 and RET, which are involved in tumor progression and overall prognosis^[22].

MEK: The MEK family consists of MEK1 and MEK2 proteins, which specifically phosphorylate tyrosine and threonine residues, and phosphorylates downstream Erk1 and Erk2^[23].

In an immunohistochemical study, MEK1/2 overexpression, ERK1/2 overexpression, and ERK1/2 phosphorylation were observed in 100% (46/46), 91% (42/46), and 69% (32/46) of HCCs, respectively. In ad-

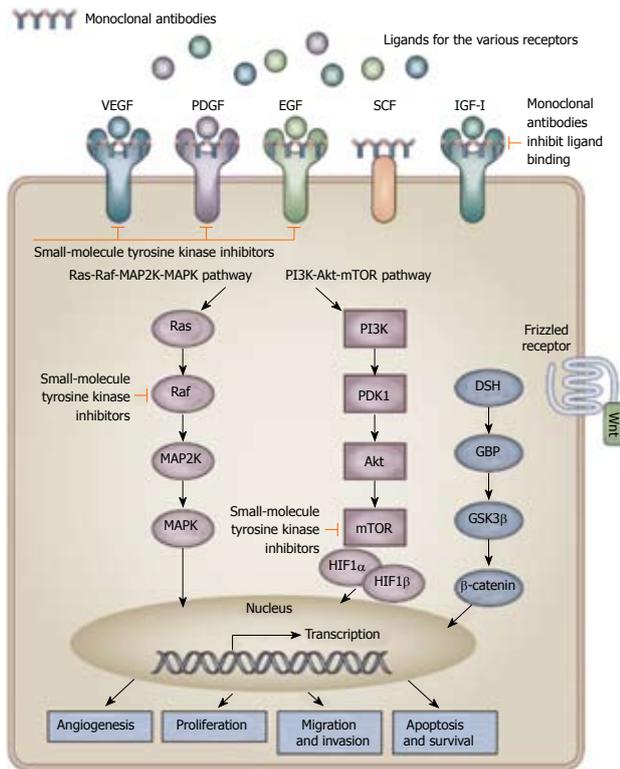


Figure 2 Signaling pathways and potential drug targets to inhibit hepatocarcinogenesis. Activation of receptor tyrosine kinases by their ligands activates downstream signaling pathways with effects on angiogenesis, proliferation, migration and invasion, and apoptosis or survival of cells. Monoclonal antibodies inhibit ligand binding to the receptor and small-molecule tyrosine kinase inhibitors inhibit propagation of the downstream signal. (Cited from Spangenberg *et al*^[11] with permission.) IGF: Insulin-like growth factor; MAPK: Mitogen-activated protein kinase; PI3K: Phosphoinositide 3-kinase; EGF: Epidermal growth factor; VEGF: Vascular endothelial growth factor; PDGF: Platelet-derived growth factor; mTOR: Mammalian target of rapamycin; HIF: Hypoxia-inducible factor; SCF: Stem cell factor.

dition, the *in vitro* treatment of HepG2 and Hep3B cells with MEK1/2 inhibitors inhibited cell growth and up-regulated apoptosis^[24].

The MEK inhibitors CI-1040, PD0325901, AZD6244, and RDEA119/BAY869766 have been tested in several cancers, including solid tumors such as HCC. Recently, the results of Phase I of AS703026 and E6201 studies against solid tumors were reported in ASCO2010. A phase II study of AZD6244 (selumetinib, ARRY-142866) and a phase I / II study of RDEA119/BAY869766 in combination with sorafenib are being conducted.

PI3K/Akt/mTOR pathway

The PI3K/Akt/mTOR pathway also plays an important role in cell growth, survival regulation, metabolism, and anti-apoptosis. The membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP₂) is phosphorylated by PI3K into phosphatidylinositol 3,4,5-triphosphate (PIP₃), which binds to and activates the serine/threonine kinase Akt. The tumor suppressor gene product phosphatase and tensin homolog (PTEN) deleted on chromosome is antagonistic to PI3K activity. PTEN is a lipid phosphatase that

dephosphorylates inositol phosphates, such as PIP₃. The inactivation of PTEN through gene deletion increases PIP₃ levels, and activates Akt, which inhibits apoptosis, leading to the development of tumors. The serine/threonine kinase mTOR is an important mediator in the PI3K/Akt pathway, which binds intracellularly to a protein called raptor or rictor, and exists as two different complexes, complex 1 and 2 (mTORC1 and mTORC2). mTORC2 (mTOR-rictor) activates Akt, while mTORC1 (mTOR-raptor) is activated downstream of Akt; thus, both molecules regulate protein synthesis (Figures 4 and 5)^[25].

Inhibiting mTOR with molecules, such as RAD001, generates additive effects that accompany upstream and downstream target inhibition. Alternatively, upstream receptor inhibition is compensated for by inhibiting the downstream pathway, even if some resistance develops against receptor inhibition regardless of initial or acquired resistance. Therefore, RAD001 is a potential targeted agent for HCC.

Besides the finding that mTOR plays a key role in cell biology, it was also demonstrated that mTOR and S6K are overexpressed in 15%-41% of HCCs. mTOR inhibitors also have antitumor effects in various HCC cell lines and animal models^[26-29]. Activation of mTOR is correlated with the development of HCC and recurrence after the excision of early HCC. Regulating this specific intracellular pathway (Ras-Raf pathway) with RAD001 is potentially more effective in suppressing sorafenib-resistant tumors.

A study of 528 HCC samples showed that the expression of pAkt, PTEN, p27 and S6 ribosomal protein (pS6) was a poor prognostic factor for survival^[30]. A tissue microarray analysis of HCC samples revealed that the loss of PTEN and overexpression of pAkt and p-mTOR were correlated with tumor grade, intrahepatic metastasis, vascular invasion, TNM stage, Ki-67 labeling index, and matrix metalloproteinase (MMP)-2 and -9 upregulation. Meanwhile, PTEN mRNA expression in the cancerous tissue was downregulated compared with that in the non-cancerous tissue. The levels of PTEN, MMP-2, and MMP-9 mRNA expression were correlated with tumor stage and metastasis, and the levels of PTEN and MMP-9 mRNA expression were inversely correlated^[31]. In an extensive analysis of 314 HCC samples in terms of mutation analysis, DNA copy number changes, mRNA levels and immunostaining, Villanueva *et al*^[32] found that activation of the IGF pathway, upregulation of EGF, dysregulation of PTEN, and aberrant mTOR signaling were present in half of the samples, and that inhibiting mTOR activity with everolimus was effective in improved survival and suppression of recurrence.

The PI3K inhibitor RG7321 and the Akt inhibitor perifosine target the PI3K/Akt/mTOR pathway and are in early stages of clinical development, while the mTOR inhibitors everolimus (RAD001), sirolimus (Rapamune), and temsirolimus (CCI-779) are at more advanced stages of development. Everolimus is used to treat sorafenib-

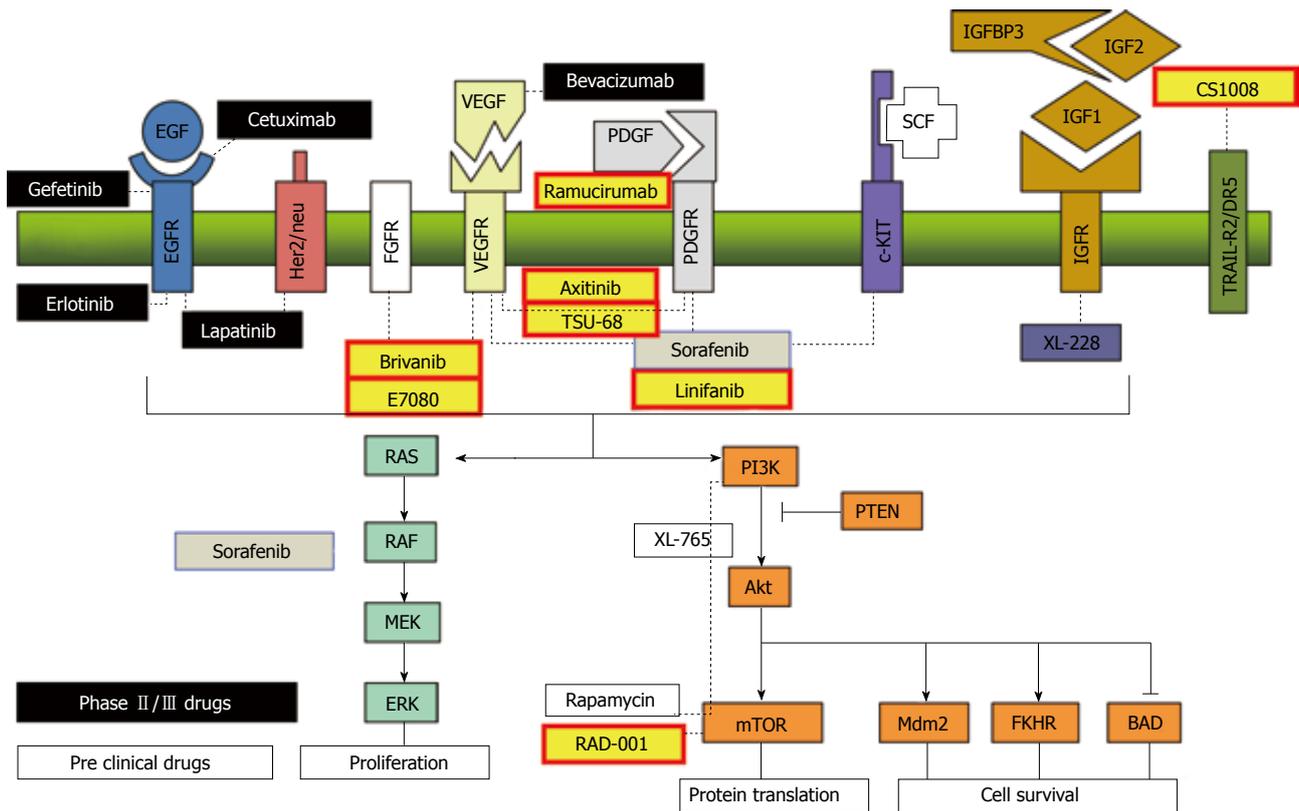


Figure 3 Target molecular and targeted agents under development. Monoclonal antibodies (VEGF: bevacizumab, EGFR: cetuximab), tyrosine kinase inhibitors (VEGFR: sorafenib, brivanib, linifanib, axitinib, TSU-68; FGFR: E7080, brivanib), EGFR: erlotinib, lapatinib), serine/threonine kinase inhibitors (Raf: sorafenib, mTOR: rapamycin and everolimus) and agonistic ligand of TRAIL-R2/DR5 (CS1008). (Cited and modified from Villanueva *et al*^[12] with permission.) IGF: Insulin-like growth factor; PI3K: Phosphoinositide 3-kinase; EGF: Epidermal growth factor; VEGF: Vascular endothelial growth factor; PDGF: Platelet-derived growth factor; mTOR: Mammalian target of rapamycin; PTEN: Phosphatase and tensin homolog; SCF: Stem cell factor; FGFR: Fibroblast growth factor receptor.

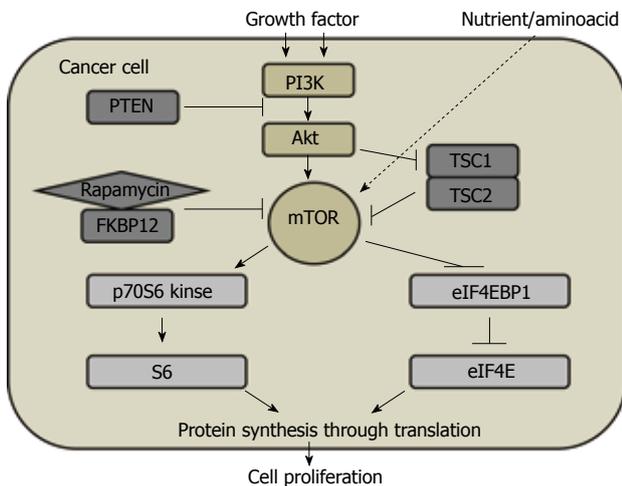


Figure 4 Phosphoinositide 3-kinase/Akt/mammalian target of rapamycin signaling pathway in cell proliferation in solid cancer. PI3K: Phosphoinositide 3-kinase; mTOR: Mammalian target of rapamycin; PTEN: Phosphatase and tensin homolog; TSC: Tuberous sclerosis; FKBP12: FK506-binding protein 12.

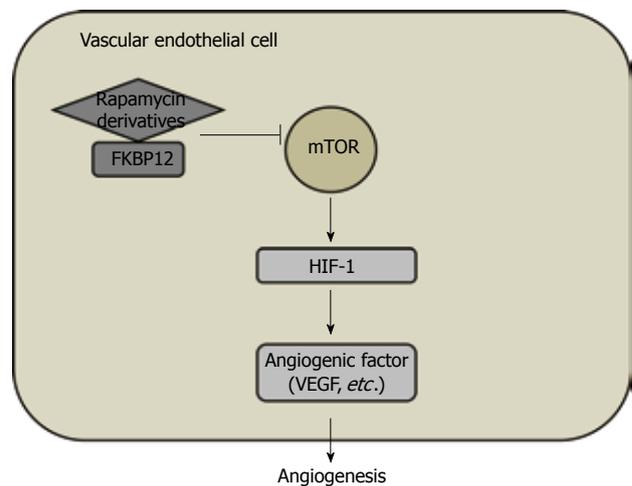


Figure 5 Mammalian target of rapamycin/hypoxia-inducible factor-1/vascular endothelial growth factor signaling pathway in angiogenesis in solid cancer. HIF: Hypoxia-inducible factor; VEGF: Vascular endothelial growth factor; mTOR: Mammalian target of rapamycin; FKBP12: FK506-binding protein 12.

intolerant patients, or for patients showing disease progression after sorafenib administration. A phase III study to compare everolimus and a placebo (EVOLVE-1: Advanced Hepatocellular Carcinoma after Disease Progression or Intolerance to Sorafenib Everolimus for Liver

cancer Evaluation) and a phase I /randomized phase II study (sorafenib + everolimus *vs* sorafenib alone) to test the efficacy and tolerance of sorafenib in combination with everolimus are underway. Since mTOR inhibitors exhibit cytostatic and antiangiogenic effects, they are

expected to be effective in combination with other angiogenesis inhibitors, such as bevacizumab, and may be appropriate for administration after transarterial chemoembolization (TACE). Furthermore, since the mTOR pathway is stimulated by factors such as EGFR, PDGFR, and TGF α , and is closely related to other signaling pathways, including the Ras/Raf/MEK/ERK pathway, they are likely to show promising efficacy when used in combination with other growth factor inhibitors^[33].

VEGF/VEGFR, PDGFR, FGFR

Angiogenesis is an important event not only for HCC, but also for cancer growth and metastasis, and occurs because of complex alterations involving promoting factors such as VEGF, angiopoietin, and FGF, and inhibitory factors including thrombospondin and angiostatin, as well as the surrounding tissue. The VEGF family consists of VEGF-A, -B, -C, -D and -E, and placental growth factor (PlGF). The VEGFR family comprises VEGFR-1 (flt-1), VEGFR-2 (flk-1/KDR), and VEGFR-3 (flt-4). VEGF-A binds to VEGFR-1 and -2 and is involved in angiogenesis and the maintenance of mature blood vessels, while VEGF-C and -D mainly bind to VEGFR-3, are involved in lymphangiogenesis^[34,35]. VEGF isoforms, such as VEGF₁₂₁ and VEGF₁₆₅, have been identified, and isoform subtypes also exist, such as EGF_{166b}. Thus, it is clear that these growth factors do not exhibit angiogenesis-promoting effects alone, and they have attracted attention as new therapeutic targets^[36].

HCC typically exhibits active angiogenesis. During the progression from early to well- and moderately-differentiated HCC, angiogenesis increases and cancer cells acquire the ability to invade vessels and metastasize. Scientific and clinical studies have revealed that, during the progression from hepatitis to cirrhosis, angiogenesis and disruption of the vascular architecture are linked to the progression of HCC, and contribute to increased hepatic vascular resistance and portal hypertension, and decreased hepatocyte perfusion^[37]. In addition, a meta-analysis has demonstrated that VEGF expression is a prognostic factor in HCC^[38].

Phase II studies have been started to test the usefulness of bevacizumab (Avastin[®]), which directly targets VEGF, in TACE-treated HCC, and the use of bevacizumab in combination with erlotinib (Tarceva[®]), an EGFR tyrosine kinase inhibitor.

Sunitinib (Sutent[®]) is a multi-kinase inhibitor that inhibits tyrosine kinases, such as VEGFR-1, -2, -3, PDGFR- α , - β and c-Kit. A phase II study of sunitinib in 37 advanced HCC patients showed that the median progression-free survival (PFS) and median overall survival (OS) were 3.7 and 8 mo, respectively. In that study, adverse events included grade 3/4 thrombocytopenia in 37.8% of patients, neutropenia in 24.3%, asthenia in 13.5%, and hand-foot syndrome in 10.8%^[39]. Since sunitinib has a lower IC₅₀ for each target than sorafenib, it is expected to exhibit greater antitumor activity. However, this factor may be responsible

for the higher incidence of adverse events with sunitinib. The main evaluation item in the above phase II trial was the response rate, which did not reach the expected value, leading to the conclusion that it was a negative study^[40]. In that study, sunitinib was administered at 50 mg/d for four weeks followed by two weeks of rest per cycle^[39], whereas Zhu *et al*^[40] used a dosing schedule of 37.5 mg/d for four weeks followed by two weeks of rest per cycle, and reported that the median PFS and OS were 3.9 and 9.8 mo, respectively. An ongoing global cooperative phase III controlled clinical trial to compare sorafenib and sunitinib head-to-head, and to seek approval for first-line indications for advanced HCC, adopted a sunitinib dosing schedule of 37.5 mg/d. However, in a "Reflection and Reaction" regarding the above trial results, Forner *et al*^[41] cast doubt on whether the drugs at this dose could maintain tolerance and ensure efficacy. Consequently, the trial was terminated on March, 2010 because of the recommendation by data monitoring committee based on interim analysis, showing relatively high toxicity and no superior efficacy to sorafenib.

Brivanib is a kinase inhibitor that selectively inhibits VEGFR-1, -2 and -3, and FGFR-1, -2 and -3. Recent studies suggest that tumor progression following treatment with antiangiogenic agents that target the VEGF signaling pathway alone may result from either evasive or intrinsic resistance^[42]. Furthermore, there is strong evidence to support the hypothesis that evasive resistance to anti-VEGF blockade is associated with reactivation of tumor angiogenesis by alternative signaling pathways. One such mechanism of resistance is the activation of the FGF signaling pathway^[43,44]. Basic FGF (FGF2) is a potent angiogenic factor. Indeed, expression of FGF2 enhances growth, invasion, and angiogenesis of many tumor types^[45,46]. Moreover, recent evidence has shown that FGF is overexpressed and activated in HCC, and that high FGF2 levels may predict a poor clinical outcome among patients with HCC^[46].

Considering the proposed importance of FGF signaling in HCC angiogenesis, it is clear that novel antiangiogenic agents that combine inhibition of FGF receptor signaling with inhibition of VEGFR signaling might provide a potential mechanism to overcome anti-VEGF resistance in HCC (Figure 6). With this in mind, it is worthwhile considering the potential future impact of brivanib on the treatment of advanced HCC. Brivanib, a small-molecule tyrosine kinase inhibitor, is the first oral selective dual inhibitor of FGF and VEGF signaling. In multiple preclinical models of human xenograft tumors, including patient-derived HCC xenografts, brivanib has shown potent antitumor activity and no overt toxicity when dosed orally^[47,48]. Furthermore, brivanib has demonstrated promising antitumor activity and acceptable tolerability in a phase II, open-label study in patients with unresectable locally advanced or metastatic HCC^[49,50]. Crucially, within this trial, brivanib showed activity both as first-line therapy (overall survival: 10 mo) or as second-

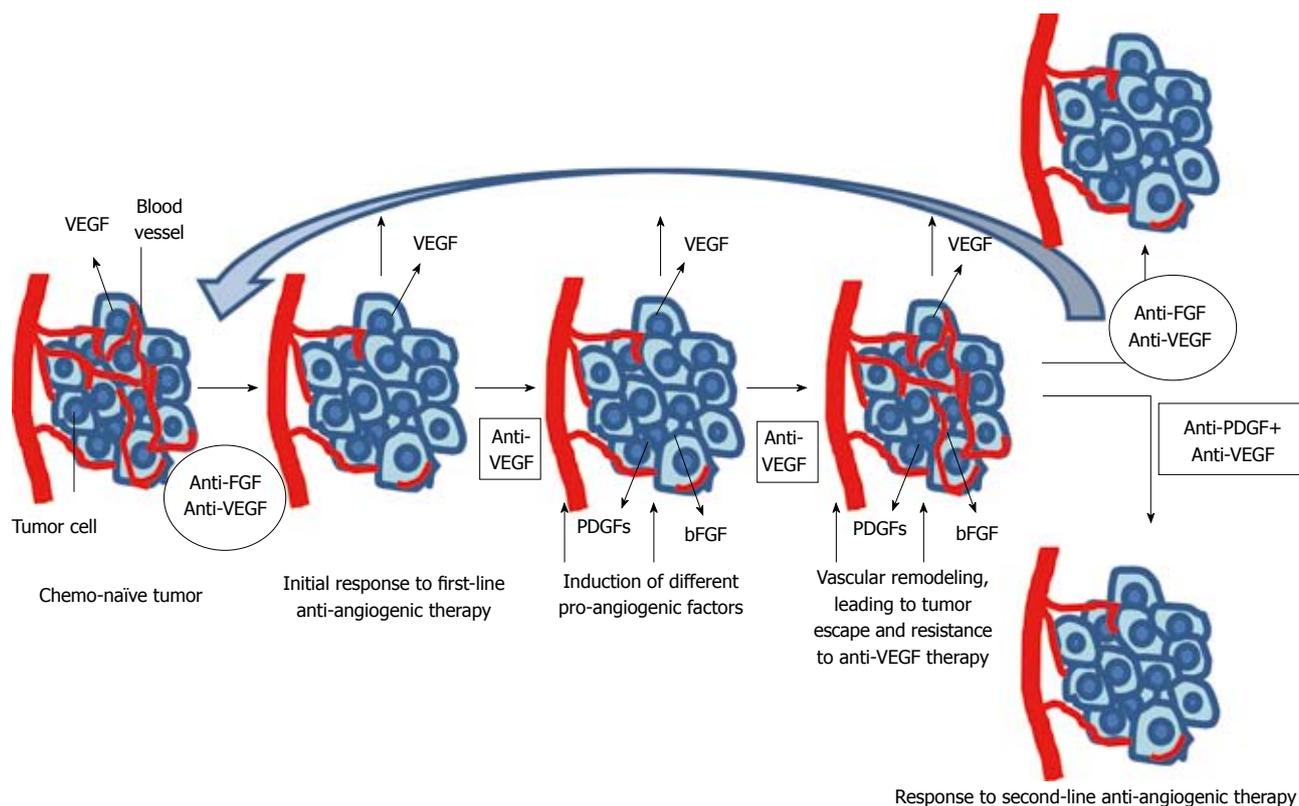


Figure 6 Brivanib may be effective for the failure or resistance of first line anti-angiogenic therapy for vascular endothelial growth factor. In addition, there is a possibility that anti-FGF agents can be first line anti-angiogenic agents. FGF: Fibroblast growth factor; VEGF: Vascular endothelial growth factor; PDGF: Platelet-derived growth factor.

line therapy in patients who had failed prior antiangiogenic treatment, primarily with sorafenib (overall survival: 9.5 mo)^[50]. Of note, the incidence of all-grade hand-foot syndrome was only 8% in this study.

As for brivanib, an international global phase III clinical trial to compare brivanib and sorafenib head-to-head and to seek approval for first-line therapy for advanced HCC has already been started, and the results are eagerly awaited. Since brivanib targets FGF and VEGF, and is associated with relatively mild adverse effects, a second-line study of brivanib in sorafenib-ineffective and -intolerant patients, and a trial to evaluate the use of brivanib in combination with TACE, are underway. Depending on the results of these trials, indications for use in HCC may be obtained; therefore, positive results are eagerly anticipated.

The results have been reported for a phase II study of brivanib in 55 patients (cohort A) who had not received systemic therapy for curatively unresectable HCC and 46 patients (cohort B) previously treated with angiogenesis inhibitors, such as sorafenib or thalidomide^[49]. The median TTP and OS were 2.8 mo and 10 mo, respectively, in cohort A versus 1.4 mo and 9.8 mo, respectively, in cohort B. Adverse events included fatigue (51.5%), diarrhea (41.6%), hypertension (42.6%), anorexia (41.6%), and nausea/vomiting (40.6%/30.7%) in total. Thus, these results demonstrated the efficacy of brivanib as a second-line treatment. The results of three phase III clinical trials,

BRISK-PS (sorafenib failure or sorafenib-intolerant patients; brivanib + best supportive care (BSC) *vs* placebo + BSC), BRISK-FL (advanced HCC; brivanib *vs* sorafenib), and BRISK-TA (patients with unresectable HCC, brivanib *vs* placebo as post-TACE adjuvant therapy) are awaited (Table 2).

Linifanib (ABT-869), which strongly inhibits VEGFR and PDGFR, is also under global phase III trial.

In a Japanese phase I / II trial of TSU-68, an oral molecular inhibitor of VEGFR, PDGFR, and FGFR, to test its safety and efficacy in 35 HCC patients, the response rate was 5.6% (CR, PR, SD, PD and NE in 1, 2, 15, 16 and 1 patients, respectively), and the disease control rate was 51.4%^[51]. The global phase III trial of TACE in combination with TSU-68 has just started on January 2011.

In addition, several phase I / II trials are being conducted to assess kinase inhibitors such as cediranib (AZD2171), which inhibit VEGFR, PDGFR, CSF-1R (cFms), Kit, and Flt3. Furthermore, a phase III global study of axitinib, which is currently being tested in renal cell carcinoma, has also been started as a second line agents on 2011.

EGF/EGFR

EGFR is a member of the HER family, which includes EGFR (erbB1), HER2/neu (erbB3), and HER4 (erb4). All members of this family, except HER3, have an intracellular tyrosine kinase domain, and the binding of a ligand to its extracellular domain triggers signal transduc-

Table 2 Ongoing clinical trials (P III)

First line
Comparison study between sorafenib and single agent (head to head): Sunitinib → endpoint not met
Brivanib
Linifanib
Combination with sorafenib and another agent: DXR, erlotinib (SEARCH), everolimus, CS-1008, <i>etc.</i>
Second line
Sorafenib failure: Brivanib, everolimus (RAD001), ramucirumab, axitinib, S-1, <i>etc.</i>
Combination with standard therapy
Adjuvant setting after surgery or RFA: STORM
Combination with TACE: SPACE, BRISK-TA, TACTICS, ECOG1208
Combination with HAIC: SILIUS

TACTICS: Phase II study: Transcatheter arterial chemoembolization therapy in combination with sorafenib (ClinicalTrials.gov ID: NCT01217034), SILIUS: Randomized controlled trial comparing efficacy of sorafenib *vs* sorafenib in combination with low dose cisplatin/fluorouracil hepatic arterial InfUSion chemotherapy in patients with advanced hepatocellular carcinoma and exploratory study of biomarker predicting its efficacy (ClinicalTrials.gov ID: NCT01214343); HAIC: Hepatic arterial infusion chemotherapy; TACE: Transarterial chemoembolization.

tion through the above-described MAPK and PI3K/Akt/mTOR pathways. Thus, these receptors are involved in cell growth, differentiation, survival, and adhesion^[52]. EGFR over expression has been reported in many cancers, and in HCC. For example, Buckley *et al*^[53] reported that EGFR, detected by immunohistochemical analysis, was overexpressed in 50 (66%) of 76 HCCs, and that fluorescence *in situ* hybridization showed extra EGFR gene copies in 17 (45%) of 38 HCCs.

EGFR-targeting drugs include anti-EGFR antibodies, such as cetuximab and panitumumab, and small-molecule inhibitors of EGFR tyrosine kinases, such as gefitinib *etc.*, and have been used widely for the treatment of several cancers other than HCC. Unfortunately, except for phase II trial data, there are little clinical data on the efficacy of these drugs for the treatment of HCC.

Similar to gefitinib (Iressa[®]), erlotinib (Tarceva[®]) is an oral EGFR tyrosine kinase inhibitor. Philip *et al*^[54] and Yau *et al*^[55] have reported the results of phase II studies of erlotinib in HCC; the median OSs in their studies were 13 and 10.7 mo, respectively. A phase III clinical study (SEARCH study: Sorafenib and Erlotinib, a Randomized Trial Protocol for the Treatment of Patients with Hepatocellular Carcinoma) for sorafenib in combination with erlotinib *vs* sorafenib plus placebo is ongoing. Since erlotinib is associated with a high incidence of skin rash, dry skin and gastrointestinal toxicity, such as diarrhea, the results of the SEARCH study should be evaluated to assess whether this combination therapy can be used in clinical settings. Thomas *et al*^[56] conducted a phase II clinical study of erlotinib in combination with bevacizumab in 40 advanced HCC patients, and reported promising results; the median PFS and OS were 9 mo and 15.7 mo, respectively. However, they noted frequent treatment-related grade 3/4 toxicities, including fatigue

(20%), hypertension (15%), gastrointestinal bleeding (12.5%), wound infection (5%), diarrhea (10%), elevated transaminase levels (10%), and thrombocytopenia (10%), which necessitates further evaluation for drug tolerance. Although a clinical study of erlotinib in combination with bevacizumab (OPTIMOX-3 study) was also conducted in colorectal cancer patients, no tolerance was observed, which led to a change in the protocol^[57,58].

After the introduction of a number of molecular-targeted drugs, strategies for the inhibition of similar or different signaling pathways (vertical or horizontal inhibition) with several drugs have been proposed. However, the combined use of molecular-targeted agents has remained largely unsuccessful, including panitumumab in combination with bevacizumab for the treatment of colorectal cancer^[59]. Similarly, the results of sorafenib in combination with bevacizumab (vertical inhibition) have been reported^[60]. Although some therapeutic responses were obtained, the combination therapy resulted in greater toxicity^[60], suggesting the need for detailed evaluation of the dosing regimen.

Lapatinib (Tykerb[®]) is a dual inhibitor of EGFR and HER-2/neu, and inhibits tumor growth by downregulating MAPK, AKT, and p70S6 kinase^[61]. In Japan, lapatinib is indicated for the treatment of breast cancer. In a phase II clinical trial of lapatinib in 26 patients with unresectable advanced HCC, the median PFS and OS were 1.9 mo and 12.6 mo, respectively, and adverse events included diarrhea (73%), nausea (54%), and skin rash (42%)^[62].

Cetuximab (Erbix[®]) is a human/mouse chimeric monoclonal antibody consisting of the variable region of a mouse anti-human EGFR monoclonal antibody and the human immunoglobulin G1 constant region. Cetuximab inhibits the binding of endogenous EGFR ligands, such as EGF and TGF α , to EGFR. In a phase II clinical trial of cetuximab in 30 patients with unresectable or metastatic HCC, the median PFS and OS were 1.4 mo and 9.6 mo, respectively, and treatment-related toxicities included grade 3 hypomagnesemia (3.3%) and grade 1/2 acne-like rash (83.3%), which was observed for the duration of anti-EGFR therapy in that study^[63].

The EGFR offers a very interesting therapeutic target. As described above, the use of erlotinib in combination with sorafenib is still in the research stage. However, based on the results of phase II studies, the efficacy of cetuximab or lapatinib as a monotherapy seems to be limited, and the results of further studies evaluating their efficacy in sorafenib-refractory or -intolerant patients are awaited with interest.

Hepatocyte growth factor/c-Met pathway

Since the hepatocyte growth factor (HGF)/Met pathway is involved in tumor growth, invasion, and angiogenesis in a wide range of neoplasms, HGF and Met have recently attracted attention as therapeutic targets. HGF is a heterodimer consisting of α and β chains bound together by a disulfate bond. The α -chain contains four

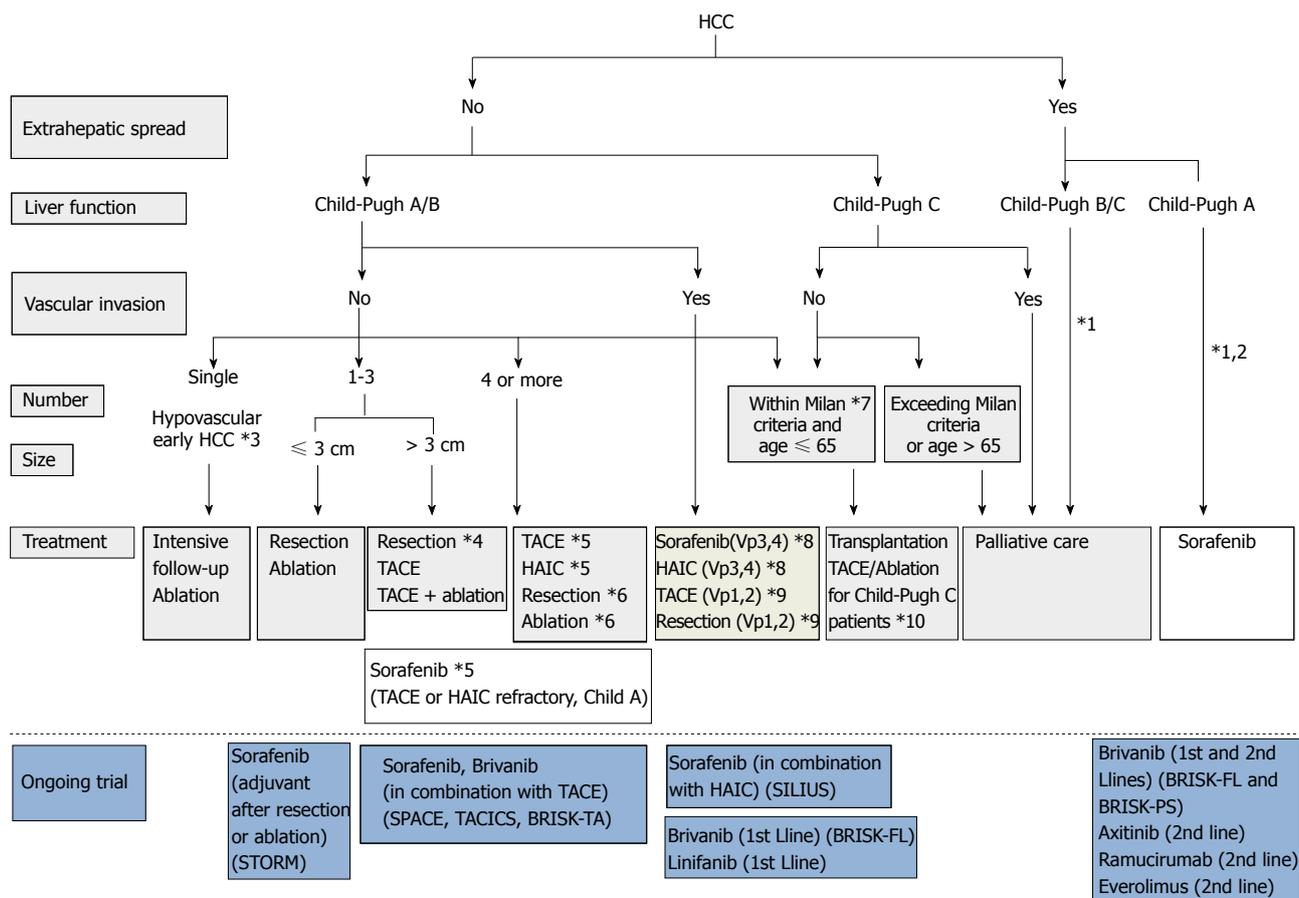


Figure 7 Consensus-based treatment algorithm for hepatocellular carcinoma proposed by Japan Society of Hepatology, revised in 2010. (Cited and modified from Kudo et al.^[67] with permission.) *1: Treatment should be performed as if extrahepatic spread is negative, when extrahepatic spread is not considered as a prognostic factor in Child-Pugh class A/B patients; *2: Sorafenib is the first choice of treatment in this setting as a standard of care; *3: Intensive follow-up observation is recommended for hypovascular nodules by the Japanese Evidence-Based Clinical Practice Guidelines. However, local ablation therapy is frequently performed in the following cases: (1) when the nodule is diagnosed pathologically as early hepatocellular carcinoma (HCC); (2) when the nodules show decreased uptake on Gd-EOB-MRI, or (3) when the nodules show decreased portal flow by CTAP, since these nodules frequently progress to advanced HCC; *4: Even for HCC nodules exceeding 3 cm in diameter, transcatheter arterial chemoembolization (TACE) in combination with ablation is frequently performed when resection is not indicated; *5: TACE is the first choice of treatment in this setting. Hepatic arterial infusion chemotherapy (HAIC) using an implanted port is also recommended for TACE-refractory patients. The regimen for this treatment is usually low-dose FP [5-fluorouracil (5-FU) + CDDP] or intra-arterial 5-FU infusion combined with systemic interferon therapy. Sorafenib is also recommended for TACE or HAIC-refractory patients with Child-Pugh class A liver function; *6: Resection is sometimes performed when more than 4 nodules are detected. Ablation is sometimes performed in combination with TACE; *7: Milan criteria: Tumor size ≤ 3 cm and tumor number ≤ 3, or solitary tumor ≤ 5 cm. Even when liver function is good (Child-Pugh A/B), transplantation is sometimes considered for frequently recurring HCC patients; *8: Sorafenib and HAIC are recommended for HCC patients with major portal invasion such as Vp3 (portal invasion in the 1st portal branch) or Vp4 (portal invasion in the main portal branch); *9: Resection and TACE are frequently performed when portal invasion is minor, such as Vp1 (portal invasion in the 3rd or more peripheral portal branch) or Vp2 (portal invasion in the 2nd portal branch); *10: Local ablation therapy or subsegmental TACE is performed even for Child-Pugh C patients when transplantation is not indicated when there is no hepatic encephalopathy, no uncontrollable ascites, and a low bilirubin level (< 3.0 mg/dL). However, it is regarded as an experimental treatment because there is no evidence of a survival benefit in Child-Pugh C patients. A prospective study is necessary to clarify this issue.

kringle domains, and the β -chain contains a serine protease-like domain. Met is a receptor tyrosine kinase for the HGF ligand, and contains a semaphorin-like domain. HGF or Met overexpression, and *Met* gene mutations and duplications, have been reported in various cancers, and abnormalities due to HGF/Met pathway activation have also been noted^[64]. These abnormalities activate the downstream signaling cascade, leading to epithelial-mesenchymal transition and increased proliferative, migratory, invasive, and metastatic potentials of cancer cells^[64].

HGF/c-MET-targeted drugs, including kinase inhibitors, HGF inhibitors and decoy c-Met receptor molecules are being developed. Of particular interest is ARQ-197, a

c-Met receptor tyrosine kinase inhibitor, which is a non-ATP-competitive molecule that binds near the ATP-binding site. A randomized phase II study of ARQ-197 vs placebo is ongoing in patients with unresectable HCC after systemic therapy failure. In addition, the results of a phase I study of ARQ-197 in combination with sorafenib was reported in ASCO 2010 (Abstract 3024).

IGF/IGFR

The IGF/IGFR system is involved in cell growth and the chemotherapeutic response. The ligands IGF- I and - II bind to their receptors IGF-1R and IGF-2R, and are involved in DNA synthesis and cell growth. Abnormali-

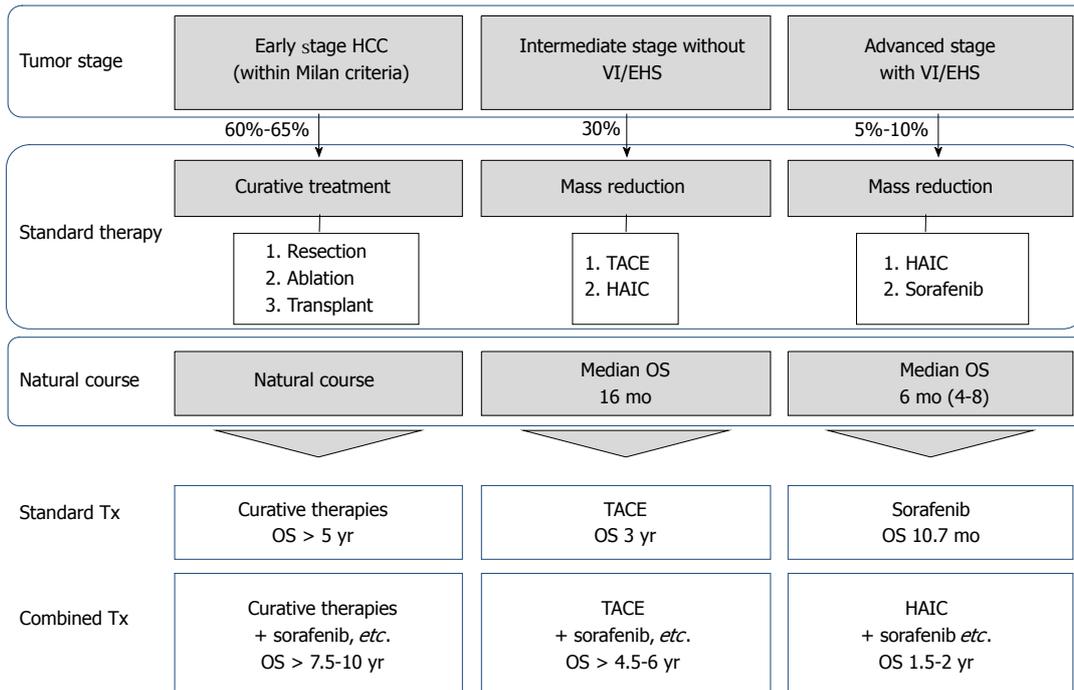


Figure 8 Outcomes of standard treatment modalities and expected future outcomes of combination therapy with molecular-targeted agents. By combining molecular targeted agents with resection or ablation, life expectancy [overall survival (OS)] is expected to be prolonged to 7.5-10 years. In addition, for intermediate stage hepatocellular carcinoma (HCC), the prognosis is expected to be improved to 4.5-6 years by combination with transarterial chemoembolization (TACE). For advanced stage HCC, the prognosis is expected to be improved to 1.5-2 years by combination with hepatic arterial infusion chemotherapy (HAIC).

Table 3 Subanalysis data of the SHARP study

	Advanced HCC with vascular invasion and/or extrahepatic spread	Advanced HCC without vascular invasion and/or extrahepatic spread
Hazard ratio	0.77 (95% CI: 0.60-0.99)	0.52 (95% CI: 0.32-0.85)
Median OS (MST) (mo)		
Sorafenib	8.9 (n = 209) (95% CI: 7.6-10.3)	14.5 (n = 90) (95% CI: 14.0-N/E)
Placebo	6.7 (n = 212) (95% CI: 5.2-8.0)	10.2 (n = 91) (95% CI: 8.6-15.5)

HCC: Hepatocellular carcinoma; OS: Overall survival; MST: Median survival time.

ties in IGF and IGF-1R, or their overexpression, have been reported in various cancers, including HCC. Their associations with disease stage, metastasis, survival^[65], and the functions of IGF and IGFR in HCC^[66] have been reported.

IGF-targeting drugs are currently being developed, and are mainly anti-IGF-1R antibodies, such as BII B022, AVE1642, and cixutumumab (IMC-A12). A phase II study of cixutumumab, a phase I b/II study of sorafenib vs sorafenib plus BII B022, and phase I/II studies of AVE1642 as monotherapy or in combination with sorafenib or erlotinib are ongoing.

COMBINATION THERAPY OF STANDARD TREATMENT WITH SORAFENIB

In addition to the pharmaceutical-sponsored clinical trials of lenvatinib and brigatinib as first- and second-line therapy in sorafenib-refractory patients, investigator initiated trials (IIT) of sorafenib in combination with hepatic arte-

rial infusion chemotherapy (SILIUS trial: trial number NCT01214343), pharmaceutical and IIT trials of sorafenib in combination with TACE [SPACE, TACICS (trial number: NCT 01217034) and BRISK-TA trials], and a trial to test the inhibitory effect of sorafenib on tumor recurrence after curative treatment (STORM trial) are ongoing. The results of these trials are eagerly awaited (Figure 7)^[67,68].

The working hypotheses in these studies can be deduced by extrapolating the median survival time (MST) and hazard ratios in overall survival (OS) calculated in a subanalysis of the SHARP study (Table 3). The results obtained suggest that starting treatment with molecular-targeted drugs at an earlier tumor stage in combination with standard treatment options such as resection, ablation, TACE, or hepatic arterial infusion chemotherapy can improve the prognosis of HCC. Thus, sorafenib has the potential to induce a paradigm shift in the treatment of HCC. For example, in a subanalysis of the SHARP trial, the hazard ratios for OS and MST ratio in intermediate stage HCC without vascular invasion or extrahepatic spread were 0.52 and 1.50, respectively (Table 3). This

suggests that survival of early stage HCC and intermediate stage HCC may be prolonged from 5 years to 7.5-10 years by using sorafenib in an adjuvant setting after curative treatment, and from 3 years to 4.5-6 years by using sorafenib in combination with TACE (Figure 8)^[68].

CONCLUSION

Several clinical trials^[39,40,49,54,63,69-74] of the molecular-targeted agents are ongoing. Angiogenesis-inhibiting drugs, particularly sorafenib, have been established for HCC, and drugs targeting several molecules are being developed.

Although sorafenib was recently approved, many issues remain to be addressed, including: (1) how to determine and define refractoriness; and (2) whether to continue TACE or hepatic arterial infusion chemotherapy (a de facto standard in Japan) in patients with TACE-refractory HCCs or portal tumor thrombi before starting sorafenib therapy. We strongly recommend that, based on the molecular-targeted agents currently under development, clinical studies (including IITs) should be conducted aggressively, and therapeutic strategies should be devised to resolve the limitations of currently used therapeutic approaches and to improve the therapeutic outcomes.

The introduction of sorafenib to treat HCC in 2007 in Western countries and in 2009 in Japan was undoubtedly the real beginning of a paradigm shift of HCC treatment, representing a significant breakthrough for HCC treatment not previously experienced for this unique tumor. Further development of survival benefit in HCC patients with new targeted agents are greatly expected.

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PNPLA3, the triacylglycerol synthesis/hydrolysis/storage dilemma, and nonalcoholic fatty liver disease

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Abstract

Genome-wide and candidate gene association studies have identified several variants that predispose individuals to developing nonalcoholic fatty liver disease (NAFLD). However, the gene that has been consistently involved in the genetic susceptibility of NAFLD in humans is patatin-like phospholipase domain containing 3 (*PNPLA3*, also known as adiponutrin). A nonsynonymous single nucleotide polymorphism in *PNPLA3* (rs738409 C/G, a coding variant that encodes an amino acid substitution I148M) is significantly associated with fatty liver and histological disease severity, not only in

adults but also in children. Nevertheless, how *PNPLA3* influences the biology of fatty liver disease is still an open question. A recent article describes new aspects about *PNPLA3* gene/protein function and suggests that the I148M variant promotes hepatic lipid synthesis due to a gain of function. We revise here the published data about the role of the I148M variant in lipogenesis/lipolysis, and suggest putative areas of future research. For instance we explored in silico whether the rs738409 C or G alleles have the ability to modify miRNA binding sites and miRNA gene regulation, and we found that prediction of *PNPLA3* target miRNAs shows two miRNAs potentially interacting in the 3' UTR region (hsa-miR-769-3p and hsa-miR-516a-3p). In addition, interesting unanswered questions remain to be explored. For example, *PNPLA3* lies between two CCCTC-binding factor-bound sites that could be tested for insulator activity, and an intronic histone 3 lysine 4 trimethylation peak predicts an enhancer element, corroborated by the DNase I hypersensitivity site peak. Finally, an interaction between *PNPLA3* and glycerol-3-phosphate acyltransferase 2 is suggested by data miming.

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Key words: Adiponutrin; Nonalcoholic fatty liver disease; miRNA; Glycerol-3-phosphate acyltransferase 2; Systems biology; Rs738409; Epigenetics

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INVITED COMMENTARY ON HOT ARTICLES

We have read with great interest the recent article by Kumari *et al.*^[1] describing new aspects about patatin-like phospholipase domain containing 3 (*PNPLA3*, also known as adiponutrin) gene function. Notably, the authors reported that human and murine *PNPLA3* exhibit increased lysophosphatidic acid acyltransferase (LPAAT) activity leading to increased cellular lipid accumulation. Kumari *et al.*^[1] concluded that the I148M substitution promotes hepatic lipid synthesis due to a gain of function, and that this property provides a plausible biochemical mechanism for the development of liver steatosis in subjects carrying the 148M variant (rs738409, allele G) as discussed below.

These results give new answers to the previous conflicting data around the question of whether the rs738409 is associated with a gain or loss of gene/protein function (Figure 1). For instance, several functional studies were elegantly performed to decipher whether the I148M substitution interferes with hepatic triglyceride (TG) hydrolysis and thus promoting hepatic steatosis^[2]. Based on an *in vitro* structural model of the patatin-like domain of PNPLA3 protein, He *et al.*^[2] showed that the 148M variant interferes with TG hydrolysis, affecting the association of PNPLA3 with the lipid droplets. Thus, the resulting mutant enzyme is inactive against its substrates. In addition, Huang *et al.*^[3] recently demonstrated that PNPLA3 plays a role in the hydrolysis of glycerolipids, and the I148M substitution causes a loss of function.

Several animal studies were conducted to evaluate the effect of the PNPLA3 loss of function by using knockout models, for instance *PNPLA3*^{-/-} mice by gene targeting^[4]. Surprisingly, loss of PNPLA3 does not cause fatty liver, liver enzyme elevation, or insulin resistance in mice under a standard or a high-sucrose/high-fat diet^[4]; a finding replicated by global targeted deletion of the *PNPLA3* gene^[5].

Interestingly, a human study demonstrated that the rs738409 PNPLA3 G allele is associated with morphological changes in adipocyte cell size^[6], and this concept strongly supports a role of the variants in the liver fat remodeling; unfortunately, these findings were not replicated.

Finally, Kumari *et al.*^[1] confirmed that PNPLA3 functions as a TG hydrolase in mice and humans but found that the specific TG hydrolase activity is much lower than that of adipose triglyceride lipase (ATGL).

Can a loss or gain of function in a single protein explain such a strong effect in the biology of nonalcoholic fatty liver disease?

The question as to whether the role of the rs738409 in the pathobiology of nonalcoholic fatty liver disease (NAFLD) is associated with a gain or loss of function is hard to answer because the biological function attributed to the PNPLA3 in lipid-related pathways is shared with other related proteins. For example, almost all members of the PNPLA family have established TG hydrolases and phospholipases activities, and PNPLA3 shares, as expected, protein domains with the PNPLA family (PNPLA1, PNPLA2, and PNPLA4-8), and also with

PLA2G6 (phospholipase A2, group VI cytosolic, calcium-independent, also known as PNPLA9). *In silico* prediction of PNPLA3 protein function by imputation of functional association data^[7] shows that only PNPLA2, PNPLA3, PNPLA8, and PLA2G6 have carboxylesterase and lipase activities ($P < 4.8$ and 9.4×10^{-5}) and PNPLA3, PNPLA8, and PLA2G6 have phospholipase A2 activity ($P < 1.6 \times 10^{-4}$). As we mentioned recently, these similarities with enzymes with the potential of releasing arachidonic acid as the precursor of potent inflammatory substances such as prostaglandins may have therapeutic implications^[8].

In addition, neutral lipid, triglyceride, or glycerolipid catabolic processes are shared by only PNPLA2 and PNPLA3 ($P < 0.05$). This finding is also consistent with the notion that PNPLA3 plays a role in the hydrolysis of glycerolipids as shown by Huang *et al.*^[3].

Furthermore, *in silico* prediction of the protein network related to PNPLA3 is beyond the PNPLA family (Figure 2), suggesting that some other interesting and still unexplored issue about the putative role of the rs738409 in liver steatosis are waiting for answers. For instance, the putative interaction between PNPLA3 and genes in the related networks, particularly glycerol-3-phosphate acyltransferase 2 (GPAT2, mitochondrial), whose protein esterifies acyl-group from acyl-ACP to the sn-1 position of glycerol-3-phosphate, an essential step in glycerolipid biosynthesis^[9]. We wonder to what extent the PNPLA3 and GPAT2 interaction modulates hepatic fat content. We used a data integration and data mining platform to explore the putative interaction between PNPLA3 and GPAT2, and as shown in Figure 3, PNPLA3 and GPAT2 are involved in shared metabolic pathways, including triglyceride biosynthetic process, glycerolipid metabolism pathways, and acyltransferase activity, which may explain an interaction in the pathogenesis of NAFLD. This prediction is biologically plausible as GPAT2 catalyzes the initial and rate-limiting step in glycerolipid synthesis, and overexpression and knock-out studies suggest that GPAT isoforms can play important roles in the development of hepatic steatosis, insulin resistance, and obesity^[10].

Impact of rs738409 on genetic risk of NAFLD

The first description about the association between a nonsynonymous single nucleotide polymorphism (SNP) of PNPLA3, the rs738409 C/G, (a coding variant that encodes an amino acid substitution I 148M as described) and fatty liver was reported by Romeo *et al.*^[11] by performing a genome-wide association study (GWAS) of nonsynonymous sequence variations ($n = 9229$) in a large multiethnic population-based epidemiological study of fatty liver. This initial finding was further replicated in several populations confirming that the G allele in the forward strand is significantly associated with increased risk of hepatic triglyceride accumulation and fatty liver disease, as reviewed recently in a meta-analysis^[12]. Likewise, the rs738409 variant was associated with fatty liver in pediatric patients with NAFLD^[13-16], and patients with NAFLD related comorbidity, such as type 2 diabetes^[17-19] or morbid

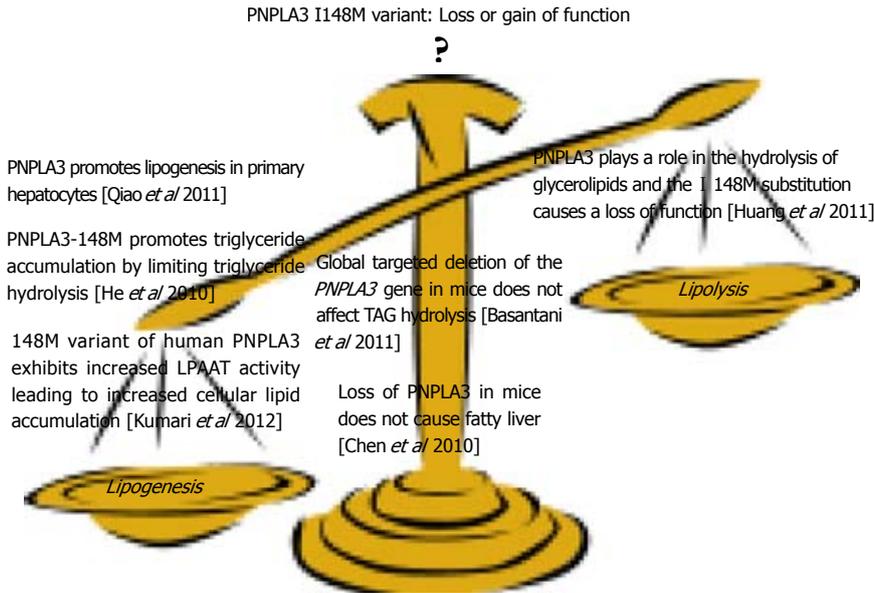


Figure 1 Patatin-like phospholipase domain containing 3 rs738409 and its role in nonalcoholic fatty liver disease. Summary of the evidence from human and rodent studies. PNPLA3: Patatin-like phospholipase domain containing 3.

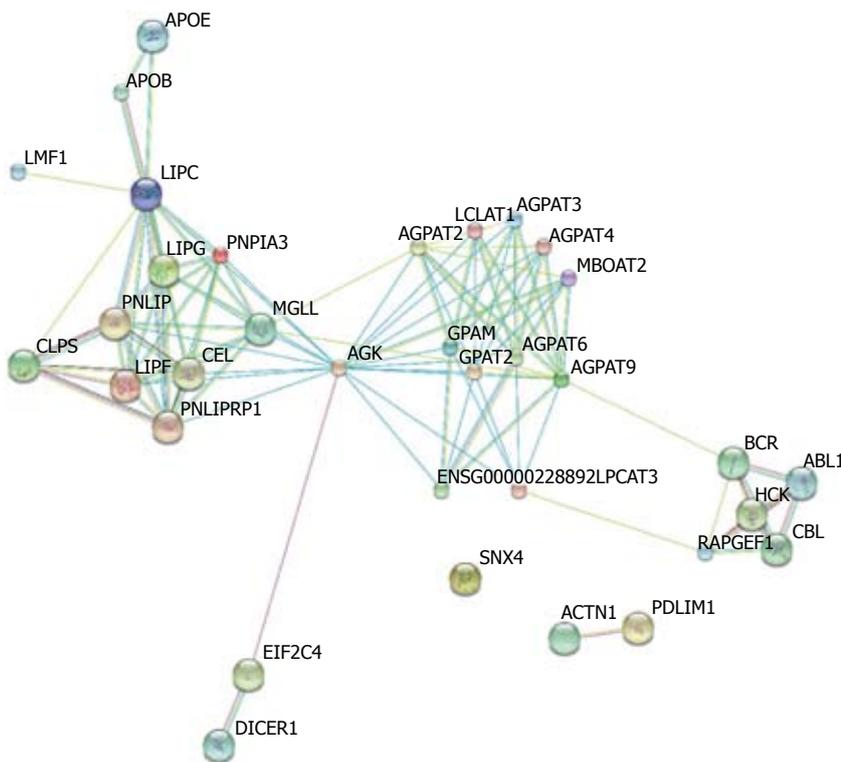


Figure 2 Protein network around patatin-like phospholipase domain containing 3 by the Search Tool for the Retrieval of Interacting Genes/Proteins resource (<http://string-db.org/>). The interactions include direct (physical) and indirect (functional) associations; they are derived from four sources: Genomic Context, High-throughput Experiments, Conserved-Coexpression and Previous Knowledge. PNPLA3: Patatin-like phospholipase domain containing 3; LIPF: Lipase, gastric; SNX4: Sorting nexin 4; AGPAT9: 1-acylglycerol-3-phosphate O-acyltransferase 9; MGLL: Monoglyceride lipase; GPAM: Glycerol-3-phosphate acyltransferase, mitochondrial; AGPAT3: 1-acylglycerol-3-phosphate O-acyltransferase 3; LIPC: Lipase, hepatic; MBOAT2: Membrane-bound O-acyltransferase domain containing 2; LCLAT1: Lysocardiolipin acyltransferase 1; AGPAT4: 1-acylglycerol-3-phosphate O-acyltransferase 4; LPCAT3: Lysophosphatidylcholine acyltransferase 3; AGK: Acylglycerol kinase; PNLIPRP1: Pancreatic lipase-related protein 1; GPAT2: Glycerol-3-phosphate acyltransferase 2, mitochondrial; PNLIP: Pancreatic lipase; PDLIM1: PDZ and LIM domain 1; AGPAT2: 1-acylglycerol-3-phosphate O-acyltransferase 2; CEL: Carboxyl ester lipase; EIF2C4: Eukaryotic translation initiation factor 2C, 4; HCK: Hemopoietic cell kinase; AGPAT6: 1-acylglycerol-3-phosphate O-acyltransferase 6; ENSG00000228892: 1-acyl-sn-glycerol-3-phosphate acyltransferase.

obesity^[15], although it remained to be explored whether these associations are truly independent of NAFLD^[12].

Interestingly, we demonstrated for the first time^[20] that the rs738409 was also significantly associated with histological disease severity; a finding that was replicated by others^[21-23]. In fact, the G allele significantly increases the risk of progressive liver disease (odds ratio 1.88 per G allele; 95% CI: 1.03-3.43; $P < 0.04$)^[20,12].

Overall, the most remarkable conclusion about the impact of the genetic variation in PNPLA3 on NAFLD-related outcomes is the strong effect that the rs738409 has on the susceptibility of fatty liver, because the pro-

portion of the total variation attributed to the SNP genotypes is about 5.3%^[20]. This effect is perhaps one of the strongest ever reported for a common variant modifying the genetic susceptibility for a complex disease.

Furthermore, the rs738409 variant not only modifies the biology of NAFLD but also has a considerable impact on the genetic susceptibility to alcoholic liver disease^[24-26], and hepatitis-C-virus-induced fatty liver^[23], and is a strong predictor of hepatocellular carcinoma occurrence among patients with cirrhosis^[27,28], indicating that these diverse liver diseases may share common physiological pathways.

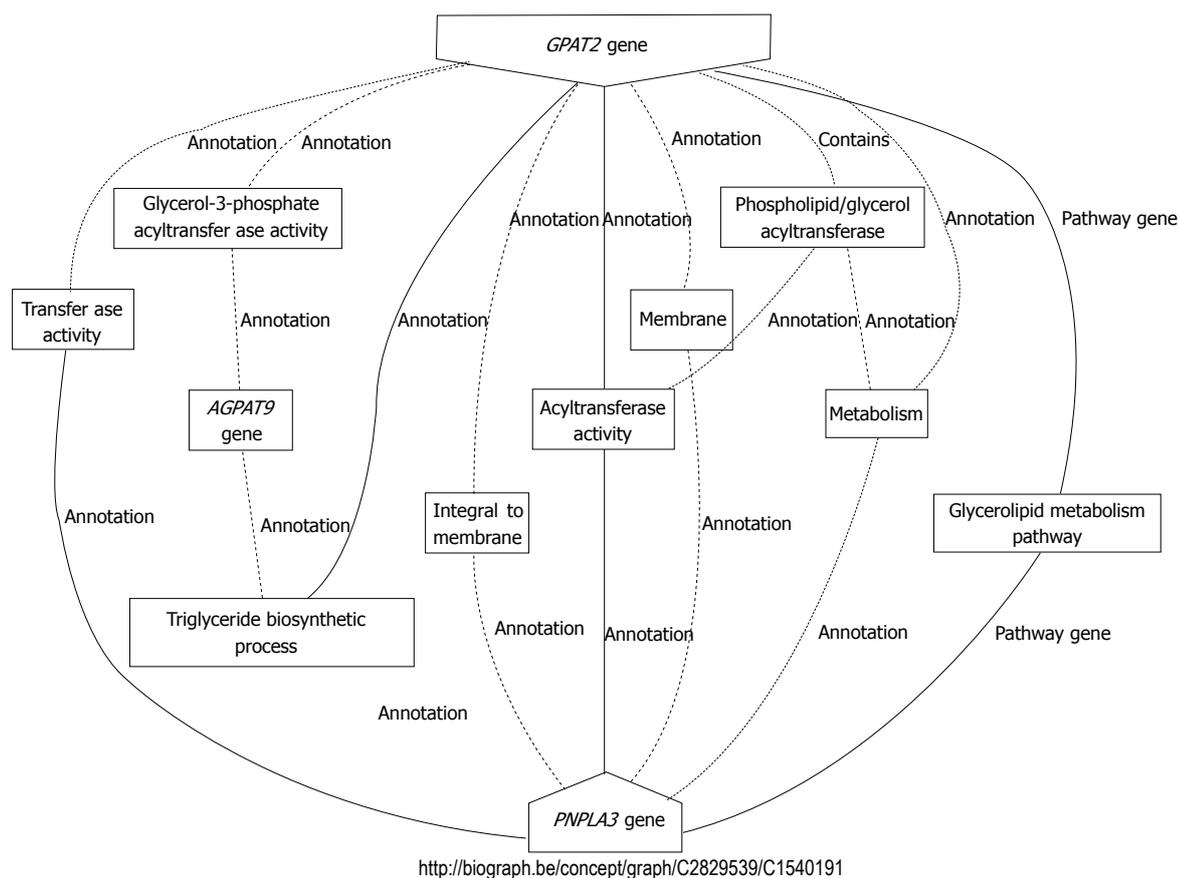


Figure 3 Interaction between patatin-like phospholipase domain containing 3 and glycerol-3-phosphate acyltransferase 2. Prediction was performed by the tool BioGraph (<http://biograph.be/about/welcome>), a data mining framework that allows for the automated formulation of comprehensible functional hypotheses relating a context to targets. *PNPLA3*: Patatin-like phospholipase domain containing 3; *GPAT2*: Glycerol-3-phosphate acyltransferase 2; *AGPAT9*: Acylglycerol-3-phosphate acyltransferase isoform.

***PNPLA3*: Short summary about gene structure and variation**

PNPLA3 gene is located in chromosome 22 (22q13.31) and has nine exons; its transcript length is 2805 bp and it is translated to a protein of 481 amino acids. There are 34 SNPs with reported frequency and heterozygosity information in the HapMap (www.hapmap.org). Nevertheless, among all known *PNPLA3* variants in coding or noncoding regions, there are three SNPs that have shown association with NAFLD-related phenotypes, including the rs738409. One is the rs6006460 (S453I) that after gene resequencing is associated with lower hepatic fat content in African Americans^[11]. The other SNP is the nonsynonymous rs2294918 (Lys434Glu) that is significantly associated with serum alanine aminotransferase levels^[20]. Haplotype analysis of *PNPLA3* shows that the rs738409 is in moderate linkage disequilibrium (LD) (r^2 : 0.65) with the other variants, including rs2294918 (Figure 4). Thus, this scenario precludes any imputation across the *PNPLA3* locus centered on rs738409, and suggests that the I148M variant might be the casual variant in the susceptibility of fatty liver. However, Figure 5 shows annotation of nearby SNPs in LD (proxies) with rs738409 based on HapMap data and a nearby locus, such as SAMM50 that is a component of the sorting and assembly machinery (SAM) complex of the outer mitochondrial membrane. The SAM complex has a role in integrating β -barrel proteins into

the outer mitochondrial membrane, and makes SAMM50 a good candidate because mitochondrial dysfunction may play a major role in NAFLD pathogenesis, as suggested by experimental models^[30] and human studies^[31]; nevertheless, this issue deserves further investigation.

Another interesting feature to highlight is the population diversity of rs738409. Genome diversity data extracted from <http://www.ncbi.nlm.nih.gov/SNP/> shows that the risk allele G is highly prevalent around the world, with an average prevalence close to 0.70 from Caucasian to Yoruban populations. Negative selection of the ancestral allele that seems to be the C allele (<http://www.ensembl.org>), which is shared by chimpanzees, orangutans, macaques, and other species, suggests that environmental pressures have exerted a strong influence. However, this picture probably reflects a sort of confusion about the reference strand and probably these annotation data refer to the minus strand when the gene is located in the plus strand, and the real frequency of the risk allele G is < 50% in all populations. In fact, published data^[12] from rs738409 association studies show that the most prevalent allele is certainly the reference allele C.

***PNPLA3*: Mechanisms of control of gene and protein expression and unexplored areas of research**

PNPLA3 is a multifunctional enzyme that has both triac-

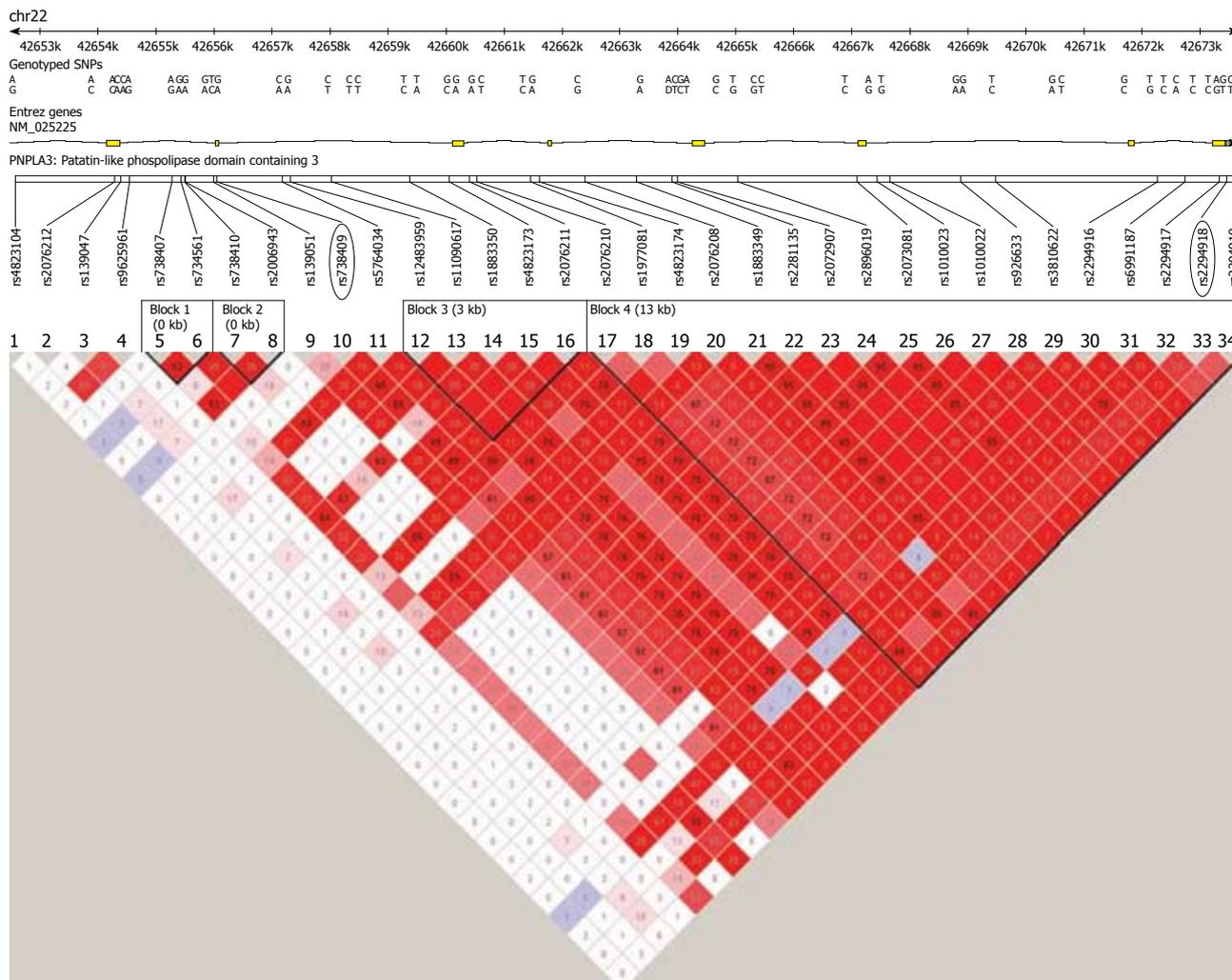


Figure 4 Linkage disequilibrium plot across the patatin-like phospholipase domain containing 3 gene. The horizontal white line depicts the DNA segment of chromosome 22q13.31. The rs738409 location is highlighted in a black ellipse. An LD plot is depicted in the bottom part of the figure: each diamond represents the magnitude of LD for a single pair of markers, with colors indicating strong LD (red $r^2 = 1.0$) and no LD (white, $r^2 = 0$) as the extremes (different red tones indicate intermediate LD). Numbers inside the diamonds stand for r^2 values. Haplotype analysis of PNPLA3 shows that the rs738409 is in moderate LD (no more than $r^2: 0.65$) with other variants. Plot was obtained by the software Haploview 2.0 freely available at www.broad.mit.edu/mpg/haploview/. LD: Linkage disequilibrium; PNPLA3: Patatin-like phospholipase domain containing 3.

Table 1 Patatin-like phospholipase domain containing 3 protein features and functions

PNPLA3 belongs to the IPLA2/lipase family. The protein encoded by *PNPLA3* is a triacylglycerol lipase that mediates triacylglycerol hydrolysis in adipocytes. Multifunctional enzyme that has both triacylglycerol lipase and acylglycerol O-acyltransferase activity (<http://genatlas.medecine.univ-paris5.fr/>) Lipid hydrolase with an unusual folding topology that differs from classical lipases^[38]

The enzyme is highly regulated by changes in energy balance: nutritional control of *PNPLA3* being affected by a feed-forward loop^[36]

PNPLA3 mRNA increases during differentiation of rat adipocytes in an insulin-dependent manner^[39]

PNPLA3 mRNA expression is upregulated by tri-iodothyronine in adipocytes *in vitro*, in humans and rats, and *in vivo* in rat WAT^[39]

PNPLA3 mRNA expression is upregulated by tri-iodothyronine in adipocytes *in vitro*, in humans and rats, and *in vivo* in rat white adipose tissue^[40]

Promoter region of the human adipoonutrin/*PNPLA3* gene is regulated by glucose and insulin^[33]

Fasting significantly downregulates *PNPLA3* mRNA expression in liver and adipose tissue^[35]

Feeding significantly upregulates *PNPLA3* mRNA in liver and fat^[34]

Liver *PNPLA3* mRNA is expressed in human liver in higher levels compared with adipose tissue^[36]

PNPLA3 mRNA is highly expressed in liver of b/ob mice^[40] and visceral and subcutaneous adipose issue in obese humans^[41]

PNPLA3 mRNA is expressed in hepatocytes but not in Kupffer cells^[35,36]

PNPLA3 is expressed in hepatocytes but not in liver endothelial and Kupffer cells; microarray-based gene profiling showed that the expression level of *PNPLA3* in hepatocytes is correlated with that of genes associated with the lipogenic pathway such as ME1, SPOT14, and SCD1^[35]

PNPLA3 is regulated in human hepatocytes by glucose *via* ChREBP^[42]

SREBP1c is able to induce *PNPLA3* expression in human immortalized hepatocytes and in HepG2 hepatoma cells^[37]

PNPLA3: Patatin-like phospholipase domain containing 3; SREBP1c: Sterol regulatory element binding protein 1; ChREBP: Carbohydrate-responsive element-binding protein; ME1: Malic enzyme; SCD1: Stearoyl-CoA desaturase 1.

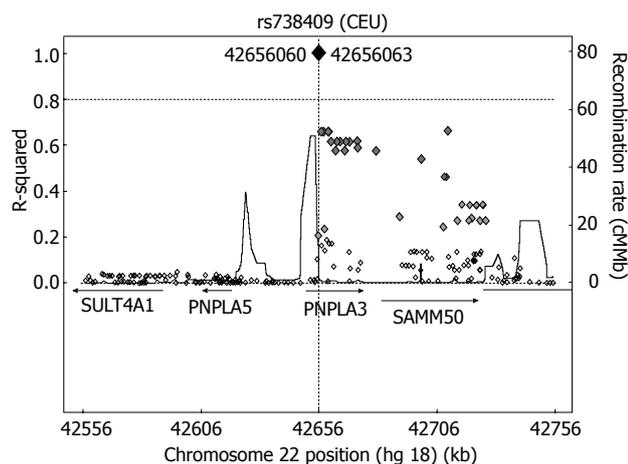


Figure 5 Regional Linkage disequilibrium plot for the rs738409 at chromosome 22 (22q13.31). The single nucleotide polymorphisms are plotted with their proxies (shown as diamonds) (based on HapMap data for CEU) as a function of genomic location, annotated by the recombination rate across the locus and nearby genes. The regional association plot was performed by the SNAP server, available at <http://www.broad.mit.edu/mpg/snap/>. SULT4A1: Sulfotransferase-4A1; PNPLA5: Patatin-like phospholipase domain containing 5; PNPLA3: Patatin-like phospholipase domain containing 3; SAMM50: Sorting and assembly machinery component 50 homolog.

ylglycerol lipase and acylglycerol O-acyltransferase activity^[32]; subfamily hierarchy of PNPLA3 protein is shown in Figure 6. A summary of PNPLA3 protein characteristics and mechanisms of gene regulation is shown in Table 1. A very interesting feature of PNPLA3 protein is that is induced by several nutritional factors, such as oleic acid, C18:2 fatty acid, palmitic acid, glucose, insulin and lactone. Moreover, it was shown that the promoter activity of PNPLA3 is upregulated by glucose concentrations in a dose-dependent manner^[33]. Adiponutrin was initially regarded as a dietary and obesity-linked protein^[34]. Furthermore, Hoekstra *et al.*^[35] suggested that PNPLA3 plays an important role in hepatic lipid metabolism under conditions of lipid excess. In the light of these findings, subsequent studies were conducted to evaluate how *PNPLA3* is transcriptionally regulated, and most of the experiments were focused to answer the question whether the lipogenic transcription factor sterol regulatory element binding protein 1 (SREBP-1) was orchestrating these events. Interestingly, Huang *et al.*^[36] demonstrated in mice that *PNPLA3* mRNA levels are regulated by SREBP-1c as they found a SREBP1c binding site mapped to intron 1 of *PNPLA3*. In particular, Huang *et al.*^[36] observed robust transcriptional upregulation of *PNPLA3* expression with carbohydrate feeding mediated by activation of liver X receptor/retinoic acid receptor (RXR) and increased levels of SREBP1c. In agreement with this finding, we observed in a high-fat-induced rat NAFLD model that liver transcript levels of RXR- α were significantly upregulated in fatty liver in comparison with normal liver (RXR- α mRNA/TATA box binding protein mRNA ratio: 11.71 ± 1.11 vs 6.48 ± 1.15 , respectively, $P = 0.008$), data adjusted by HOMA-IR (homeostatic model assessment-insulin resistance) using analysis of covariance, (unpublished data).

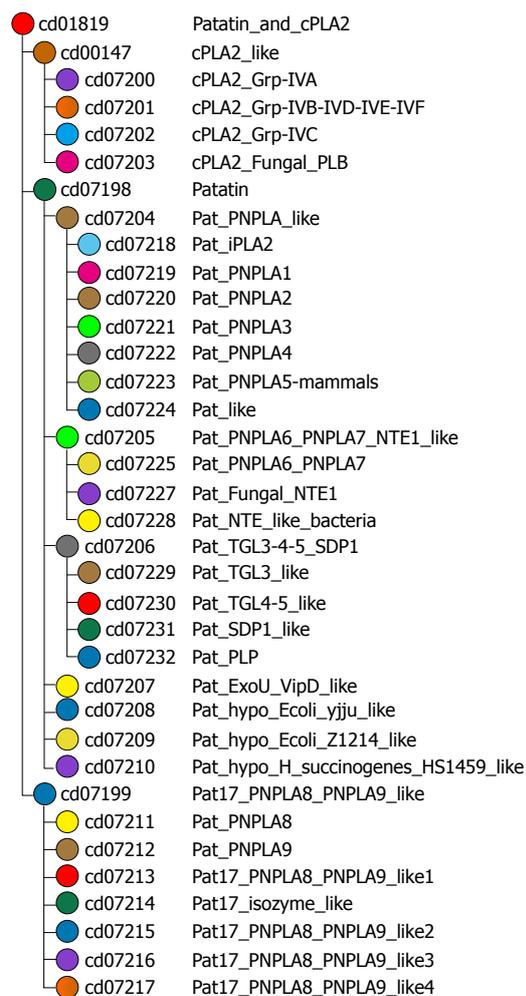


Figure 6 Domain family hierarchy of patatin-like phospholipase domain containing 3 protein. Data extracted from NCBI-curated domains at <http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>. This picture provides data about how patterns of residue conservation and divergence in a family relate to functional properties. In this particular case, picture shows the patatin-like phospholipase family. Patatin is a storage protein, but it also has the enzymatic activity of a lipid acyl hydrolase, catalyzing the cleavage of fatty acids from membrane lipids. Members of this family have also been found in vertebrates. This family also includes the catalytic domain of cytosolic phospholipase A2 (PLA2; EC 3.1.1.4) hydrolyzes the sn-2-acyl ester bond of phospholipids to release arachidonic acid. At the active site, cPLA2 contains a serine nucleophile through which the catalytic mechanism is initiated. PNPLA3: Patatin-like phospholipase domain containing 3; TGL: Triglyceride lipase; PLP: Pyridoxal phosphate.

In addition, *in vitro* data show that mouse but not human *PNPLA3* gene expression is under the transcriptional control of carbohydrate-responsive element-binding protein (ChREBP)^[37].

Unexplored mechanism of gene expression regulation

As shown in Figure 7, the promoter of *PNPLA3* gene has a typical chromatin structure [a peak of histone 3 lysine 4 trimethylation (H3K4me3) between the bimodal peaks of H3K4me1] and is DNase hypersensitive. The gene lies between two CCCTC-binding factor (CTCF)-bound sites that could be tested for insulator activity. An intronic H3K4me1 peak predicts an enhancer element, corroborated by the DNase I hypersensitivity site peak. The presence of a CpG island is typical of an active

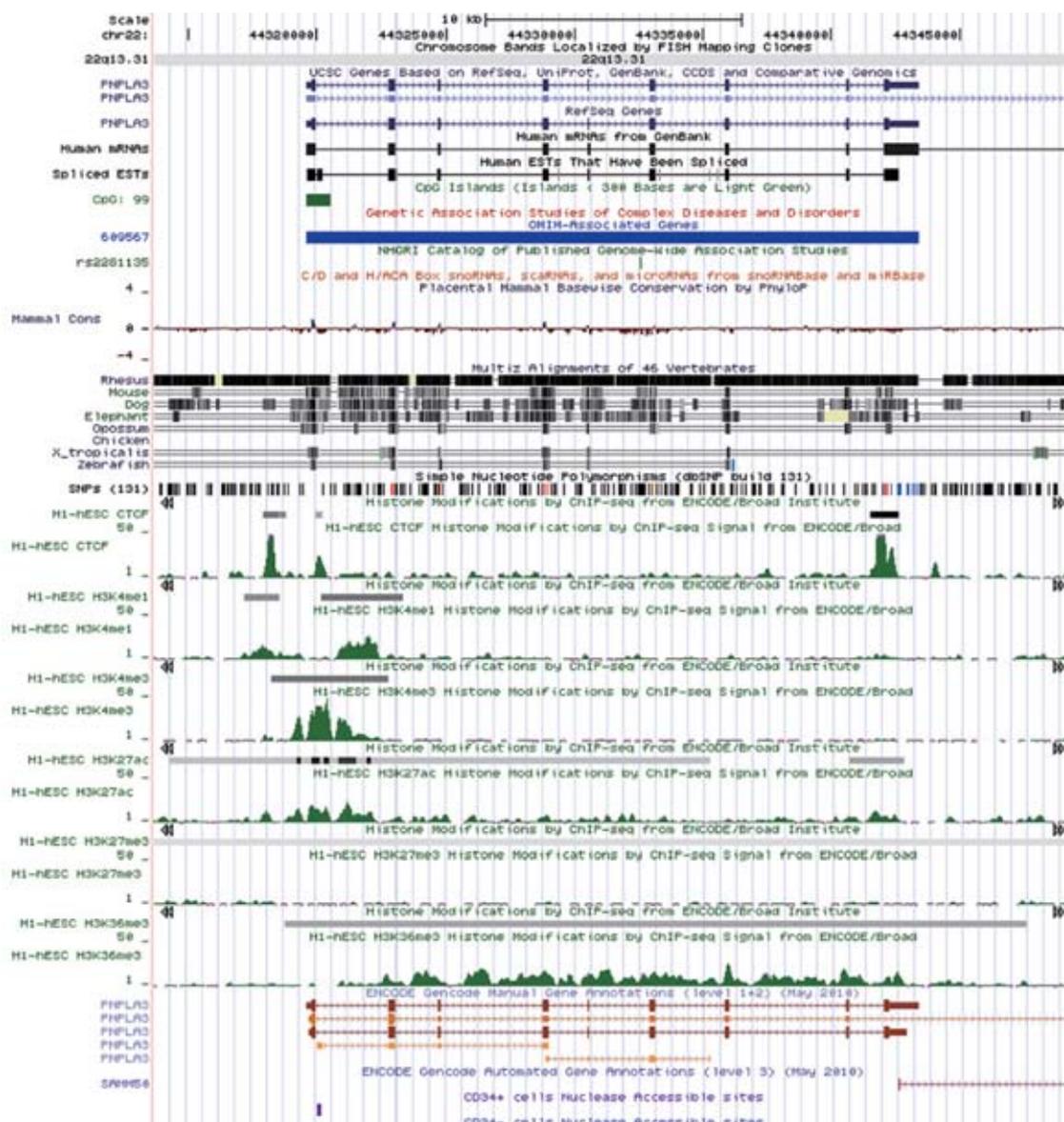


Figure 7 Prediction of patatin-like phospholipase domain containing 3 gene structure extracted from The University of California-Santa Cruz (UCSC) Genome Browser. <http://www.genome.ucsc.edu/>.

Table 2 *In silico* prediction of rs738409 C or G alleles as putative and differential target sites of microRNA

rs738409-C miRNA	rs738409-G miRNA
hsa-miR-1233	hsa-miR-1181, hsa-miR-1295, hsa-miR-1298, hsa-miR-133a, hsa-miR-133b
hsa-miR-1249	hsa-miR-135a, hsa-miR-136, hsa-miR-139-5p, hsa-miR-190b, hsa-miR-222
hsa-miR-129-5p	hsa-miR-297, hsa-miR-324-5p, hsa-miR-331-5p, hsa-miR-34c-5p, hsa-miR-362-5p
hsa-miR-155	hsa-miR-370, hsa-miR-370, hsa-miR-376b, hsa-miR-384, hsa-miR-412, hsa-miR-432
hsa-miR-365	hsa-miR-449a, hsa-miR-449b, hsa-miR-452, hsa-miR-455-5p, hsa-miR-509-3-5p
hsa-miR-433	hsa-miR-511, hsa-miR-513a-5p, hsa-miR-513b, hsa-miR-515-3p, hsa-miR-516a-3p
hsa-miR-498	hsa-miR-516b, hsa-miR-518d-5p, hsa-miR-519b-5p, hsa-miR-519c-5p, hsa-miR-519e
hsa-miR-517a	hsa-miR-526a, hsa-miR-550, hsa-miR-552, hsa-miR-554, hsa-miR-557, hsa-miR-579
hsa-miR-517c	hsa-miR-582-5p, hsa-miR-584, hsa-miR-593, hsa-miR-600, hsa-miR-601
hsa-miR-578	hsa-miR-609, hsa-miR-613, hsa-miR-623, hsa-miR-623, hsa-miR-623, hsa-miR-629
hsa-miR-586	hsa-miR-632, hsa-miR-636, hsa-miR-642, hsa-miR-642, hsa-miR-654-5p, hsa-miR-659
hsa-miR-874	hsa-miR-661, hsa-miR-663b, hsa-miR-664, hsa-miR-668, hsa-miR-760, hsa-miR-875-5p

Prediction was assessed by PITA miRNA prediction tool. Each allele was represented by at least 60 bp around the polymorphic base using a seed of 7 bp.

promoter. The coding sequence is well conserved across species and there is no known alternative splicing of the

Table 3 Results of search for miRNA target sites on the 3' untranslated region of the patatin-like phospholipase domain containing 3 gene by DIANA-microT v3.0 prediction tool

miRNA name	Binding category	UTR start	UTR stop	UTR binding NTs
hsa-miR-769-3p	8mer (pos 1)	89	117	GCC GAC U G GGAUCCAG
hsa-miR-769-3p	7mer (pos 2)	389	417	GAGA GAUCCA
hsa-miR-769-3p	7mer (pos 1)	699	727	G CAG GGC CGG AUCCAG
hsa-miR-769-3p	7mer (pos 1)	834	862	G CA CCUG AUCCAG
hsa-miR-516a-3p	8mer (pos 1)	128	156	UGAA AGGAAGCA
hsa-miR-516a-3p	7mer (pos 2)	216	244	GCU UUGGG AGGAAGC
hsa-miR-516a-3p	8mer (pos 2)	591	619	UG AAGGAAGC

mRNA. PNPLA3 is an OMIM-related gene (number 609567). These data were extracted from The University of California-Santa Cruz (UCSC) Genome Browser.

Concluding remarks and future research directions: Possible role for rs738409 alleles in modifying miRNA target sites

SNPs associated with polygenetic disorders, such as NAFLD, can destroy or create miRNA binding sites. We hypothesize that disruption of miRNA target binding by rs738409 alleles may play a role in the effect of the gene variant on fatty liver disease susceptibility. To test this, we analyzed *in silico* whether the rs738409 C or G alleles have the ability to modify miRNA binding sites and miRNA gene regulation by using the PITA microRNA prediction tool (http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html). We observed that rs738409 alleles show potentially different miRNA binding sites (Table 2), suggesting a putative different role in regulation of gene regulation; a hypothesis that has to be proven experimentally. Finally, although these data do not explain the association of the rs738409 variant with NAFLD, it is worth noting that prediction of PNPLA3 target miRNA by DIANA-microT v3.0 (<http://diana.cs-lab.ece.ntua.gr/microT/>) shows two miRNAs potentially interacting in the 3'UTR region (hsa-miR-769-3p and hsa-miR-516a-3p) (Table 3).

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Human endogenous retroviruses and cancer: Causality and therapeutic possibilities

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Abstract

A substantial part of the human genome is derived from transposable elements; remnants of ancient retroviral infections. Conservative estimates set the percentage of human endogenous retroviruses (HERVs) in the genome at 8%. For the most part, the interplay between mutations, epigenetic mechanisms and post-transcriptional regulations silence HERVs in somatic cells. We first highlight mechanisms by which activation of members of several HERV families may be associated with tumor development before discussing the arising chances for both diagnosis and therapy. It has been shown that at least in some cases, tumor cells expressing HERV open reading frames (ORFs) thus gain tumor-promoting functions. However, since these proteins are not expressed in healthy tissues, they become prime target structures. Of potential pharmacological interest are the prevention of HERV transposition, the inhibition of HERV-encoded protein expression and the interference with these proteins' activities. Evidence from recent studies unequivocally proves that HERV ORFs represent a very interesting source of

novel tumor-specific antigens with even the potential to surpass entity boundaries. The development of new tumor (immune-) therapies is a very active field and true tumor-specific targets are of outstanding interest since they minimize the risk of autoimmunity and could reduce side effects. Finally, we postulate on main future research streams in order to stimulate discussion on this hot topic.

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Key words: Human endogenous retroviruses; Gastrointestinal cancer; Therapeutic targets; Tumor-specific antigens; Tumorigenesis

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HUMAN ENDOGENOUS RETROVIRUSES: AN INTRODUCTION

Human endogenous retroviruses (HERVs) are remnants of ancient retroviral infections. Many insertions into the genome have taken place tens of millions of years ago^[1,2]. Since the first set of data on the human genome project has been published in 2001, it is well established that about 8% of the genome consists of HERVs and their total number is approximately 3×10^5 copies^[3]. Generally, HERVs are classified into three groups:

class I [gamma (like) retroviruses], class II [beta (like) retroviruses] and class III [spuma (like) retroviruses]^[11]. The most common nomenclature utilizes the single-letter amino acid code corresponding to the tRNA primer that is used for reverse transcription of the HERV genome^[14]. For the most part, their canonical structure of a single open reading frame (ORF) consists of the *gag*, *pol* and *env* genes flanked by 5' and 3' long terminal repeats (LTR)^[2,5]. The latter features are what endogenous and exogenous retroviruses have in common.

ENDOGENIZATION

Following a retroviral infection, the fate of the host depends on the pathogenicity of the infectious virus. Highly pathogenic ones will kill the host whereas ones with only weak pathogenicity may manage to infect many different cell types, including reproductive tissue cells. Subsequently, an endogenous retrovirus will establish if virus and host proceed to fixation of the virus' sequences in the host genome. This process has been termed endogenization or molecular domestication^[6]. In most cases, HERV activities have been silenced by a variety of mechanisms. Mutational inactivation includes deletions as well as point mutations and probably has been triggered by specific regulatory proteins such as APOBEC^[7,8]. Furthermore, epigenetic mechanisms including methylation and histone modification contributed to HERV inactivation^[9]. Besides directly silencing the expression, posttranscriptional regulation further protects the host's genome^[10]. It has been argued that the presence of such a high number of HERV copies must be advantageous for the host, too^[11]. An amazing idea suggests that over time of evolution, retroelements like HERVs actively contribute to the development of novel physiological capacities^[12,13]. It is for example easily imaginable that a *de novo* ORF which basically encodes a membrane protein may give rise to a protein with novel functions when mutated. If such a protein is beneficial to the host, it will be fixed. Thus, HERVs are together with other mobile genetic elements drivers of the (human) evolution by providing material for genomic evolution, variation and natural selection^[6,12,14]. This argumentation adds another level of complexity to the relationship of humans (and all other vertebrates) and retroviruses^[15]. Consequently, HERVs must not be considered as parasites but as true symbionts - on the population level. However, the individual risk for *de novo* insertions is rather low with estimated rates of only 1 in 100 births^[16].

PRESERVED FUNCTIONS

Although the vast majority of HERV sequences have been inactivated over time as outlined above, there are some examples of HERVs with potentially useful functional modules; comparable to the proviruses of their exogenous counterparts. Among the cellular functions

influenced by HERVs are enhancement and promotion of gene expression. In a study on primate evolution ERV-9 LTR sequences were found in higher primates and humans. In the latter, tissue specific enhancer activity could be detected in hematopoietic cells and even stronger in embryonic cells^[17]. HERV-E LTR functions as enhancer for endothelin B receptor and apolipoprotein C- I genes in humans^[18]. Furthermore, HERV sequences also give rise to novel or alternative splicing and polyadenylation sites^[19]. They also can be involved in membrane fusion, with Syncytin in the placenta being the most prominent representative here of^[20].

HERV AND CANCER

A variety of oncogenic mechanisms have been attributed to animal oncogenic retroviruses^[21,22]. Moreover, it has been suggested, that failures and errors in single somatic cells' efficiency to control HERV activity potentially results in genome damage and may thus contribute to the formation of cancer^[14]. The possible oncogenic mechanisms of HERVs include (Figure 1): (1) the general or more specific (re)activation of HERV sequences by hypomethylation^[23-25]; (2) the expression of HERV encoded oncogenes such as Rec and NP9^[26]; (3) the inactivation of tumor suppressor genes by *de novo* insertion or translocation of retroelements within the genome^[26]; (4) the regulation of nearby (proto-) oncogenes or growth factors by the regulatory sequences of LTRs^[27,28]; and (5) the potential of Env proteins to induce cell fusions, which may contribute to tumor progression or even aid in metastasizing processes^[29]. Far from being complete, this is already a quite impressive list. An additional aspect comes from the observation that Env proteins of the mouse leukemia virus, the Mason-Pfizer monkey virus and also of HERV-K have strong immunosuppressive properties and may thus help tumor cells evade an antitumoral immune response^[26,30,31].

Methylation

As a general rule, all human regulatory genomic sequences become methylated unless specific factors prevent methylation^[23]. In addition, methylated sites are more prone to mutations^[23] and by this means, virus inactivation is further strengthened. Demethylation of regulatory regions is possible in the context of normal physiological processes by strong transcriptional activators. Re-expression of methylated sites is also possible during cell stress dependent on chromatin remodeling as a reaction to this stress^[32]. Obviously, the maintenance of methylation patterns and status must play a central role in HERV transcriptional control. In healthy somatic and mature germ cells HERV sequences are generally (hyper-) methylated. Thus, HERV transcriptional activity is mainly restricted to germ cell development or the desensitization of check-point activation in meiotic cells. This mechanism may also be responsible for a high(er) retroelement expression in germ cell tumors^[23]. In somatic cells, severe global hypomethylation

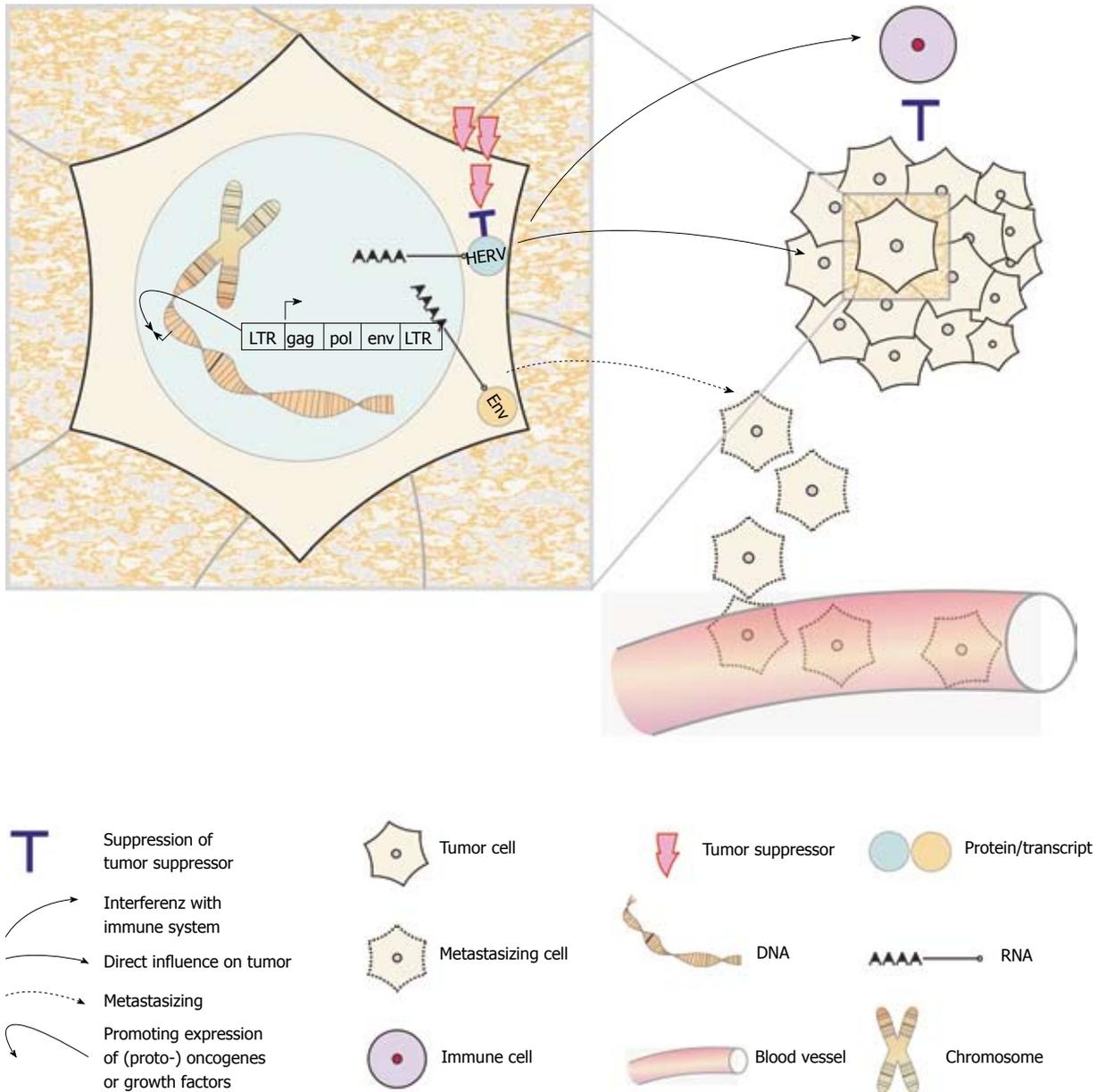


Figure 1 Possible mechanisms by which human endogenous retroviruses contribute to oncogenesis. Human endogenous retroviruses (HERVs) transcripts or proteins may directly have tumor promoting properties. The long terminal repeat (LTR) elements can function as promoters or enhancers for nearby (proto-) oncogenes or growth factors. Especially Env proteins might attract regulatory immune cells and thus provide an immunosuppressive microenvironment. And finally, the Env proteins may be directly involved in the metastasizing process.

leads to apoptosis induction mediated by TP53 and other tumor suppressive factors^[33]. Premalignant and malignant cells are typically insensitive to apoptosis induction^[34] and aberrant expression from normally methylated promoters is a main oncogenic force. In line with this, a general hypomethylation of HERV sequences can be found in the cancer cells of different entities, including testicular germ cell cancer, teratocarcinomas, colorectal, breast and ovarian cancer^[35-39]. However, methylation analyses are biased by a lack of accuracy of the bisulfite sequencing technique^[40]. When not highly standardized, this may account for a number of false positive or negative results in methylation analyses. Thus, it is always recommended to combine methylation analyses together with an investiga-

tion of mRNA or superior protein expression.

HERV ORF expression

Of interest, Syncytin-1 is the only expressed HERV sequence with a presumable physiological function. Syncytin-1 expression takes place in the placenta in the context of syncytiotrophoblast generation by cellular fusion of precursor cells, the cytotrophoblasts^[41,42]. This expression follows after a general hypomethylation of a HERV-W *env* sequence and the Env protein is considered to contribute to this cellular fusion process. It may be a coincidence, but for many tumor entities, naturally occurring cellular fusions have been described^[43-45] and this may hint towards the expression of similar HERV Env proteins.

Expression of HERV sequences has been described for several tumor entities including melanoma, breast, ovarian, prostate and colon cancer^[37-39,46,47]. Active retrotranspositions cause DNA strand-breaks and will thus lead to an activation of check-point signaling, e.g., TP53. Thus, transpositions as another mechanism for HERV re-expression may consequently occur especially in tumors with defect check-points and TP53 mutations^[23].

Tumor induction/promotion

Beside sheer tumor specific expression, HERVs have repeatedly been discussed to induce or promote tumorigenesis. Potential mechanisms have been outlined in the preceding paragraphs. Here we want to gather the bits of evidence that have been obtained so far.

Several groups could show the production of HERV-derived proteins or even of viral particles in tumor cells^[48-54]. In a mouse study, Howard and coworkers could directly link genome hypomethylation to ERV up-regulation^[55]. Further research could make the connection between hypomethylation of (H)ERVs and chromosomal instability; it is by mediating ectopic recombination^[56]. These HERV-induced recombination events have been found to produce large scale chromosomal anomalies^[57], a hallmark of most tumors^[34]. Finally, Lamprecht and colleagues could link the deregulated expression of the colony-stimulating factor 1 receptor (CSF1R) in B cell-derived Hodgkin's lymphoma cells to hypomethylation of an up-stream HERV-derived LTR, which promotes ectopic expression of the CSF1R proto-oncogene^[58]. However, the question if the reactivation of a (pro-) virus could actively promote cellular transformation or at least contribute to tumor progression is formally unsolved for human cancer. Similarly, it is unknown, if HERV activation is an early or a late step in tumor formation. Still, when considering the above listed bits of evidence, it seems reasonable to conclude that HERVs' contribution to the multi-step process of tumor development in humans is very likely^[14].

Immune responses towards HERV sequences

The human immune system's capability to recognize HERV sequences has so far only scarcely been analyzed. However, some examples can be found in the literature. In patients with kidney cancer, cytotoxic T lymphocytes (CTLs) reactive to a HERV-E sequence encoded on chromosome 6q were found^[59]. Serological responses and CTLs reactive to HERV-K sequences were detected in melanoma patients^[60,61]. Anti-Env antibodies for HERV-K, -E and ERV3 were present in sera of patients with ovarian cancer^[38] and in male patients with germ cell tumors^[62]. Similarly, in breast cancer patients, anti-HERV-K serum antibodies were detected together with HERV-K-specific CTLs^[63]. The orchestrated activation of both arms of the adaptive immune system in the latter cases is a strong indicator of HERV sequences' high immunogenicity. Consequently, one may conclude that at least no strong tolerance towards HERV encoded sequences is induced during lymphocyte development.

Future studies will have to analyze whether the immunological recognition of HERV sequences is executed by highly avid or only by intermediate avid T cells and antibodies. Also, it must be carefully analyzed, which HERV sequences give rise to strong immune responses when aberrantly expressed in tumor cells. We would like to state that immune recognition is a strong indicator for endogenous expression of a given HERV protein, as has been shown for other tumor antigens^[64]. Of note, T cell reactions against HERVs, such as HERV-K, HERV-L and HERV-H, were associated with successful control of human immunodeficiency virus (HIV) in a subset of HIV patients^[65]. It can be anticipated that this association of successful HIV control by HERV specific immune reactions will be translated into the tumor field. One of the major questions with clinical relevance is whether HERV-specific immune signatures can be associated with better prognosis or not.

THERAPEUTIC STRATEGIES

Assuming that in the normal physiology of adult tissues, HERVs do not play a vital role and following the line of evidence that HERV sequences are of significance in tumor formation, development and metastasis, HERVs recommend themselves as prime targets for tumor therapy. Several targeting strategies have been suggested (Figure 2 for an overview) and first experimental results can be found in the literature.

Inhibition

In the light of the tremendous success in HIV control with infected people treated by antiretroviral combination therapies, it would make sense to simply reverse the expression of HERV sequences in human tumor cells. The group of Carlini analyzed the effect of a reverse transcription inhibitor (Abcavir) on prostate cancer cell lines^[66]. It showed a strong anti-proliferative capacity and even triggered senescence in the cancer cells. Interestingly, the authors found an up-regulation of transcripts from LINE elements in the treated cells but unfortunately, they did not analyze HERV expression^[66].

A direct targeting of HERV proteins by small molecular inhibitors or via RNA interference would also be worth trying. However, this has not yet been done. Therapeutical use of natural inhibitors of retroviruses such as APOBEC^[67,68] or TRIM5^[69] would be another possible future option. First, detail knowledge on how and when such retroviral restriction elements act on HERVs must be build up.

Passive immune therapy

Only very recently, Wang-Johanning and coworkers designed a monoclonal antibody (mAb) recognizing a HERV-K Env protein. They described that HERV-K Env protein expression was substantially higher in malignant breast cancer cell lines than in non-malignant breast cells. Furthermore, HERV-K expression was detected in 148

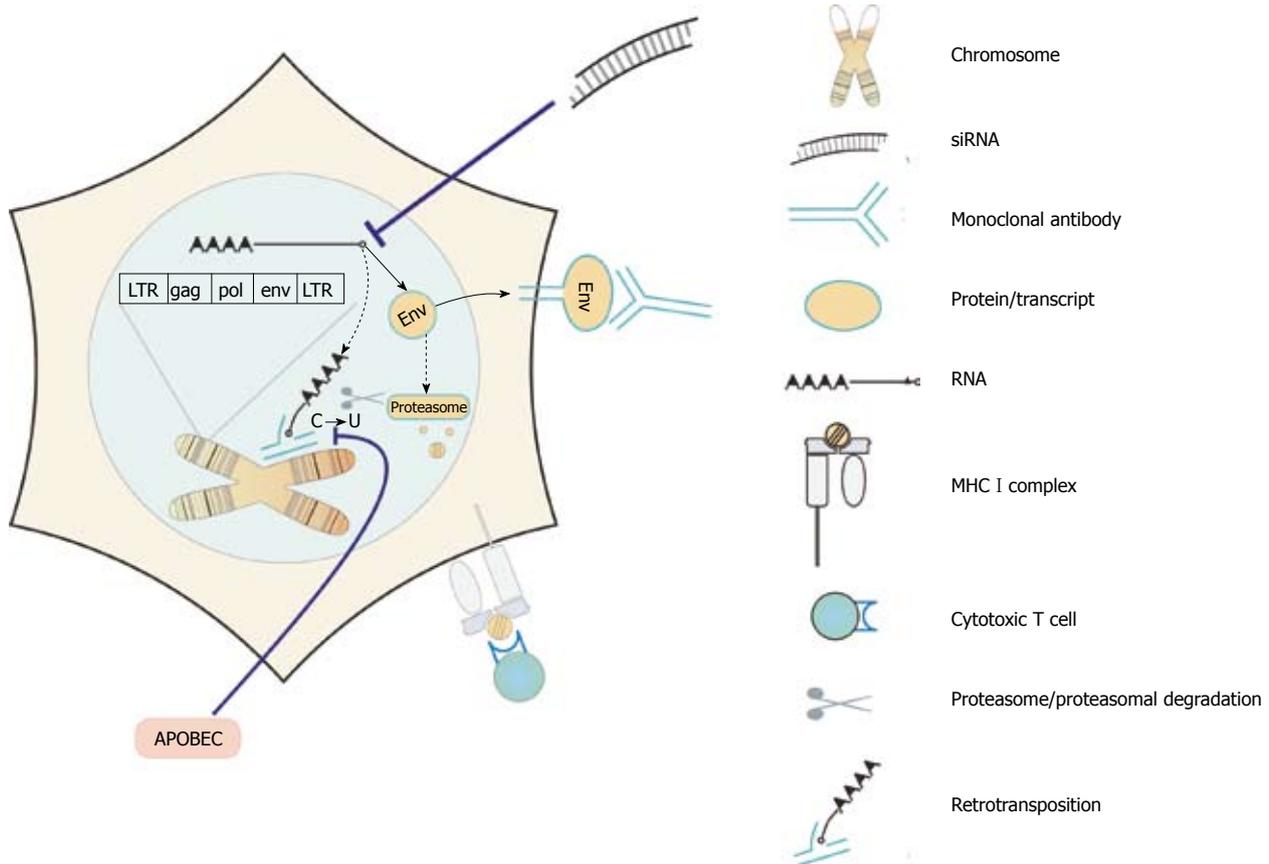


Figure 2 Therapeutic possibilities to target tumor cells with active human endogenous retroviruses. Expressed Env proteins may be targeted by therapeutic monoclonal antibodies. (Re-) activation of retroelement-controlling proteins may help to reduce human endogenous retrovirus (HERV) activities. Small molecule inhibitors of HERV proteins or inhibitory targeting of expressed HERV sequences potentially will prevent oncogenic properties of HERV and harm or kill tumor cells with activated HERV oncogenesis. HERV proteins with tumor-specific antigen properties can be targeted by specific T cells. LTR: Long terminal repeat; MHC: Major histocompatibility complex.

(66%) of 223 primary breast tumors. And a higher rate of lymph node metastasis was associated with HERV-K-positive tumors. Anti-HERV-K-specific mAbs inhibited tumor growth and induced apoptosis of breast cancer cells *in vitro*. Mice treated with these mAbs showed significantly reduced growth of xenograft tumors. *In vitro*, this treatment resulted in an over-expression of several proteins involved in the apoptotic signaling pathways in malignant breast cells^[70]. In principle, targeting HERV Env proteins by therapeutical antibodies should be exploitable to all individual tumors expressing HERV Env. Moreover, passive immune therapies may well be applied in combination with active immune therapies.

Active immune therapy

The ideal cancer therapeutic agent should be able to discriminate between cancer and normal cells (i.e., specificity) and be potent enough to kill small or large numbers of tumor cells (i.e., sensitivity). A feature that makes immunotherapies unique is that an ideal cancer immunotherapy should be able to prevent recurrence of the tumor (i.e., durability). In the last decades it became increasingly apparent that this durability in prevention of tumor recurrences is due to persistent recognition of

tumor antigens by lymphocytes.

Researchers distinguish between tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs). TAAs are antigens that are expressed in normal tissue but to a much higher extent in malignant cells. Contrary to this, TSA are truly specifically expressed in tumor cells alone. Beside specific point^[71] or frameshift mutations^[72], proteins from tumor-inducing viruses^[73] for the most part form this class of tumor antigens. Most of the features an ideal TSA should possess have been assigned to HERV encoded proteins. This being beside exclusivity also the necessity of expression for maintenance of the cancer cells' transformed state. Thus, immune-escape by simple down-regulation of expression is prevented^[74]. Moreover, to ease therapy development, ideal TSA expression should be not only present in single tumors but shared between individual tumors of a given entity or even superior between tumors of different entities^[75,76]. Finally, the immune system should be able to mount both a cellular and a humoral response^[63]. When summing up these desired properties of TSAs attributable to (at least some) HERV-encoded proteins, one may conclude that they might indeed be ideal targets for tumor immunotherapy. Because of the multitude of HERV-

encoded sequences one can even expect that the development of a polyvalent (i.e., containing many epitopes) vaccine basing only on HERV epitopes may be possible. Even more visionary, actual bioinformatics approaches will allow the identification of immunogenic core epitopes shared between different HERV copy ORFs active in different tumor entities in order to design a universal HERV-based vaccine. As a first step in that direction, we recently described two CD8⁺ T cell epitopes encoded by a HERV-H copy located on Xp22.3^[77].

FUTURE PERSPECTIVES

At the moment, in the field of HERVs more questions are open than answered. Are there human (tumor-) cells producing virus particles? If so, are those particles infectious? Further analyses on expression of HERV sequences and proteins - and in especially of Env proteins - would add to the full picture and understanding of the relationship of tumors and endogenous retroviruses. In a first step the mutual interaction between HERV Env and the immune system, also in a suppressive manner, has been addressed^[26,30,31]. These analyses should be expanded. Especially a broader knowledge on tumor infiltrating cells specific for HERV epitopes and their prognostic value would be interesting. Furthermore, it would be very beneficial to know if there is a correlation between tumor grade, stage, progression or outcome and the expression of HERV sequences.

CONCLUSION

Our understanding of HERVs has come a long way. They must be considered as domesticated retroviruses with even main functions in evolution. On the level of an individual human being, however, their activities most likely are tightly controlled. Heavy genetic disorders, as present in tumor cells, generally seem to be linked with HERV activities. The tumor-specific expression of HERV-encoded proteins opens the way to diagnostically and therapeutically interesting opportunities: (1) The targeting of HERV proteins either biochemically or immunologically as TSAs; (2) Immune recognition of tumor cells takes place already early in tumor development. HERV-encoded ORF-derived proteins are likely candidates of this early recognition. Consequently, they may be ideal for screening people at risk to develop cancer as we suggested for frameshift mutations in lynch syndrome^[72,78]; (3) the recognition of expressed HERV sequences by the adaptive immune system is likely to result in a better prognosis for patients raising to-be-defined minimum levels of immune responses. Such HERV-specific responses may well be suited for prognostic purposes.

POSTULATES

We would like to take the chance and hypothesize on

some of the open questions and obvious tasks in the HERV/tumor field: APOBEC and other retroelement controlling factors are likely to be inactivated in cancer cells with active HERV-driven oncogenesis. If this is frequently the case, they must be considered tumor suppressor genes and screening for their inactivation would possibly hint towards specific HERV activation.

HERV-encoded TSAs are released into the circulation^[79] and thus screening of HERV-TSA blood levels will become an interesting field of investigation. Similarly, HERV-specific (immune-) therapies will be developed in the near future for several tumor entities. For these immunotherapies, beside knowledge about expression in different tumors, the level of tolerance towards HERV-TSAs will guide the decision on which candidates to investigate in clinical trials.

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Celiac disease: Prevalence, diagnosis, pathogenesis and treatment

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Abstract

Celiac disease (CD) is one of the most common diseases, resulting from both environmental (gluten) and genetic factors [human leukocyte antigen (HLA) and non-HLA genes]. The prevalence of CD has been estimated to approximate 0.5%-1% in different parts of the world. However, the population with diabetes, autoimmune disorder or relatives of CD individuals have even higher risk for the development of CD, at least in part, because of shared HLA typing. Gliadin gains access to the basal surface of the epithelium, and interact directly with the immune system, *via* both trans- and para-cellular routes. From a diagnostic perspective, symptoms may be viewed as either "typical" or "atypical". In both positive serological screening results suggestive of CD, should lead to small bowel biopsy followed by a favourable clinical and serological response to the gluten-free diet (GFD) to confirm the diagnosis. Positive anti-tissue transglutaminase antibody or anti-endomysial antibody during the clinical course helps to confirm the diagnosis of CD because of their over 99%

specificities when small bowel villous atrophy is present on biopsy. Currently, the only treatment available for CD individuals is a strict life-long GFD. A greater understanding of the pathogenesis of CD allows alternative future CD treatments to hydrolyse toxic gliadin peptide, prevent toxic gliadin peptide absorption, blockage of selective deamidation of specific glutamine residues by tissue, restore immune tolerance towards gluten, modulation of immune response to dietary gliadin, and restoration of intestinal architecture.

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Key words: Celiac disease; Demography; Diagnosis; Pathogenesis; Treatment

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INTRODUCTION

Celiac disease (CD) is a life-long gluten-sensitive autoimmune disease of the small intestine affecting genetically susceptible individuals worldwide. CD individuals may present gastrointestinal symptoms, extraintestinal symptoms or no signs of symptoms. The classical symptoms include gastrointestinal-related symptoms such as diarrhea, steatorrhea and weight loss due to malabsorption. About 50% of CD patients present extraintestinal or

atypical symptoms, such as anemia, osteoporosis, dermatitis herpetiformis, neurological problems and dental enamel hypoplasia^[1-3]. The variable clinical picture of CD is due to having both genetic and immunological bases, with age of onset, extent of mucosal injury, dietary habits, and gender^[4], affecting the clinical manifestation of the disease.

CD diagnosis is based on presence of predisposing genetic factor human leukocyte antigen (HLA) DQ2/8, with positive biopsy and serological antibodies upon gluten contained diet. The spectrum of CD may present in different forms^[5]. The classical form may be diagnosed at any age of life and is often characterized by crypt hyperplasia and villous atrophy along with features of malabsorption. The atypical form is characterized by positive celiac serology, limited abnormalities of the small intestinal mucosa or no intestinal symptoms, but associated extraintestinal conditions such as osteoporosis, peripheral neuropathy, anemia and infertility. The latent form is defined by presence of predisposing gene HLA-DQ2 and/or HLA-DQ8, normal intestinal mucosa and, possible positive serology. Extraintestinal features and biopsies of the small bowel show alterations with gluten intake (i.e., gluten-sensitive). Rarely, usually after age 50 years^[6], some that have initially responded to a gluten-free diet (GFD), develop recurrent symptoms and biopsy changes despite a GFD. This is the refractory form^[7]. If no response to GFD was initially documented, however, the use of sprue-like intestinal disease or unclassified sprue has been used.

PREVALENCE

CD originally thought to almost exclusively affect white Europeans, is now known to be widely distributed worldwide^[8]. Epidemiological studies conducted in areas supposedly free of CD, including Africa, the Middle East, Asia, and South America, show that the disease was previously underdiagnosed^[9]. This provides evidence that CD is one of the most common genetic diseases, resulting from both environmental (gluten) and genetic (HLA and non-HLA genes) factors.

The world distribution of CD seems to have followed the mankind wheat consumption and the migratory flows. Man originally fed on meat, fruit and vegetables, with no exposure to gluten-containing cereals. It was only about 10 000 years ago in a small region called the "Fertile Crescent" of the Middle-East (including Anatolia (Southern Turkey), Lebanon, Syria, Palestine and Iraq) where wild wheat and barley grains successfully cultivated due to favorable environmental conditions. In the Fertile Crescent some tribes changed from nomadic to stable settlement style of living because land cultivation permitted food storage, and later migrated westwards to obtain new lands for cultivation. These persons spread through the Mediterranean area (Northern Africa, Southern Europe) and Central Europe. The expansion continued from 9000 to 4000 BC by which

time the cultivation of wheat and barley had spread all over the Old Continent, also reaching Northern Europe (Ireland, Denmark and the Scandinavian countries). This expansion in farming was due to the diffusion of agricultural practices and replacement of local inhabitants by descendants from the Middle-East^[10]. Hence, the European and North-African populations share genetic background with the peoples of Middle-East origin.

In the last few years a number of studies in different populations have been carried out using molecular genetics methods to identify genes causing CD. CD-predisposing genetic loci are *CELLAC1* on chromosome 6, *CELLAC2* on chromosome 5q31-33^[11], *CELLAC3* on chromosome 2q33^[12], and *CELLAC4* on chromosome 19p13.1^[13]. *PAR3* and *MAGI2* tight junction genes associations have also been reported in Dutch CD or ulcerative colitis patients, suggesting a common intestinal defect in these two conditions^[14]. Another gene expressed in major histocompatibility complex (MHC) I antigen presenting cell is *HLA B8*, was found to be associated with CD in Algeria^[15], Iraq^[16,17] and Turkey^[18]. Moreover, atypical CD Saharawi patients were found to over-express the MHC class I chain-related gene A (MICA) allele 5.1^[15], which have also been reported in Western countries^[19]. Increased prevalence of HLA-A25 in Turkish children with CD was also reported, suggesting that this genotype is particularly encountered among this population^[18], with no association described in Western countries.

Useful Background: Genes causing CD *CELIAC1* on chromosome 6 (*HLA-DQ2* and *HLA-DQ8*); *CELIAC2* on chromosome 5q31-33^[11]; *CELIAC3* on chromosome 2q33 (containing T lymphocyte regulatory genes *CD28*, *CTLA4* and *ICOS*)^[12]; and *CELIAC4* (myosin IXB gene, *MYO9XB*) on chromosome 19p13.1^[13].

HLA genotype contributes to the genetic risk for CD at 30%-50%^[20,21]. Non-HLA genes contribute more evidence to the CD genetic background than the HLA genes, but each by itself contributes only a modest to the disease development. Hence, it is reasonable to assume that the susceptibility to CD involves with polymorphic genes that influence the immune response to gluten, as shown for the HLA-linked genes^[22].

Ninety percent of European patients with CD carry the HLA-DQ2 molecule, encoded either in cis on the HLA-DQA1*0501-DQB1*0201 haplotype (HLA-DQ2.5cis) or in trans on the HLA-DQA1*0505 DQB1*0301/DQA1*0201-DQB1*0202 haplotypes (HLA-DQ2.5trans). Approximately 5% express HLA-DQ8, encoded by HLA-DQA1*0301-DQB1*0302. The majority of the remainder carry the HLA-DQA1*0201-DQB1*0202 haplotype^[20]. With genetic testing, DQ2 is almost synonymous with DQB1*02, a gene with two common alleles designated DQB1*0201 and DQB1*0202. The DQ2 frequency in Caucasian in Western Europe populations has been estimated at 20%-30%, and relatively high frequencies also occur in Northern and Western Africa, the Middle East and central Asia^[23]. Thereafter, the overall frequency of DQ2 declines from West to East with low frequencies in

Table 1 Frequency of human leukocyte antigen-DQ2, encoded by human leukocyte antigen-DQB1 *02 and human leukocyte antigen-DQ8, encoded by human leukocyte antigen-DQA1 *0301-DQB1 *0302

< 5%	5%-20%	20%
HLA-DQ2		
Albania	Belarus	Algeria
Canada BC (Athabaskans)	Algeria	Australia
Cook Islands	Cameroon	Belgium
Indonesia	Congo	Central African Republic
	Costa Rica	Croatia
Japan	China	England
Jordan	Cuba	Equatorial Guinea
Papua New Guinea	Ecuador Africans	Bioko Island
	France	Ethiopia
Philippines	India	Germany
Samoa	Malaysia	Greece
	Mexico	Iran
	Poland	Ireland South
	Russia	Israel
	Singapore	Italy
	South Korea	Mongolia
	Spain	New Zealand
	Sri Lanka	Pakistan
	Sweden	Saudi Arabia
	Taiwan, China	Slovenia
	Thailand	Tunesia
	Turkey	United States
	Uganda	
	Ukraine	
	Vietnam	
HLA-DQ8		
Australia	Algeria	Argentina
China	Belgium	Ecuador
Georgia	Brazil	Ethiopia
Greece	Canada BC (Athabaskans)	Mexico
North India	Croatia	Venezuela
Spain	England Caucasoid	
Uganda	France	
	South India	
	Israel	
	Italy	
	Japan	
	Russia	
	South Korea	
	Tunisia	
	Turkey	
	Ukraine	
	United States	
	European American	

Estimates are based on studies included in a comprehensive Internet website^[24]. In several countries, the frequency is not known. HLA: Human leukocyte antigen.

populations in South-East Asia and the virtual absence of DQ2 in Japan (Table 1). DQ8 frequency has a worldwide distribution, whereas DQ2.5, is common in South and Central America; approximately 90% of Amerindian populations carry DQ8 and may display the celiac phenotype^[24,25]. The frequency of DQ8 population is shown in Table 1.

In the past, the prevalence of CD had been underestimated, but it is now regarded one of the most common genetic disorders in the West with 1% prevalence^[26-28]. In-

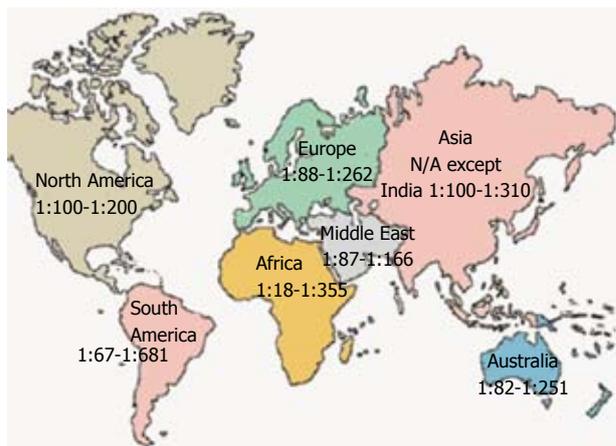


Figure 1 Prevalence of celiac disease worldwide. N/A: Not available.

terestingly, there is increased prevalence of CD amongst women compared to men with male:female ratio of 1:2.8^[29]. This could be due to the finding that men with CD were diagnosed at an older age^[30]. Indeed, there have been reported CD cases among immigrant children native of Eastern Europe, Northern, West and East Africa, the Middle East and Southern Asia, according to their acquisition of Western dietary practices (i.e., short period or lack of breast feeding and early weaning with a great amount of gluten intake)^[31]. This suggests that many persons may have the genetic predisposition to CD but the clinical presentation only occurs when there is sufficient gluten in the diet.

Normal at-risk persons

In several parts of the world, the presence of the combination of antibody (serum tissue transglutaminase and endomysial autoantibodies) positivity and an HLA haplotype associated with CD is predictive of small-bowel abnormalities indicative of CD. For the majority of countries, the CD prevalence is unknown. Figure 1 shows a range of estimated normal at-risk CD prevalence in continents and nations around the globe. It must be noted that some studies report prevalence of CD based on serology, others on celiac compatible small bowel biopsies and a few on serology, biopsy and response to gluten challenge.

North America

CD prevalence in North American and Europe were found to be similar in symptomatic patients and not-at-risk subjects. In the United States, CD is believed to affect 0.5%-1.0% of the general population^[32]. The study by Fasano *et al*^[28] on serum antibody and biopsy screening were performed for a total of 13 145 United States subjects: first-degree (*n* = 4508) and second-degree relatives of patients (*n* = 1275) with biopsy-proven CD, symptomatic patients (*n* = 3236) (with either gastrointestinal symptoms or a disorder associated with CD), and not-at-risk individuals (*n* = 4126). The overall CD prevalence is 1:133 in the not-at-risk groups, whereas in the at-

Table 2 Prevalence of celiac disease in Europeans based on unselected population serological screenings^[36-38] (adapted)

Countries	Prevalence
Czechoslovakia	0.193
Estonia	0.103
Finland	0.110
Hungary	0.101
Ireland	0.126
Italy	0.115
Norway	0.224
Portugal	0.135
Spain	0.124
Sweden	0.174
Switzerland	0.133
Netherlands	0.179
United Kingdom	0.111

risk group, the prevalence is 1:22 in first-degree relatives, 1:39 in second-degree relatives, and 1:56 in symptomatic patients^[28].

South America

In South America, CD had been historically considered a rare disorder and the prevalence investigations have not been extensively studied. However, during the last few years studies in Brazil disclosed a prevalence of 1:681 in healthy blood donors^[33] and 1:473 among adult out-patients attending a clinical laboratory for routine blood testing^[34]. In an urban area of Argentina, the overall prevalence of CD, among 2000 adults from the general population (996 women; median age 29 year, range 16-79 year) was 1:167, with prevalence in women double that for men^[35]. The high CD prevalence in Argentina could be correlated with HLA DQ8 (> 20%) in the Argentina population.

Europe

The overall prevalence of CD in Western populations is close to 1% (1:100) and may be higher in Northern European countries (Table 2)^[36-38]. The Scandinavian countries, Ireland, and the United Kingdom population tended to show a higher prevalence of CD of approximately 1.0%-1.5%, although there also were studies that showed a lower prevalence in these countries. A study of small-intestinal biopsy obtained from healthy Dutch blood donors at Arnhem and Nijmegen Blood Donation Centers shows that the prevalence of CD-compatible biopsies of 1:330^[39]. The prevalence of CD among 3654 children (age range, 7-16 years) in Finland was at least 1:99 based on serum autoantibodies and small-bowel abnormalities^[40]. The prevalence of CD in Northern Spain in the general population was 1:389^[41], 1:132 (0.75%) in Eastern Switzerland adolescents^[42].

Africa

In Northern African populations (including Morocco, Algeria, Tunisia, Libya and Egypt) higher incidences of 0.28%-5.6% of CD have recently been reported in the

general population^[43-46]. The prevalence of CD in asymptomatic Tunisian school children was estimated to be about 1:157, which is close to the European prevalence. In this respect, the highest world frequency of 16.4% is reported in the CD associated with Insulin Dependent Diabetes Mellitus^[47], in Oran (Algeria). A recent serological screening in 2500 Tunisian healthy blood donors^[45], showed similar that the prevalence of anti-endomysium antibodies in the general population of 1:355, to that of Europeans. Due to high wheat and barley consumption in the North American countries^[45], as well as high frequency of CD predisposing DR3-DQ2 haplotypes in these populations^[48-50], these high CD frequencies are not surprising.

Saharawi population in North Africa, who are of Arabian and Berberian origin, having a high degree of cognation and live as refugees in the Sahara desert (Algeria), has the highest prevalence of CD (5.6%) known in the world today^[43,49]. This elevated prevalence in this population may be explained both by genetic factors: very high frequency of the DR3-DQ2 haplotype, and by environmental factors: changed of dietary habits in the last few decades. The reduced rates and duration of breast-feeding and increased consumption of gluten in early life as part of the staple diet, supplied by Western countries as humanitarian aids^[51], may have played a role in this elevated CD prevalence. However, there are other unknown genetic and environmental factors that explain such a high frequency of CD in the Saharawi people, because there is a much lower prevalence of CD in Sardinia population with similar staple diet consumption and frequencies of DR3-DQ2^[52].

Australia and New Zealand

Australia and New Zealand are the two countries having the highest proportion of individuals from Caucasian background, with high prevalence of HLA DQ2 and per capita wheat consumption of > 150 and 75-150 kg per person per year, respectively^[23]. Only two prevalence studies have been carried out in these two countries. From a random population of 1064 adults in Christchurch, New Zealand (96% Caucasian), CD was confirmed histologically in all patients with positive serology giving an overall prevalence of 1:82 (1.2%)^[53]. A larger study in 3011 adults from a large Caucasian community in Western Australia, revealed an overall prevalence of CD of 1:251 (0.4%) of the population^[54].

Asia

CD is likely to be rare in Indonesia, South Korea, Philippines and many smaller Pacific islands because of their low wheat consumption and a low frequency of HLA-DQB1*02. In South-East Asia, HLA-DQB1*02 is often present in more than 5% of the population but CD is predicted to be rare, as staple diets are based on rice. In contrast, prevalence rates that are similar to those in Europe are likely to apply from Pakistan in the South to Kazakhstan in the North. Ancient migration patterns

Table 3 High risk populations for celiac disease^[73] (adapted)

Relatives, especially first-degree
Anemia, especially iron deficiency
Osteopenic bone disease
Insulin-dependent diabetes (type 1), especially children
Liver disorders, especially Autoimmune hepatitis and primary biliary cirrhosis
Genetic disorders, including down and Turner's syndrome
Autoimmune endocrinopathy, especially thyroid disease
Skin disorders, particularly dermatitis herpetiformis
Neurological disorders, including ataxia, seizures, myasthenia gravis
Others, including immunoglobulin A nephropathy

that determine the frequency of DQB1*02 would also predict more patients with CD in Western China than in Eastern China^[23]. Interestingly, there is one report of CD in three adult descendants of Chinese and Japanese families who migrated to Canada^[55].

In genetic studies of CD in India, the appearance of HLA associations is similar to those in Western countries with a frequency of HLA-DQB1*02 of close to 100%^[56]. This association is more frequent in the population of Northern and North-Eastern India (16%-27%)^[57], than in groups of adults in the Southern state of Tamil Nadu (9%-14%)^[58]. The prevalence of CD in India is nearly the same as that in Western Caucasian populations^[59]. In Punjab (North-west India) school children, CD frequency was estimated to be 0.3%^[60]. This prevalence is probably an underestimation. A retrospective analysis of confirmed cases of CD between 1995 and 2000 in Dayanand Medical College and Hospital (Ludhiana, Punjab) from a total of 202 cases showed an initial of 10 positive cases with a significant growth rate of 79.43% annually with a trend equation increase of 15.49 cases/year^[61]. These studies showed that CD is relatively common in Northern India where there has been a history of wheat cultivation from before 1000 BC^[62]. Hence, the relative rarity of CD in Southern India reflects the effect of both genetic and environmental factors.

The prevalence in first-degree relatives of North Indian children with CD diagnosed as per the European Society for Pediatric Gastroenterology and Nutrition criteria is 4.4% of the first-degree relatives (85% positive for HLA DQ2/DQ8), which is 14 times higher than that of the general population^[63]. There have been reports on clinical experience of biopsy-defined CD in 10 North Indian Immigrants or descendants born in Canada out of 14 Asians diagnosed since 1988 in a single Canadian teaching hospital^[55]. Several studies, particularly from Northern and North-West India, have also documented the presence of CD in children presenting with chronic diarrhea^[64].

Middle East

It seems likely that the prevalence of CD in the Middle East is similar to that of Europe^[65]. CD is a relatively common cause of chronic diarrhea in Iran, Iraq and Kuwait and has been diagnosed in 2%-8% of patients with type 1 diabetes in Iran, Israel and Saudi Arabia^[39]. Many

of these countries have a per capita wheat consumption that ranks among the highest in the world (> 150 kg per person per year)^[23]. Although only a limited number of genetic studies have been carried out, the population of countries such as Iran, Israel, Saudi Arabia and Turkey have a high frequency of HLA-DQB1*02. The prevalence of CD in adult blood donors in Iran, Israel, Syria, Turkey and Anatolia are 1:166^[66], 1:157^[67], 1:62^[68], 1:87^[69], 1:100^[70], respectively. Similar prevalence rates were determined in surveys of Iranian children (1:165, 0.6%)^[71], and Turkish children (1:115, 0.9%)^[72].

The prevalence of CD is approximately 0.5%-1% in all parts of the world, except for populations with very low and very high intake of gluten in their diet.

High at-risk persons

In the general celiac population (without classical CD symptoms, e.g., diarrhea or weight loss), there are high risk groups that may have higher CD prevalence rates (Table 3). Among factors that denote a higher risk for CD, the most important factor is familial history of biopsy proven CD with an estimate of 20% or more of first-degree relatives having CD^[73]. Some authors observed a higher prevalence in CD siblings as compared to parents^[74-76]. A study in Swedish youth (< 20 years old) diagnosed with type 1 diabetes (T1D) confirmed the low prevalence (0.7%) of diagnosed symptomatic CD at initial onset of clinical diagnosis, but document by screening an increasing prevalence of silent CD during a 5-year follow-up to reached an overall prevalence of 10%^[77]. Thus, the prevalence of an association with CD in high risk groups may increase over time.

The overall prevalence of CD is highly dependent on the HLA DQ2/DQ8 typing and gluten consumption. The population with positive HLA typing for celiac have high chances of developing celiac symptoms when on high gluten consumption. However, the population with diabetes, autoimmune disorder or relatives of CD individuals) have even higher risk for the development of CD, since they share the same HLA typing.

PATHOGENESIS

CD is an intestinal enteropathy triggered by the ingestion of gliadin and of other related prolamins in genetically predisposed individuals^[22,78]. Wheat and related species such as barley and rye also induce CD^[79]. A small minority of CD patients also react to oat^[80]. Gliadin peptides exert damaging effects since they are resistant to gastrointestinal enzymes^[81], they have amino acid sequences that are specific for HLA-DQ2, which is a class II major histocompatibility complex, they also have preferred glutamine residues for tissue transglutaminase (tTG)-mediated deamidation^[82], and lastly, they affect intestinal permeability^[83]. Hence the pathogenesis of CD is dependent on genetic and environmental factors. The environmental factor is mainly ingestion of gluten, while several genes contribute to the genetic predisposition^[13]. CD commonly

Table 4 Possible clinical manifestations of celiac disease^[81] (printed with permission)

Typical symptoms	Atypical symptoms	Associated conditions
Chronic diarrhea	Secondary to malabsorption	Possible gluten dependent
Failure to thrive	Sideropenic anemia	IDDM
Abdominal distention	Short stature	Autoimmune thyroiditis
	Osteopenia	Autoimmune hepatitis
	Recurrent abortions	Sjogren syndrome
	Hepatic steatosis	Addison disease
	Recurrent abdominal pain	Autoimmune atrophic gastritis
	Gaseousness	Autoimmune emocytopenic diseases
	Independent of malabsorption	Gluten independent
	Dermatitis herpetiformis	Down syndrome
	Dental enamel hypoplasia	Turner syndrome
	Ataxia	Williams syndrome
	Alopecia	Congenital heart defects
	Primary biliary cirrhosis	IgA deficiency
	Isolated hypertransaminasemia	
	Recurrent aphthous stomatitis	
	Myasthenia gravis	
	Recurrent pericarditis	
	Psoriasis	
	Polyneuropathy	
	Epilepsy	
	Vasculitis	
	Dilatative cardiomyopathy	
	Hypo/hyperthyroidism	

IgA: Immunoglobulin A; IDDM: Insulin dependent diabetes mellitus.

appears in early childhood, with severe symptoms including chronic diarrhea, abdominal distension, and failure to thrive. In many patients, symptoms may not develop until later in life, when the disease symptoms include fatigue, diarrhea, and weight loss due to malabsorption, anemia, and neurological symptoms (Table 4). Celiac disease is a life-long disorder, and if untreated, it is associated with increased morbidity and mortality^[84, 85].

Possible triggers

Genetic predisposition association (HLA, MYO9B), exogenous trigger (gluten), pro-autoimmune genetic background, viral infections, tissue damage, early termination of breastfeeding and gender contribute to the development of CD (Table 5)^[86].

Apart from introduction of gluten during the first year of life, infectious agents may play a role in development of CD. Several studies have implicated infections with Adenovirus type 12^[87-89], hepatitis C virus^[90,91], *Campylobacter jejuni*^[92], *Giardia lamblia*^[93], Rotavirus^[94] and Enterovirus infection^[95] as triggers for the development of CD. The immunologic response in persons genetically susceptible

Table 5 The most important factors contributing to the development of celiac disease^[86] (printed with permission)

Factors contributing to the onset of celiac disease	Mechanism
Gluten	Elicit T cell responses Induces cytokine production and intestinal lesion
Age of introduction of gluten	Weak gut immune during early childhood
HLA-DQ2 or HLA-DQ8	Gluten presentation
MYO9Bo	Increased permeability of the intestine
Pro-autoimmune genetic background	Shift in Th1/Th2 balance towards Th1
Viral infections	Defect in generation of active tolerance (e.g., regulatory T cells) IFN production
Tissue damage	Tissue damage Increased level of tTG Danger signals
Early termination of breastfeeding	Decreased protection against infections
Gender	Hormone-related pro-autoimmune status

Th1: T helper 1; Th2: T helper 2; tTG: Tissue transglutaminase; HLA: Human leukocyte antigen; IFN: Interferon.

CD may be triggered due shared viral sequence of 8 to 12 amino acids with the toxic gliadin fraction^[89]. Other factors such as timing of gluten ingestion and breast feeding cessation may involve in the pathogenesis and disease development of CD^[96]. Some initiating factors, such as gluten overload, gastric surgery “unmasking”, giving up smoking, and infections can also trigger the disease, which can become apparent in an abrupt manner^[97,98].

Prolamin trigger

Gluten is a protein that appears in wheat, barley, rye and oat, compositing of prolamin and glutelin. The majority of the proteins in food responsible for the immune reaction in CD are the prolamins. Prolamins is found in several grains, such as wheat (gliadin), barley (hordein) and rye (secalin), corn (zein) and as a minor protein, avenin in oats. Because of their high glutamine content and specific sequence patterns, prolamins are resistant to gastrointestinal proteolytic enzymes and are excellent substrates for deamidation by tissue transglutaminase.

The incomplete gastrointestinal digestion of gluten leads to the appearance of gluten-derived gliadin peptides such as 33mer (LQLQFPQPQLPYPQPLPYPQPQLPYPQPQPF) with a variety of characteristics^[81]. It contains overlapping T-cell epitopes, and its deamidated form is a potent T-cell stimulator, generating the glutamic acid residues essential for binding to HLA-DQ2 in celiac patients^[99]. The ingestion of prolamins from wheat, barley, rye and possibly oats causes histological changes in the small intestine mucosa of celiac patients, leading to a malabsorption syndrome^[100]. Clinical symptoms of an autoimmune attack after ingestion of the gluten containing food include digestive symptoms and skin reactions.

Gliadin peptides cause stimulation of the innate and adaptive immune system^[83,101,102]. The prototype of pep-

tides effective on innate response is peptide 31-43/49, which has been proved both *in vitro* and *in vivo* to be toxic for CD patients^[103,104]. Peptide 31-43 (p31-43) stimulates the synthesis and release of interleukin (IL)-15, a proinflammatory cytokine, that promotes the adaptive immune response^[101], involving CD4+ T cells that recognize several deamidated gliadin peptides^[82]. Unlike p31-43 which is not immunogenic for T cells, peptide 57-68 (p57-68), which binds to HLA-DQ2/8 molecules, is one of the dominant epitopes recognized by T cells isolated from the intestine of CD patients^[82]. The so-called toxic peptides, of which p31-43 is probably the most fully studied, modulate the small-intestinal mucosal biology *via* an innate immune mechanism.

Time of trigger

Several studies related the rise in childhood CD to infant feeding practices^[96,105]. Consumption of wheat, barley, and rye in the first 3 mo children have significantly increased the risk of developing CD-associated autoantibodies, as compared with exposure during first 4 to 6 mo^[105,106].

Although CD can be diagnosed at any time of life, it is present mostly in either early childhood (between 9 and 24 mo) or in the third or fourth decade of life^[8,85,107,108]. In contrast to the 1/1 sex ratio in children, in adulthood it is diagnosed twice in females. Interestingly, celiac disease is also becoming more frequently recognized in the elderly, and in this population, a 1/1 sex ratio has also been noted^[109]. Although the “classical” gastrointestinal malabsorption syndrome characterized by diarrhea, steatorrhea, weight loss, fatigue, and anemia may occur in severe cases, most patients have a milder symptoms such as abdominal discomfort, bloating, indigestion, or non-gastrointestinal symptoms (or no symptoms at all)^[8,85,107,108]. Mäki *et al.*^[2] reported a shift of 5-6 years of age at diagnosis in Finland, with less than 50% of new cases presenting typical gastrointestinal symptoms. In England^[110], Scotland^[111], Canada^[112], and the United States^[28], reports have also shown that almost 50% of newly diagnosed CD patients do not present with gastrointestinal symptoms.

GENETICS

Genetics play a strong role in CD, indicated by the high disease concordance in monozygotic twin^[113]. The CD prevalence rose to 17.6% in sisters, 10.8% in brothers, and 3.4% in parents^[21]. CD is associated with HLA alleles as well as more than 250 other MHC and non-MHC genes. The main genetic factor is the given HLA-DQ genes, i.e., the genes encoding DQ2 or DQ8 in the HLA complex on 6p21. Approximately 95% of celiac have a DQ2 comprised of DQB1*302 and DQA1*03. A small number of individuals lacking either of those heterodimers have DQB1*02 and DQA1*05 alone^[20,114]. Gene dosage also affect CD susceptibility; individuals with the heterodimer comprised of DQB1*02 and DQA1*05 and most of the remaining 5% have a DQ8 heterodi-

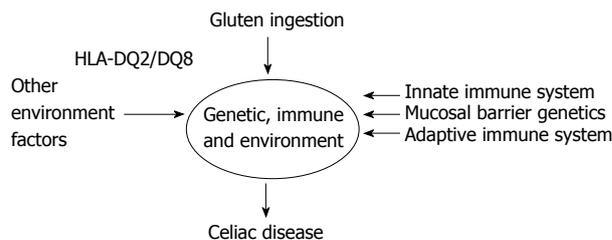


Figure 2 Factors necessary for celiac disease development^[96] (adapted). HLA: Human leukocyte antigen.

mer. Homozygous individuals who carry DQB1*02 and DQA1*05 in cis on both chromosomes have a great risk developing complicated forms of CD^[115]. A significant higher risk for CD of 1:7 for DQ2 and DQ8 individuals and 1:2518 for subjects lacking all predisposing factors have been determined^[21].

It is found that 30% of the Caucasian populations carry HLA-DQ2 and most will eat wheat, while only 1 in 100 will develop disease^[116]. The remaining susceptibility is thought to be due to a combination of genetic and environmental factors (Figure 2).

HLA-DQ2.5 carriage is necessary for disease development, but it is not sufficient by itself. A combination of other genetic factors influencing the mucosal barrier, the adaptive and the innate immune system also impact the likelihood of disease development. Wheat ingestion is a known environment factor that is necessary for disease development but on top of this, a number of factors such as timing of gluten ingestion and breast feeding cessation may influence disease development^[96].

Studies using twins, which are assumed to share environmental factors, have estimated the percentage of non-HLA genetic variants which predispose to disease as approximately 60%^[117]. To date a large list of variants have been suggested to predispose to CD through a combination of linkage and association studies, a large number of variants, however, do not stand up to further scrutiny. Only those that have been validated with convincing evidence in multiple populations are mentioned here.

In CD, like many common diseases, this genome wide linkage approach has been fairly unsuccessful at locating variants. Linkage was found to various regions including 5q and 19p, however, the only genomic region that was replicated with some reliability in other populations was 2q33, a region that contains the *CTLA4*^[118], *ICOS* and *CD28* genes^[117]. *CTLA4* is an excellent candidate gene for involvement in CD not only due to its integral involvement in the suppression of immune responses but also because it has been implicated as a genetic variant that increases susceptibility to T1D.

The prevalence of CD in patients diagnosed with T1D has been estimated at up to 15% in children and 6% in adults^[119]. The reason for this association has never been fully elucidated, but common mechanisms within the pathogenesis and genetics of the two conditions may provide some insights. IL-21 region displays CD associations to T1D^[120], rheumatoid arthritis^[121], Grave's

disease^[122] and psoriatic arthritis^[123]; but genetic involvement in all these conditions is not currently understood. There is possibility of shared genetic susceptibility to autoimmunity through *IL-2*, *IL-21* locus, both inside and outside of the HLA region, with almost no function identified thus far^[116]. Like the studies associated with the HLA-DQ2.5 variant, further identification of the causal variant and its function will provide a unique insight into CD and other autoimmune disease biology.

Immune function

CD is an autoimmune disease associated with the genetic predisposition HLA and tTG autoantigen. tTG is a calcium dependent enzyme that plays a crucial role in CD pathogenesis^[124]. tTG mediates ordered and specific deamidation of gliadins, creating an epitope that binds efficiently to DQ2 and is recognized by gut-derived T cells^[125]. During gluten consumption, these tTG autoantibodies are produced by the mucosa of the small intestine, and detected in patients' serum but disappear slowly from the patient's circulation on a GFD^[126]. Extraintestinal CD symptoms may be associated with immunoglobulin A (IgA) deposits on extracellular tTG in the liver, kidney, lymph nodes and muscles of CD patients^[126].

The toxic peptides, such as the 19-mer, trigger an innate immune response^[101], characterized by the production of IL-15 by epithelial cells and lamina propria dendritic cells^[127]. There is some evidence that this response is a generalized response in all individuals, but is amplified in CD patients (possibly due to a lower threshold to IL-15) who only get disease as a result of adaptive immune system involvement^[128]. IL-15 affects the epithelial barrier, both by increasing the permeability through disruption of the tight junctions^[129,130] and acting on intraepithelial lymphocytes (IELs) promoting interferon γ (IFN- γ) production as well as a potent cytotoxic activity particularly by NKG2D⁺ cells^[131,132]. Therefore, immunoadaptive peptides, like the 33-mer, can now reach the lamina propria, where they are presented by dendritic cells to gluten-specific T cells^[133,134].

Other autoantigens that are normally "cryptic" can be unmasked and cause a self-aggressive immunologic response following the gliadin-initiated inflammatory process^[8]. In fact, persistent stimulation by some proinflammatory cytokines (IFN- γ and tumor necrosis factor α) can cause further processing of autoantigens and their presentation to T lymphocytes by antigen-presenting cells. The mucosa is expanded by increased numbers of lymphoid cells both in the intraepithelial compartment, in which there is an increase in $\gamma\delta$ T cells, and in the lamina propria, which is expanded by lymphocytes and plasma cells. The intestinal crypts are elongated because of an increase in dividing epithelial cells, and villi are shortened or even completely absent because of rapid loss of mature epithelial cells from the villus tip.

Intestinal epithelium function

Intestinal epithelium plays a central role in CD disease

pathogenesis. It modulates the intestinal immune system that is acutely altered by gliadin. This indicates that gliadin can gain access to the basal surface of the epithelium, and therefore interact directly with the immune system, *via* both trans- and paracellular routes of absorption (Figure 3).

Retrotranscytosis

The protected retrotransport of secretory IgA into the intestinal lumen *via* the transferrin receptor CD71, allows the entry of intact and thus harmful gliadin peptides into the intestinal mucosa by a transcellular route. The overexpression of the transferring receptor CD71 in patients with active CD by transportation of gliadin across the intestinal mucosa through retrotranscytosis of secretory immunoglobulin-gliadin complexes is shown in Figure 4^[135,136].

Transcytosis of α 2-gliadin-33mer (an important trigger of CD) by apical-to-basal is stimulated by IFN- γ , which is a key cytokine involved in CD immunopathogenesis^[137].

Paracellular route

There have been recent hypothesis associated with non-digested gliadin absorption in the intestinal lumen during the early event in CD pathogenesis by stimulation of the innate and adaptive immune system^[83,101,102]. Zonulins provide information on the regulation of intercellular tight junctions (TJs) and increased intestinal permeability^[138-142]. It is released by the enterocyte upon apical exposure to α -gliadin digests^[129,143]. Lammers *et al.*^[143] have identified that MyD88 induces release of zonulin upon gliadin binding to CXCR3 on enterocytes, as a result inducing greater epithelial permeability and subsequent paracellular gliadin passage to the gut mucosa.

After binding to its surface receptor, gluten is internalized^[144] and subsequently triggers a series of intracellular events including phospholipase C and Protein kinase Ca activation and actin polymerization, which lead to the opening of TJs^[139,145] through Zot/Zonulin receptor (Figure 5).

Other pathways

There are several pathways including cellular signals that may be involved in the mucosal damage in CD. Deamidation of gluten peptides by tissue tTG reinforces presentation of gluten peptides by HLA-DQ2 or HLA-DQ8 molecules of plasmacytoid dendritic cells (pDCs) to T cells, which activate gluten-reactive Th1 cells and produce high levels of proinflammatory cytokines. IL-21 is overproduced in the mucosa of CD patients, where it helps sustain T-bet expression and IFN- γ production^[146]. Th1 cytokines promote increased cytotoxicity of IELs and natural killer (NK) T cells which cause apoptotic death of enterocytes by the Fas/Fas ligand system, or IL-15-induced perforin/granzyme and NKG2D-MICA signaling pathways. IFN- α released by activated pDCs perpetuates the inflammatory reaction by inducing Th1

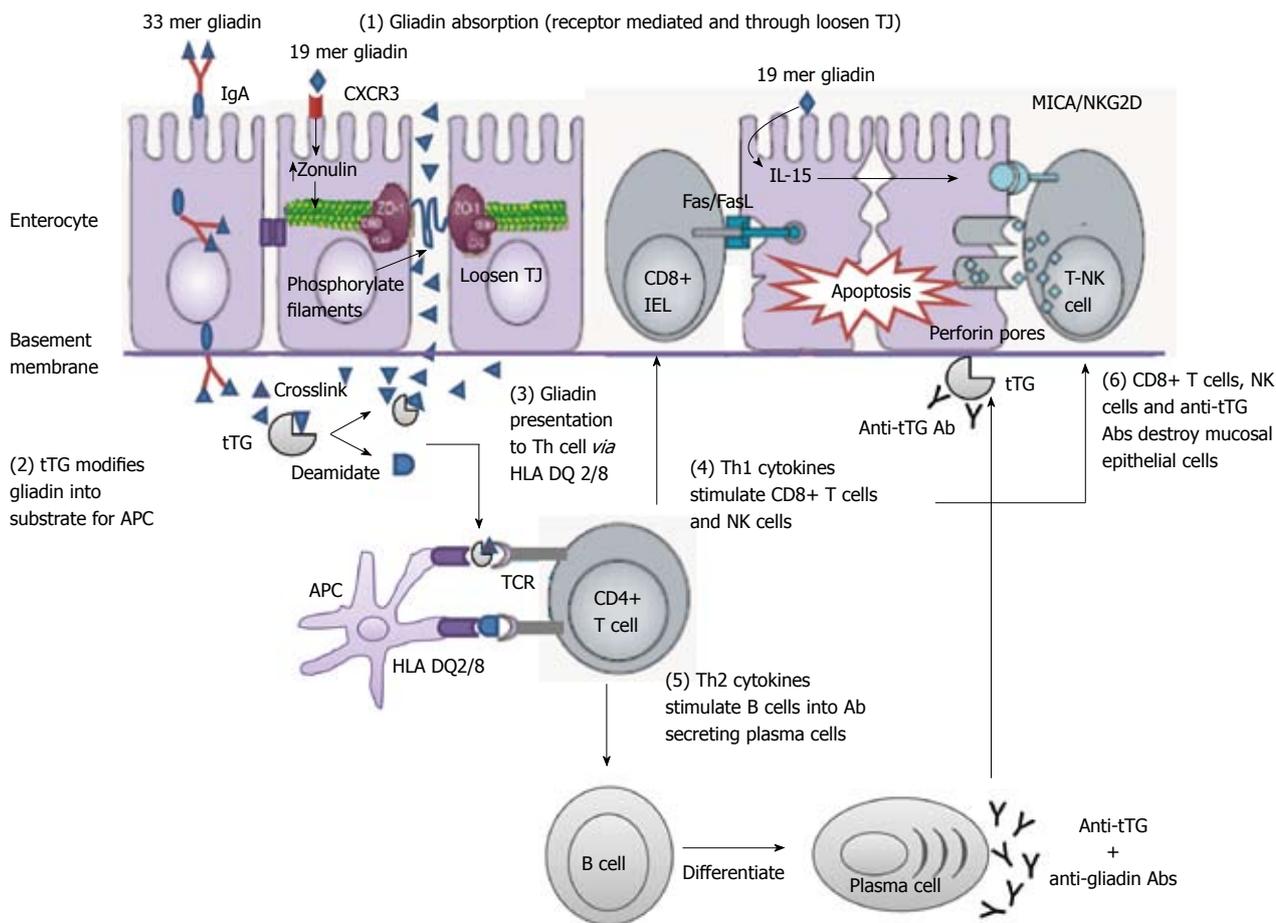


Figure 3 Mechanisms of mucosal damage in celiac disease^[80] (adapted). Gliadin peptides crosses the enterocyte by paracellular tight junctions (TJ) as a consequence of increased release of zonulin leading to impaired mucosal integrity upon 19 mer gliadin binding to chemokine (C-X-C motif) receptor 3 (CXCR3) receptor, or via transcytosis, or retrotranscytosis of secretory immunoglobulin A (IgA) through transferrin receptor CD71. Tissue transglutaminase (tTG) deamidates or crosslinks 33 mer gliadin which is then recognized by human leukocyte antigen (HLA)-DQ2 or -DQ8 molecules of antigen presenting cell (APC). APC presents the toxic peptide to CD4+ T cells. Activated gluten-reactive CD4+ T-cells produce high levels of pro-inflammatory cytokines. T helper 1 (Th1) cytokines promote increased cytotoxicity of intraepithelial lymphocytes (IELs) and natural killer (NK) T cells which cause apoptotic death of enterocytes by the Fas/Fas ligand (FasL) system, or interleukin 15 (IL-15)-induced perforin/granzyme and homodimeric natural killer-activating receptor-major histocompatibility-class I chain-related gene A complex (NKG2D–MICA) signaling pathways. The production of T-helper2 (Th2) cytokines activate and induce clonal expansion of B cells, which differentiate into (antigliadin and anti-tTG) antibody secreting plasma cells. Interaction between with the extracellular tTG and anti-tTG-autoantibody may induce epithelial damage. TCR: T cell receptor.

cells to produce IFN- γ . IL-21 and IL-15 produced by DCs and intraepithelial cells also inhibit transforming growth factor beta signaling and regulatory T cells (Tregs) function. Additionally, the production of Th2 cytokines, Th2 cells drives the activation and clonal expansion and differentiate of B cells into plasma cells secreting anti-gliadin and anti-tissue transglutaminase antibodies^[147], which interact with extracellular tTG, and may induce epithelial damage.

Hence in CD, there is impaired suppressor activity of Tregs. This defect in Tregs function could play a role in the pathogenesis of CD and in CD autoimmunity^[148].

DIAGNOSIS

Approach to initial CD diagnosis

In 1970, the European Society of Paediatric Gastroenterology laid down criteria for the diagnosis of CD in children, entailing three biopsies of an initial flat mucosa in the upper small intestine, restoration of the mucosa to

normal on a GFD, and a deterioration of the mucosa after gluten challenge^[149]. Given the current availability of serological tests being highly sensitive and specific, the European Society of Paediatric Gastroenterology, Hepatology, and Nutrition has proposed a revised CD diagnostic protocol^[150]. Based on this protocol, if the symptoms (either “classical” or “atypical”) and serological tests are suggestive of CD, small bowel biopsy followed by a favourable clinical and serological response to the GFD is now considered sufficient to definitely confirm the diagnosis. In asymptomatic patients improvement in mucosal appearance may be required to confirm the diagnosis, but in majority symptomatic patients, continual abnormality of mucosa at the second biopsy is more likely to indicate slow /partial mucosal recovery^[151]. This may also reflect that the site of re-biopsy (proximal small intestine) is often the last site to improve.

The current approach to evaluating CD has been modified by the advent of highly sensitive and specific serological tests. An algorithm for diagnosing CD is

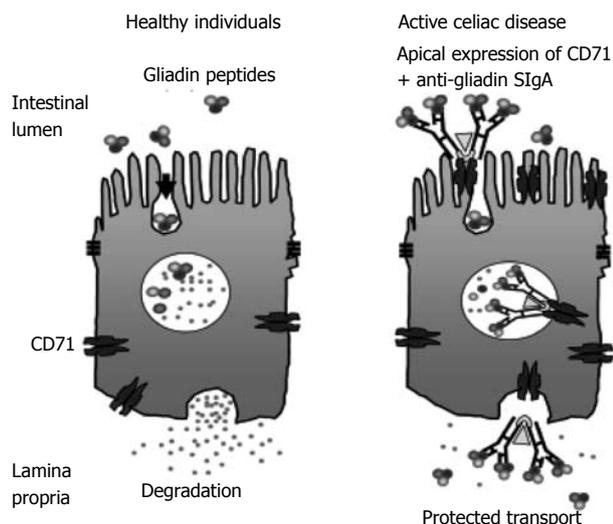


Figure 4 CD71 receptor-mediated transport of immunoglobulin A-gliadin complexes in celiac disease^[136] (adapted). Gliadin bound to apically expressed CD71 receptor in active celiac disease individual allows protected transport of gliadin into the lamina propria. SIgA: Secretory immunoglobulin A.

given in Figure 6. Assays for IgA anti-tissue transglutaminase (TGA) and IgA anti-endomysial (EMA) have both the highest specificities and sensitivities, and are therefore regarded as being superior serological screening tools for diagnosis of CD^[152].

Initial CD evaluation is based on a combination of positive CD-specific serological tests, histological findings in the intestinal biopsy, CD-predisposing gene encoding HLA DQ2 or DQ8, family and medical history of CD, and clinical or histological response to GFD^[26,80]. However, CD diagnosis can be challenging in some non-responsive patients to GFD^[7]. Practically all patients with CD carry HLA-DQ2 or HLA-DQ8. Thus the absence of these gene pairs reflects a very high negative predictive value for CD and should prompt consideration of other causes of small bowel-related symptoms and pathological changes^[153,154]. Positive TGA or EMA at initial diagnosis of CD or at any time in the clinical course of the disease helps to confirm the diagnosis of CD because of their excellent specificities of over 99% when small bowel villous atrophy is present on biopsy^[155].

However, false positive serological assays may also occur^[156], in liver disease and small-bowel inflammation^[157], so documentation of gluten sensitivity is important. A combination of biopsy and serological antibody can also be used to support diagnosis to reduce false positive results. A validated subjective Celiac Dietary Adherence Test, a patient-completed tool, can also be used in conjunction with biological markers to assess dietary adherence and disease activity in individuals with CD^[158].

Diagnosis of refractory CD

The influence of noncompliance to a GFD and the substantial number of patients being undiagnosed are of greatest concern, as these factors could possibly contribute to the refractory form of CD and to the devel-

opment of malignancies. These patients' CD symptoms do not revert on GFD. The first evaluation step of a potential RCD case is to confirm correct initial diagnosis of CD^[7]. Sometimes neglected in this determination is the documentation of an initial and convincing response to a GFD, i.e., demonstration that the disease was truly a "gluten-sensitive" small bowel disorder. Otherwise, it may be difficult to ascertain if CD was initially present. More precise terms in this clinical setting include "sprue-like intestinal disease" or "unclassified sprue".

Some patients with developed RCD are likely to have negative TGA and EMA^[6,159], demonstrating that negative CD specific serology does not exclude the diagnosis of CD. A family history of CD in first-degree relatives (especially siblings) further supports the diagnosis of CD in patients, having 14% positive tTG test and 10% positive EMA with an estimated prevalence of 11% where 54% had "silent" disease, most with severe intestinal villous atrophy^[76]. The diagnosis of CD by histological findings or clinical improvement after GFD without confirmation with other diagnostic criteria may not be entirely reliable because CD is just one of many causes of villous atrophy. Clinical response to GFD or exacerbation after gluten re-introduction have low sensitivity of 59% and specificity of 92% for CD^[160], which account to a positive likelihood ratio of 7.37 (means that individuals with positive histology upon gluten re-introduction are 7.37 times more likely to have CD than those with negative histology upon gluten re-introduction) and a negative likelihood ratio of 0.44 (means that individuals with positive histology upon gluten re-introduction are 0.44 times less likely to lack CD than those with negative histology upon gluten re-introduction). Thus, a critical review of prior tests and villous histology is crucial to determine the accuracy of a prior diagnosis of CD. Ideally, documented normalization of biopsies after a GFD and then demonstration of recurrent symptoms with histological relapse best defines refractory CD (RCD). Obviously, this is not always possible.

RCD is believed to affect approximately 5% of patients with CD. It is subdivided into types I and II, with normal and aberrant (expressing cytoplasmic) CD3, but lacking surface expression of the T-cell markers CD3, CD4, CD8^[161], and the T-cell receptor, intraepithelial T lymphocytes in the small intestinal mucosa, respectively^[162]. Enteropathy-associated T-cell lymphoma (EATL) occurs in more than half of patients with RCD II within 4-6 years after RCD II diagnosis, and is the main cause of death in this group of patients^[76,163]. RCD type 1 only rarely evolves into EATL. Recent data indicate a relative risk for patients with (untreated) CD to develop EATL^[164,165].

DIAGNOSTIC TESTS

Serological tests

HLA typing: The contribution of HLA type to the genetic risk for CD has been variously estimated at

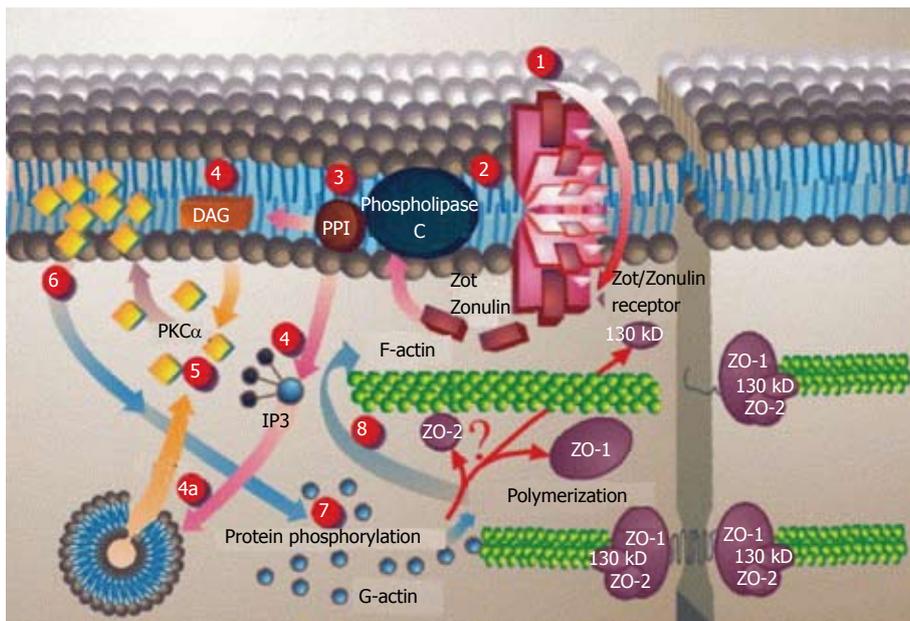


Figure 5 Proposed Zot intracellular signal mediated opening of intestinal tight junctions^[146] (printed with permission). 1: Zot interacts with a specific Zot/Zonulin intestinal surface receptor; 2: Leading to protein internalization; 3: Activation of phospholipase C; 4: Hydrolyzes phosphatidyl inositol to release inositol 1,4,5-tris phosphate (PPI-3) and diacylglycerol (DAG), either via DAG or (4a) through the release of intracellular Ca²⁺ via PPI-3; 5: Protein kinase C alpha (PKCα) is then activated; 6: Membrane-associated, activated PKCα catalyzes the phosphorylation of target protein(s); 7: With subsequent polymerization of soluble G-actin in F-actin; 8: This polymerization causes the rearrangement of the tight junctions (TJ) filaments and displacement of proteins [including zonula occludens-1 (ZO-1)]. As a result, intestinal TJ becomes loosened. IP3: Inositol triphosphate.

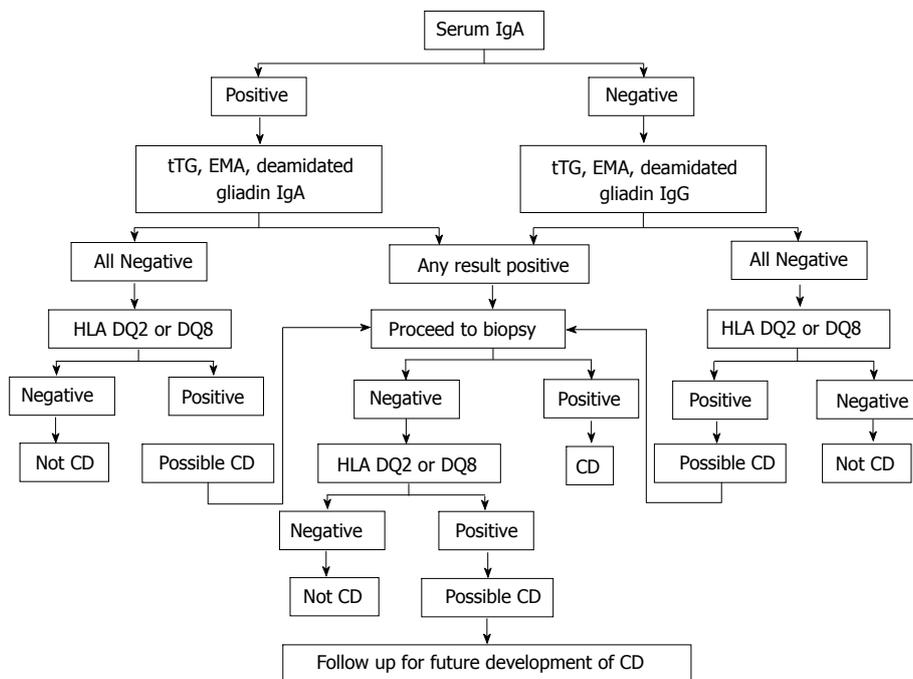


Figure 6 Celiac disease diagnostic testing algorithm (adapted from Mayo Medical Laboratories, Mayo Foundation for Medical Education and Research). IgA: Immunoglobulin A; IgG: Immunoglobulin G; tTG: Tissue transglutaminase; EMA: Endomysial; HLA: Human leukocyte antigen; CD: Celiac disease.

30%-50%^[20,21]. Many of the polymorphic genes are involved in susceptibility to CD encode products that influence the immune response upon gluten ingestion, as shown for the HLA-linked genes^[22]. Although Non-HLA genes contribute more than HLA genes to the genetic background of CD, each of them adds only a minor contribution to the disease development.

There is strong association between CD and the presence of HLA DQA1*0501-DQB1*02 (DQ2) and DQA1*0301-DQB1 [0302 (DQ8) haplotypes. Approximately 90% to 95% of patients with CD carry DQ2 and those patients that are negative for HLA-DQ2 are usually positive for HLA-DQ8^[166,167], indicating a strong genetic risk for the disease^[100]. Several studies also have confirmed that the absence of HLA-DQ2, HLA-DQ8, or both vir-

tually excludes the diagnosis of CD^[168-170]. However, the modest sensitivity (HLA-DQ2, 70%-99.8%; HLA-DQ8, 1.6%-38%) and specificity (HLA-DQ2, 69%-77%; HLA-DQ8, 77%-85%) of the test means that a positive result is not sufficient to diagnose the disease [having a low positive predictive values (HLA-DQ2, 6.3-18; HLA-DQ8, 0.28-8.1) and likelihood ratios (HLA-DQ2, 2.25-4.33; HLA-DQ8, 0.07-2.53)]^[171]. Even the presence of HLA-DQ2 or HLA-DQ8 in patients with positive serologic test results is strongly suggestive but not pathognomonic for CD. Antibody screening to identify participants with preclinical CD may be reduced by preselecting HLA risk group from the large populations with long-term follow-up for CD^[172]. Hence HLA-DQ genotyping could be included in the algorithm of selecting large populations prospec-

Table 6 Operating characteristics of serological markers to detect the celiac disease in adults^[178] (adapted)

Serological tests	Sensitivity	Specificity	Predictive value		Likelihood ratio	
	95% CI (%)	95% CI (%)	Positive	Negative	Positive	Negative
IgG AGA	57-78	71-87	0.2-0.9	0.4-0.9	1.96-6	0.25-0.61
IgA AGA	55-100	71-100	0.3-1.0	0.7-1.0	1.89-∞	0-0.63
IgA EMA	86-100	98-100	0.98-1.0	0.8-0.95	43-∞	0-0.14
IgA TGA	77-100	91-100	> 0.9	> 0.95	8.55-∞	0-0.25
IgA TGA and EMA	98-100	98-100	> 0.9	> 0.95	49-∞	0-0.02

IgG: Immunoglobulin G; IgA: Immunoglobulin A; AGA: Anti-gliadin antibodies; EMA: Endomysial; TGA: Transglutaminase.

tively screened for CD.

Antibody level: Several serum antibodies have been used to initially evaluate patients with suspected CD, monitor adherence and response to GFD, and screen asymptomatic individuals. Anti-gliadin antibodies (AGA) detection has low sensitivity and specificity, leading to high false-positive rate in patients^[175]. Recent reports of deamidated gliadin peptide AGA (DGP-AGA) have suggested a much improved accuracy^[174]. The sensitivity and specificity for IgA DGP-AGA is 84.3% and 79.8%, whereas for IgG DGP-AGA the sensitivity and specificity are 82.3% and 98.9%, respectively^[175]. As shown in Table 6, EMA and TGA have been found to be superior to AGA and gives highest sensitivity and specificity of greater than 95% when used in combination^[173,176,177]. EMA testing, however, produces a subjective and highly observer-dependent result, whereas TGA testing is quantitative.

Small intestinal mucosal biopsy

Histopathological analysis: Although the diagnosis of CD can be suspected on clinical or laboratory grounds, or as a result of serological tests, histology of the proximal small intestinal mucosa is still the diagnostic gold standard and must always be performed. Small intestinal histopathology of CD biopsy samples are characterized by typical architectural abnormalities. Marsh^[178] has pioneered the theory of a sequence of progression of the CD lesion in the small intestinal mucosa.

According to the modified Marsh classification: normal mucosa is classified Marsh 0, intraepithelial lymphocytosis as Marsh I, intraepithelial lymphocytosis and crypt hyperplasia as Marsh II, and intraepithelial lymphocytosis, crypt hyperplasia and villous atrophy as Marsh III^[179]. Later the Marsh-Oberhuber system was developed, where stage 3 was split into three sub stages (a, b and c)^[178,180]. The Marsh-Oberhuber classification was based on a 6-stage grading, namely (1) type 1 infiltrative lesions, characterized by normal mucosal architecture with an increased number of IELs; (2) type 2 hyperplastic lesions, characterized by an increase in crypt depth without villous flattening; (3) type 3a, 3b, and 3c destructive lesion, characterized by mild, marked, and complete villous flattening, respectively; and (4) type 4 hypoplastic lesions, characterized by villous atrophy with normal crypt height and IEL count.

Considering the broad spectrum of lesions possibly present in CD, the Marsh-Oberhuber system is undoubtedly valid under optimal clinical conditions, but the considerable number of diagnostic categories involved makes it prone to a low inter-observer and intra-observer agreement.

False-positive and false negative test results may occur due to patchy mucosal damage, inter-observer variability, low-grade histopathological abnormalities and technical limitations. Hence, reliance on standard histological findings alone may result in failure to diagnose CD^[181]. Several other limitations may be evident in high-volume, service-oriented laboratories with limited attention to quality control. Poorly oriented biopsies fixed in the endoscopy suite may be prone to difficult interpretation. Inter-observer variation in pathological interpretation may occur, especially if there is limited access to a pathologist with expertise focused on interpretation of small intestinal biopsies. Some patients with low-grade histopathological abnormalities (Marsh I /Marsh II) can present with gluten-dependent symptoms or disorders before overt villous atrophy occurs. Furthermore, some patients with isolated intraepithelial lymphocytosis (Marsh I), who are not clinically suspected of having CD, may develop CD during follow-up^[182]. Although the mucosal changes in CD are thought to develop gradually, whether minor mucosal lesions in asymptomatic patients indicate CD in an early stage is not yet clear^[183].

In case of strong clinical suspicion of CD, duodenal biopsy must be performed regardless of serological analysis^[184]; in cases of low suspicion of disease or screening, duodenal biopsy probably only needs to be performed in seropositive patients. Hence, the new system for routine use of simplified grading system with uniform diagnosis and increase validity of the pathologic diagnosis of CD was developed by using only three categories (A, B1 or B2) with A representing normal villous with lymphocytic infiltration and B1 and B2 representing partial and complete villous atrophy, respectively^[185]. The new proposed grading system classified the CD lesions into non-atrophic (grade A) and atrophic (grade B)^[186]. Grade A was characterized by the isolated increase of IELs (> 25/100 enterocytes)^[187], whereas grade B was split into B1, in which the villous/crypt ratio is less than 3/1, with still detectable villi, and B2, in which the villi are no longer detectable. A comparison between the Marsh-Oberhuber and the new

(1) Marsh system (2) Marsh-Oberhuber system (3) New grading system

Type I → Type 1 → Grade A
 Type II → Type 2

Type III → Type 3a → Grade B1
 Type 3b

Type 3C → Grade B2

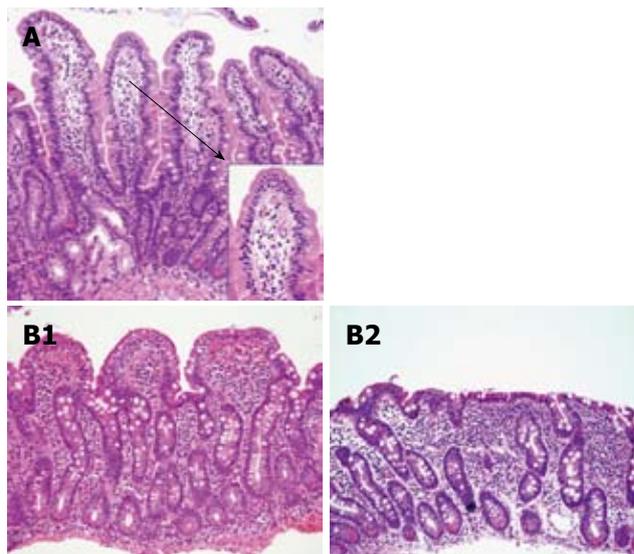


Figure 7 A comparison between the Marsh classification for celiac disease. 1: Marsh-Oberhuber; 2: Grading system for celiac disease, and the new grading system; 3: Representative pictures of the grades A (original magnification, 20×; insert, 60×), B1 (20×), and B2 (20×), proposed in the new grading system. An alternative classification may simply describe “mild”, “moderate” or “severe (flat)” architectural changes^[186] (printed with permission).

Table 7 Factors that support the diagnosis of celiac disease in patients with an increased density of intraepithelial lymphocytes but no villous shortening ^[194] (printed with permission)	
Family history of celiac disease	15% of first-degree relatives are affected
Concomitant autoimmune conditions	Risk of coeliac disease approximately 5-fold
Increased density of $\gamma\delta$ + IELs	Sensitivity 0.84, specificity 0.91
Increased density of villous tip IELs	Sensitivity 0.84, specificity 0.95
HLA DQ2 or DQ8	High sensitivity, low specificity Negative predictive value high
Gluten dependence	Should be ascertained by gluten challenge or gluten-free diet

IELs: Intraepithelial lymphocytes; HLA: Human leukocyte antigen.

grading criteria is shown in Figure 7. Figure 7C represents pictures of the grades proposed in the new histologic grading criteria.

Recently, quantitative measurements of villous height, apical and basal villous widths, and crypt length (morphometry) have been used to determine changes in duodenal morphology, particularly after the introduction of a GFD, in correlation with Marsh grade, self-reported adherence to GFD, and changes in serology. GFD resulted in increase in villous area and a progressive decrease in crypt length, with a plateau after 6-12 mo and mean villous area half that of control subjects^[188].

Intraepithelial lymphocyte: The presence of aberrant IELs appears to be a reliable prognostic marker to differentiate between RCD type I and type II patients, with characteristic normal and aberrant IELs, respectively. IELs are considered aberrant when there is cytoplasmic CD3 expression, but no expression of surface CD3, CD4

and CD8 T-cell markers^[189,190]. The current methods for double CD3/CD8 T cell receptor clonal from intestinal tissue can be done by immunohistochemistry, polymerase chain reaction or flow cytometry^[161,162,189]. The presence of these IELs is associated with a significant increase in EATL development^[161,163,191,192]. Increased IELs may be used to support or exclude diagnosis of CD, and may be useful for follow up as mentioned in Table 7^[193].

In 95% of non-refractory CD and control patients, the highest percentage of aberrant T-cells in duodenal biopsy specimens is in agreement with the cut-off of the % T cells which are aberrant. Such a cut-off has been previously suggested in the RCD group based on the clinical observation that none of the RCD patients with less than 20% aberrant T-cells eventually developed EATL^[163]. Clonal T-cell population can be found in the intestinal mucosa of RCD patients, which relates to the development of EATL^[161,194]. Immunophenotyping using flow cytometry^[162], gives significant higher negative predictive value and sensitivity (both 100%) for aberrant T-cells were found with regard to EATL development in RCD, when compared to clonality in a duodenal biopsy specimen (75% and 78%, respectively). The positive predictive values (59%) and likelihood ratios (1.85) of these tests for EATL development in RCD are almost comparable.

Aberrant T-cells is quantified by flow cytometry is well suited to identify RCD patients at risk for EATL as it has a higher predictive value and sensitivity than T-cell clonality analysis of duodenal biopsy specimens. A cut-off value of 20% appears reliable for early risk stratification^[159], and targeted therapeutic options in RCD patients^[6,27,195]. This is particularly important since once overt T-cell lymphoma has developed, treatment outcome and survival are very poor^[159,196]. Additionally, quantification of aberrant T-cells is useful for the subsequent follow-up of treated RCD II patients^[27].

Table 8 Future therapeutic approach for celiac disease treatment

Mechanism	Therapeutic agent	Stage of study
Hydrolysis of toxic gliadin	ALV003	Glutenases and endoprotease
	AN-PEP	Prolyl endopeptidase
		<i>Lactobacilli</i>
Prevention of gliadin absorption	VSL#3	<i>Lyophilised bacteria</i> , including <i>Bifidobacteria</i> , <i>Lactobacilli</i> and <i>Streptococcus salivarius</i>
	Larazotide	Hexapeptide derived from zonula occludens toxin of <i>Vibrio cholera</i>
		Synthetic polymer poly (hydroxyethylmethacrylate-co-styrene sulfonate)
tTG2 inhibitor		Anti-gliadin IgY
		Dihydroisoxazoles
		Cinnamoyltriazole
Peptide vaccination	Nexvax2	Aryl β -aminoethyl ketones
		Three deamidated peptides derived from wheat α -gliadin, ω -gliadin and β -hordein
		Human hookworm (<i>Necator americanus</i>) inoculation
Modulate immune response		HLA-DQ2 blocker
		Interleukin blocker
		NKG2D antagonist
Restore intestinal architecture		R-spondin-1

tTG2: Tissue transglutaminase 2; PEP: Prolyl endopeptidases; NKG2D: Homodimeric natural killer-activating receptor; HLA: Human leukocyte antigen; IgY: Immunoglobulin Y.

Useful background for the diagnosis of CD: The HLA class II molecules DQ2 and DQ8 are required for but are not sufficient for the development of CD: 50% of Americans are positive of one of those molecules, but only 1% develop CD. Negative HLA DQ2 or DQ8 may rule out CD as a cause of the enteropathy; IgA TGA serology is > 95% sensitive for CD, especially when there is a high titre, but false positive tests can occur; Anti-gliadin antibodies have a relationship high false negative rate, and have been replaced by IgG DGP assays that appear to have a sensitivity compared to TGA; The endoscopic features of CD (scalloping of mucosal folds, less prominent folds, fissures, and a nodular/mosaic pattern) are 59% sensitive but 92% specific for CD. For example, other small bowel disorders, including Crohn disease in the duodenum, may cause mucosal scalloping and other endoscopic features of CD.

TREATMENT

Existing treatment

GFD: Currently, the only effective treatment available for CD individuals is a strict life-long GFD^[197]. In reality, total avoidance of gluten intake is extremely difficult, due to hidden gluten from food contamination^[198]. For safety purposes, United States Food and Drug Administration has set the limit (August 2011) of < 20 ppm gluten (equivalent to 10 ppm gliadin) for gluten-free foods. The total daily consumption of gluten-free foods must be taken into account as it may exceed the tolerable limit of each celiac individual. It has been estimated that the threshold of prolonged gluten ingestion in some CD individuals may be lower than 50 mg/d^[199]. However, some CD individuals can conceivably be more sensitive. The presence of hidden gliadin in contaminated food products represents an imminent risk for celiac consumers, because of long-term effect of regular ingestion of small amounts of gliadin^[200], on causing positive tTG and char-

acteristic small bowel biopsy.

Gluten modification: Approaches to modify dietary gluten have been made to render gliadin non-toxic, since it is a non-invasive approach to CD patients. This approach has been less appealing due to loss of baking characteristic, public refusal for genetically modified crops, contamination of genetically modified crops with gluten contained crops grown nearby and heterogeneous uncharacterised immunostimulatory epitopes in gluten, and difference among patients response to epitopes and gluten levels^[201].

A greater understanding of the pathogenesis of CD allows alternative future treatments to be designed. A number of preliminary studies have been published that illustrate from a conceptual perspective future possible approaches that may be pursued in more detail (Table 8).

FUTURE TREATMENT APPROACHES

Hydrolysis of toxic gliadin peptide

Prolyl endopeptidases: Prolyl endopeptidases (PEPs) are endoproteolytic enzymes expressed in micro-organisms and plants. These enzymes cleave proline-rich gluten to smaller peptides that are ready for digestion by intestinal brush-border enzymes (aminopeptidases and carboxypeptidases). Limited efficiency was found, since PEP required 3 h preincubation with gluten containing foods to achieve full detoxification of peptides and to prevent intestinal transport of active gluten fragments^[202]. This is unlikely to be achieved by co-administration of PEP and gluten-containing diet.

A two-stages cross-over phase II clinical trial was performed using asymptomatic CD patients eating, a slice of bread daily and a slice of bread pre-treated with PEP daily^[203]. After 2 wk of PEP treated gluten challenge, majority of patients did not develop malabsorption, measured by faecal fat excretion and D-xylose

malabsorption tests. The tests likely lacked the necessary sensitivity to assess minor malabsorption resulting from active CD, since no histological confirmation was performed to determine deterioration in the Marsh grading^[201]. When PEPs were consumed as jam spread on a slice of gluten-containing bread by CD patients, villous blunting was seen in small bowel biopsy histological evaluation in most patients^[204]. Further studies are needed to determine the appropriate dose of enzyme and time of administration relative to the quantity of ingested gluten.

ALV003: ALV003, a mixture of two glutenases, an endoprotease from germinating barley and PEP, was pre-treated with wheat flour and tested in CD patients^[205]. Symptoms typically associated with gluten ingestion were not significantly reduced by ALV003 pre-treatment, but ALV003 abolished immune responses induced by gluten in CD patients. A randomized controlled phase IIa clinical trial has been performed where CD patients received either ALV003 or placebo daily for 6 wk at the time of 2 g gluten contained bread. This proof-of-concept study demonstrated that ALV003 can attenuate gluten-induced small intestinal mucosal injury in CD patients^[206]. After six weeks period, biopsies proved lower small intestinal mucosal injury in patients treated with ALV003 than placebo-treated patients despite of persistent intestinal inflammation in many patients on a strict GFD. Placebo-treated patients were found to have suffered more adverse events, most commonly including abdominal distention, flatulence, eructation, abdominal pain and diarrhea^[206].

Lactobacilli: Lactobacilli added to sourdough for fermentation are able to lyse the proline-/glutamine-rich gluten peptides and thus decrease immunotoxicity^[207-210]. A mixture of fermented wheat flour with oat, millet and buckwheat allows sourdough bread to retain its baking characteristics. A pilot study in patients with CD suggested that this bread was well tolerated^[209]. However, these patients were challenged for only 2 d, which is clearly not sufficient to draw any firm conclusions. Hence, another 60-d diet of fully hydrolyzed wheat flour with sourdough lactobacilli and fungal proteases (8 ppm residual gluten; $n = 5$) was further studied. The pretreated flour was rendered non-toxic by serological, morphometrical, and immunohistochemical analysis^[211]. A larger group of subjects in the trial and palatability of digested flour baked products needs to be taken into consideration.

VSL#3: VSL#3 is a probiotic containing lyophilised bacteria, including bifidobacteria (*Bifidobacterium longum*, *Bifidobacterium infantis* and *Bifidobacterium breve*), lactobacilli (*Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus delbrueckii subsp.*, *Lactobacillus bulgaricus* and *Lactobacillus plantarum*) and *Streptococcus salivarius subsp.*, *Thermophiles*. It is used to hydrolyse gliadin peptides in pre-treated flour and tested for efficacy in rat intestinal cell line and celiac jejunal biopsies^[207]. VSL#3 pre-digested gliadins did not show an increase of the infiltration of CD3+ intraepi-

thelial lymphocytes and caused a less pronounced effect on intestinal mucosa permeability (determined by lower F-actin rearrangement and zonulin release). Hence, VSL#3 may have importance during food processing to produce pre-digested gluten-free products.

Prevention of toxic gliadin peptide absorption

Larazotide: Larazotide (AT-1001, Alba Therapeutics, Baltimore, MA), is a synthetic hexapeptide derived from Zonula Occludens toxin of *Vibrio cholera*^[212]. It is used to inhibit the opening of tight junctions of the small intestine epithelial cells. Clinical trial phase I in CD patients suggested that Larazotide therapy is well tolerated by patients and reduces intestinal barrier dysfunction, pro-inflammatory cytokine production, and gastrointestinal symptoms in CD individuals after gluten exposure^[213]. Encouraging results were obtained from a 6-wk phase II b trial in terms of symptoms and antibody titers^[214], showing larazotide acetate a promising drug candidate. This drug inhibits the paracellular route of gliadin absorption through tight junctions, which is not the only mechanism of gliadin absorption. Indeed, gliadin may gain access to the mucosa through transcellular pathways in addition to paracellular route^[135,137]. Hence, this strategy might be best exploited in combination with other treatments.

Synthetic polymer poly (hydroxyethylmethacrylate-co-styrene sulfonate): Poly (hydroxyethylmethacrylate-co-styrene sulfonate) [P (HEMA-co-SS)] forms supra-molecular particles upon gliadin complexation in gastric and intestinal conditions^[215,216], and deteriorates gliadin's effect on epithelial cells^[217]. This complexation decreases the effect of gastrointestinal (GI) digestive enzymes on gliadin absorption, and thus the formation of immunogenic peptides is reduced. Gluten-sensitive HLA-HCD4/DQ8 mice co-administered with P (HEMA-co-SS) showed attenuated gliadin-induced changes in permeability and inflammation^[217]. Low side effect, cost and possibility to be taken, occasionally with gluten-containing food, makes it an attractive option. Further investigation of the mechanisms of action and its interaction with human tissues is required before its efficacy is investigated in human trials^[218].

Anti-gliadin egg yolk antibody: Oral antibody passive immunotherapy may be of value due to the advantages of reduced cost, ease of administration, and potential to treat localized conditions in the gastrointestinal tract^[219]. Among antibodies, chicken egg yolk immunoglobulin (IgY), is ideal for passive immunotherapy, as it may be readily obtained in large quantities from egg yolk, presenting a more cost-effective, convenient, and hygienic alternative to mammalian antibodies. Oral immunotherapeutic IgY is a promising alternative to neutralize gliadin in the GI tract and prevention it from absorption^[220]. Mannitol contained antibody preparation is highly resistant against GI enzymes and proved to effectively

neutralized gliadin under simulated GI conditions in the presence of food. *In vivo* study; BALB/c mice fed with IgY formulation and gliadin ratio of 1:5 (w/w), demonstrated that gliadin absorption in the gastrointestinal tract was minimal at < 1%^[221]. Further investigations in CD patients is requires to prove its efficacy and determine dosing regimen of antibody relative to the amount of gliadin ingestion.

Blockage of selective deamidation of specific glutamine residues by tissue transglutaminase 2 inhibitor

Transglutaminases (a family of eight enzymes) have diverse functions in human and are involved in several biological and pathological processes^[222]. tTG2 is an enzyme that has a pro-inflammatory effect and increases the immunostimulatory epitopes present in the lamina propria of the small intestine. Blockage of tTG2 may be a promising approach to inhibit the inflammatory process upon gluten ingestion. There are two essential classes of tTG2 inhibitors; irreversible and reversible inhibitors^[223]. Irreversible inhibitors form a stable covalent bond with this enzyme, and thus prevent deamidation of gliadin peptides^[223,224]. Reversible inhibitors are more desirable to minimize possible side effects. These include aldehyde-bearing tTG modulators^[225], cinnamoyl triazole derivatives^[226], and the highly specific modified peptide targeting the active cysteine site of tTG2^[227]. Since a few gluten T-cell epitopes can be recognized without being deamidated by tTG2^[228,229], this approach will not inhibit the innate response^[101], or the immune response induced by non-deamidated peptides^[82]. To be able to use tTG2 inhibitors clinically, it is critical to design highly specific inhibitors, since all human tTG share high sequence homology.

Vaccine application to restore immune tolerance towards gluten

Autoimmune enteropathy in CD has been proposed to be due to impairment of immunoregulatory mechanisms that controls oral tolerance. Systematic peptide mapping of T-cell was performed to determine gliadin reactive epitopes recognized by approximately 90% of CD patients. A clinical trial phase I study has been initiated as Nexvax2[®] (Nexpep Pty, Ltd., Australia) peptide vaccine-containing mixture of immunotoxic α - and ω -gliadins and B-hordein^[230].

Engineered *Lactococcus lactis* secreting a DQ8-restricted gliadin peptide administered orally^[231], or recombinant α -gliadin in HLA-DQ8 administered intranasally in transgenic mouse model^[232], have been studied to modulate immune response to gluten. However, it is difficult to appreciate how the vaccine or the intranasal peptide administration can modulate the Tr1 response. More work is needed to assess the effect of these therapies on the spectrum of gluten peptides presented to the gut.

Dermal inoculation of human hookworm (*Necator americanus*) has also been used to modulate the immune response to gluten^[233]. A phase II trial with CD patients suggested that hookworm infection on its own may not

obviate the necessity for a restricted diet in CD, but appears to be safe and might impact on immune pathology^[234]. Here in, hookworm infection is expected to reduce gluten sensitivity and immune reactivity.

Modulation of immune response to dietary gliadin

HLA-DQ blocker: HLA-DQ blocker is used to block the binding sites of HLA-DQ2 or DQ8 for it to be unrecognized by T cells as well to block the presentation of the antigen. This is not a new concept that was developed without much success to treat type 1 diabetes mellitus and rheumatoid arthritis, due to difficulties in effective drug delivery^[235,236]. By amino-acid substitution of gliadin T-cell stimulatory sequence, the epitope can be converted to an agonist or antagonist, abolishing the inflammatory cascade^[237]. IFN- γ production by peripheral blood lymphocytes was prevented when either an alanine or lysine amino acid was substituted through the immunodominant α -gliadin peptide, corresponding to the peptide's anchor to the HLA-DQ cleft^[238]. To develop this as a new therapeutic agent, more studies need to be performed, looking at the mass T-cell action of the gut towards these modified peptides.

Interleukin blocker: Modulation of cytokine production has been evaluated for the treatment of several autoimmune diseases, although their side effects may be severe. Modulation of proinflammatory IL-15 and anti-inflammatory IL-10 cytokines has been suggested to influence the immune balance between tolerance and autoimmunity^[127,239-241]. Blocking IL-15 may promote maintenance of epithelial integrity, limit epithelial destruction, leading to decreased passage of dietary gliadin.

NKG2D antagonists: MICA molecules, strongly expressed on active CD epithelial cell surface upon gliadin challenge^[132], interact with the NKG2D-activating receptor on human natural killer cells and CD8 T cells, leading to villous atrophy due to an IEL-mediated damage to enterocytes^[131,132]. Thus, NKG2D antagonists^[131] and anti-NKG2D antibodies^[242], have been proposed as therapeutics in CD.

Restoration of intestinal architecture by R-spondin-1

R-spondin-1 is an intestinal mitogen, shown to stimulate crypt cell growth, accelerate mucosal regeneration and restore intestinal architecture in mouse models of colitis^[243]. This agent has yet to be tested in human to be considered as a therapeutic agent in CD.

CONCLUSION

CD has been kept in the dark for decades with very little known about what is a relatively common medical condition. It is only recently that we have greater understanding of its prevalence, diagnosis and pathogenesis, which has supported the development of new therapeutic approaches to treat CD. There are several future

Table 9 Key points from recent findings

Cause
Environmental (gluten) and genetic factors (HLA and non-HLA genes)
Prevalence
0.5%-1% worldwide in normal at-risk population
Higher risk in the population with diabetes, autoimmune disorder or relatives of CD individuals
Pathogenesis
Gliadin gains access <i>via</i> trans- and para-cellular routes to the basal surface of the epithelium, and interact directly with the immune system
Types of CD symptoms: "typical" or "atypical"
Diagnosis
Positive serological (TGA or EMA) screening results suggestive of CD, should lead to small bowel biopsy followed by a favourable clinical and serological response to the GFD to confirm the diagnosis
Current treatment
Strict life-long GFD
Alternative future CD treatments strategies
Hydrolysis of toxic gliadin peptide
Prevention of toxic gliadin peptide absorption
Blockage of deamidation of specific glutamine residues by tissue
Restoration of immune tolerance towards gluten
Modulation of immune response to dietary gliadin
Restoration of intestinal architecture

HLA: Human leukocyte antigen; CD: Celiac disease; EMA: Endomysial; TGA: Transglutaminase; GFD: Gluten-free diet.

directions to follow to treat CD, which if successful will supplement or even replace the current only effective treatment, the use of a GFD. A greater understanding of the pathogenesis of CD allows alternative future CD treatments to hydrolyse toxic gliadin peptide, prevent toxic gliadin peptide absorption, blockage of selective deamidation of specific glutamine residues by tissue, restore immune tolerance towards gluten, modulation of immune response to dietary gliadin, and restoration of intestinal architecture (Table 9).

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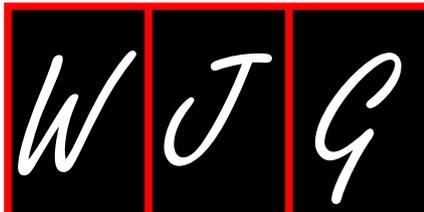
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Current progress in the treatment of chronic hepatitis C

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Abstract

Over the last decade, the standard of care for the treatment of chronic hepatitis C has been the combination of pegylated-interferon-alfa (PEG-IFN) and ribavirin (RBV) which results in sustained virological response (SVR) rates of 75%-85% in patients with genotypes 2 or 3 but only of 40%-50% in patients with genotype 1. Currently, there are rapid and continuous developments of numerous new agents against hepatitis C virus (HCV), which are the focus of this review. Boceprevir and telaprevir, two first-generation NS3/4A HCV protease inhibitors, have been recently licensed in several countries around the world to be used in combination with PEG-IFN and RBV for the treatment of genotype 1 patients. Boceprevir or telaprevir based triple regimens, compared with the PEG-IFN/RBV combination, improve the SVR rates by 25%-31% in treatment-naïve genotype 1 patients, by 40%-64% in prior relapsers, by 33%-45% in prior partial responders and by 24%-28% in prior null responders. At the same time, the application of response-guided treatment algorithms according to the on-treatment virological response results in shortening of the total therapy duration to only 24 wk in 45%-55% of treatment-naïve patients. There are, however, several

challenges with the use of the new triple combinations in genotype 1 patients, such as the need for immediate results of HCV RNA testing using sensitive quantitative assays, new and more frequent adverse events (anemia and dysgeusia for boceprevir; pruritus, rash and anemia for telaprevir), new drug interactions and increasing difficulties in compliance. Moreover, the SVR rates are still poor in very difficult to treat subgroups of genotype 1 patients, such as null responders with cirrhosis, while there is no benefit for patients who cannot tolerate PEG-IFN/RBV or who are infected with non-1 HCV genotype. Many newer anti-HCV agents of different classes and numerous combinations are currently under evaluation with encouraging results. Preliminary data suggest that the treatment of chronic HCV patients with well tolerated combinations of oral agents without PEG-IFN is feasible and may lead to a universal HCV cure over the next 5-10 years.

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Key words: Chronic hepatitis C; Pegylated interferon; Ribavirin; Protease inhibitors; Nucleos(t)ide analogue inhibitors; Non-nucleos(t)ide analogue inhibitors; Hepatitis C virus polymerase; NS5A inhibitors; Cyclophilin inhibitors

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INTRODUCTION

Chronic hepatitis C virus (HCV) infection affects approximately 170 million people worldwide^[1]. Chronic hepatitis C may lead to the development of cirrhosis, liver decompensation and hepatocellular carcinoma and is a major indication for liver transplantation, particularly in Western countries^[2]. HCV is classified into 6 major genotypes. Some genotypes have a restricted geographical distribution (genotypes 4-6), while others (genotypes 1-3) are more broadly disseminated. Genotype 1 (subtypes 1a and 1b) is the most prevalent genotype in the world. Genotype 2 is found in clusters in the Mediterranean region, genotype 3 is most prevalent among intravenous drug users and genotype 4 is found mostly in Egypt, while genotypes 5 and 6 are less frequent^[3,4]. The HCV genotypes strongly affect the likelihood of the response to treatment.

During the last decade, the standard of care (SOC) for chronic HCV patients consisted of pegylated interferon-alfa (PEG-IFN)-2a or -2b combined with ribavirin (RBV). The treatment duration has been based on the on-treatment virological responses, mainly estimated at 4 wk [rapid virological response (RVR)] and 12 wk of therapy (early virological response)^[3,5]. Recently, polymorphisms of the interleukin 28b (*IL28B*, interferon lambda 3) gene were strongly associated with the rates of sustained virological response (SVR) to PEG-IFN/RBV therapy and therefore their determination may be useful to identify a patient's likelihood of response to treatment, but the predictive value is low^[5]. In patients with HCV genotypes 2 or 3, the combination of PEG-IFN/RBV is usually given for 24 wk, achieving rates of SVR, i.e., absence of HCV RNA at 6 mo or more after cessation of therapy, of about 75%-85%. In patients with HCV genotypes 1 and 4, the combination of PEG-IFN and RBV is usually given for 48 wk, resulting in SVR rates of 40%-50% for genotype 1 and 55%-65% for genotype 4 patients^[5,6]. The SVR rates are substantially lower in previous non-responders to PEG-IFN and RBV, in whom the proportion of genotype 1 patients is higher due to the lower initial SVR rates. It has been reasonable, therefore, that new treatments with improved efficacy were mostly needed for patients with genotype 1. In addition and regardless of HCV genotype, there are chronic HCV patients who cannot be treated with PEG-IFN and RBV for several reasons. First and of most clinical relevance, PEG-IFN therapy is contraindicated in patients with decompensated liver disease. Second, patients may not tolerate and/or may have other contraindication(s) to PEG-IFN or RBV. Thus, there has definitely been a need for new antiviral drugs with better efficacy, improved tolerance and good safety profiles for chronic HCV patients.

The current review focuses on the recent rapid and continuous developments in the management of chronic HCV infection, which have been based on a better understanding of the structure of the HCV genome and the key viral enzymes.

HCV GENOME ORGANIZATION AND NEW ANTIVIRALS

HCV has a positive-sense, single-stranded RNA genome of some 9.6 kilobases that encodes a polyprotein of about 3000 amino acids^[7,8]. The open reading frame for the polyprotein is flanked by 5' and 3' untranslated regions, which contain elements that regulate translation and replication. The polyprotein is generated by the host cell translation machinery and cleaved co- and post-translationally by viral and host proteases to yield the mature viral proteins. The N-terminal segment of the polyprotein encodes the structural components of the virus (Core, E1, E2 and p7). Core protein forms the capsid shell into which the virus genome is packaged, while the glycoproteins are considered to locate to the lipid envelope surrounding the capsid. P7 is required for the virus assembly^[9,10].

The C-terminal component of the polyprotein contains non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B). NS2 and NS3 are viral proteases required for the processing of the HCV polyprotein^[11]. NS3 is a multifunctional enzyme, which provides a serine protease and helicase/nucleotide triphosphatase activity and forms a stable heterodimeric complex with its cofactor NS4A, which is essential for protein folding. The NS3/NS4A complex cleaves the junction between NS3/4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B. NS3 has also a helicase activity and is necessary for HCV replication supporting the unwinding of HCV^[12]. NS4B is the presumed central organizer of the HCV replicase complex and a main inducer of intracellular membrane rearrangements^[13]. NS5A is a RNA-binding phosphoprotein required for RNA replication and assembly of infectious virus particles^[14]. NS5B RNA-dependent RNA-polymerase is required for viral replication.

The nonstructural proteins mentioned above have been the major targets for the evolving directly acting antivirals (DAAs) or specifically targeted antiviral therapy for hepatitis C. In particular, DAAs under development mainly include NS3/4A protease inhibitors, NS5B polymerase inhibitors and NS5A inhibitors. In addition, inhibitors of cyclophilin, which is a host protein with an important role in the HCV lifecycle, are also under development (Table 1).

NS3/4A PROTEASE INHIBITORS

Two first-generation, linear NS3/4A protease inhibitors, boceprevir and telaprevir, have recently been approved in several countries around the world for clinical use in patients with genotype 1, while many new NS3/4 protease inhibitors are currently under evaluation in clinical trials (Table 1). Boceprevir and telaprevir have high antiviral potency only against genotypes 1 and 2^[15], but a low barrier to resistance. In particular, resistant HCV strains develop within a few days of monotherapy with one of these two agents^[16,17], while most mutations confer

Table 1 New agents against hepatitis C virus currently evaluated in phase II or III trials

Drug	Company
NS3/4A protease inhibitors	
First generation, linear	
Boceprevir (Approved)	MERCK
Telaprevir (Approved)	JANSSEN
Narlaprevir	MERCK
First generation, macrocyclic	
BI201335	BOEHRINGER Ingelheim
TMC435	TIBOTEC/JANSSEN
Danoprevir	ROCHE
Vaniprevir	MERCK
Asunaprevir	BRISTOL-MYERS SQUIBB
ABT-450	ABBOTT
GS-9451	GILEAD
GS-9256	GILEAD
ACH-1625	ACHILLION
Second generation, macrocyclic	
MK-5172	MERCK
Nucleos(t)ide analogue inhibitors of HCV polymerase	
PSI/GS-7977	PHARMASSET/GILEAD
Mericitabine	ROCHE
IDX-184	IDENIX
Non-nucleoside analogue inhibitors of HCV polymerase	
Tegobuvir	GILEAD
Filibuvir	PFIZER
ANA-598/Setrobuvir	ANADYS/ROCHE
BI207127	BOEHRINGER Ingelheim
ABT-333	ABBOTT
ABT-072	ABBOTT
VX-222	VERTEX
NS5A inhibitors	
Daclatasvir	BRISTOL-MYERS SQUIBB
GS-5885	GILEAD
GSK2336805	GSK
Cyclophilin inhibitors	
Alisporivir ¹	NOVARTIS
SCY-635	Scynexis

¹On hold by the United States Food and Drug Administration because of toxicity (pancreatitis cases with one death). HCV: Hepatitis C virus.

cross-resistance to both drugs (V36A/M, T54S/A, V55A, R155K/T/Q, A156S, A156T/V)^[18]. HCV subtype 1a develops resistance more frequently and more rapidly than subtype 1b^[19], as just one (instead of two in subtype 1b) nucleotide change (R155K) is enough for an amino acid replacement and emergence of a resistant strain. Because of the low barrier to resistance, boceprevir and telaprevir should always be used in triple combinations together with PEG-IFN/RBV. Since viral resistance may develop even in triple combinations with PEG-IFN/RBV, strict stopping rules are recommended to be applied with the use of boceprevir- or telaprevir-based regimens. Newer, first-generation NS3/4A inhibitors under development are mainly macrocyclic (danoprevir, vaniprevir, asunaprevir, *etc.*), and are expected to have better pharmacokinetics and tolerability compared with boceprevir and telaprevir. Second-generation NS3/4A inhibitors (MK-5172) are expected to have pan-genotype antiviral activity and improved resistance profiles.

Efficacy data from phase III clinical trials with boceprevir- or telaprevir-based regimens in treatment-naïve patients with genotype 1

SPRINT-2 was a randomized, double-blind, placebo-controlled trial designed to evaluate the efficacy and safety of boceprevir-based regimens compared with the previous SOC, as well as the efficacy of boceprevir-based response-guided therapy (RGT) compared with a fixed 48-wk boceprevir regimen^[20]. In total, 1097 (938 nonblack and 159 black) treatment-naïve genotype 1 patients were randomly assigned to one of three treatment arms (1:1:1). They all received PEG-IFN-2b and RBV for the first 4 wk (lead-in period) followed by (1) placebo plus PEG-IFN-2b/RBV for 44 wk (control arm); (2) boceprevir plus PEG-IFN-2b/RBV for 24 wk with or without an additional 20-wk course of placebo plus PEG-IFN-2b/RBV (boceprevir RGT arm); or (3) boceprevir plus PEG-IFN-2b/RBV for a standard period of 44 wk (boceprevir fixed arm). PEG-IFN-2b was administered subcutaneously at a dose of 1.5 µg/kg per week, RBV orally at a total daily dose of 600-1400 mg according to body weight and boceprevir orally with food at a dose of 800 mg every 7-9 h. In the boceprevir RGT arm, treatment was stopped at 28 wk in patients who achieved an extended RVR (eRVR) defined as undetectable HCV RNA between 8 and 24 wk, or continued until week 48 in patients who did not achieved such an eRVR.

The SVR rate was significantly lower in the control arm (38%) compared with both boceprevir arms (RGT: 63%, fixed: 66%; $P < 0.001$) (Table 2). In all arms, black patients achieved lower SVR rates compared with non-black patients (control arm: 23% *vs* 40%, boceprevir RGT arm: 42% *vs* 67%, boceprevir fixed arm: 53% *vs* 69%). In the RGT arm, a total of 44% of patients were eligible to receive only 28 wk of treatment having an excellent SVR rate of 96% (97% for nonblacks and 87% for blacks). The relapse rates were 22% in the control arm and 9% in the two boceprevir arms. In conclusion, boceprevir-based regimens, compared with the PEG-IFN/RBV combination, offer a 25%-28% benefit in the likelihood of SVR in treatment-naïve genotype 1 patients, while a RGT-based duration of boceprevir-based therapy has excellent results in patients with an eRVR and is not overall inferior than a fixed 48-wk boceprevir-based regimen.

ADVANCE was a randomized, double-blind, placebo-controlled trial designed to evaluate the efficacy and safety of telaprevir-based regimens compared with previous SOC as well as the optimal duration of telaprevir triple combination^[21]. In total, 1088 treatment-naïve genotype 1 patients were randomized to one of three treatment arms (1:1:1): (1) telaprevir plus PEG-IFN-2a/RBV for the first 12 wk followed by 12 or 36 wk of PEG-IFN-2a/RBV (T12PR arm); (2) telaprevir plus PEG-IFN-2a/RBV for the first 8 wk and placebo plus PEG-IFN-2a/RBV for another 4 wk followed by 12 or 36 wk of PEG-IFN-2a/RBV (T8PR arm); or (3) PEG-IFN-2a/RBV for 48 wk together with placebo

Table 2 Sustained virological response rates in phase III clinical trials with hepatitis C virus protease inhibitor-based regimens in treatment-naïve and treatment-experienced patients

Name of trial	SVR
Naïve patients - Treatment group	
SPRINT-2	
BOC/RGT	63%
BOC44/PR48	66%
PR48	38%
ADVANCE	
T12PR	75%
T8PR	69%
PR	44%
ILLUMINATE	
T12PR24 (eRVR+)	92%
T12PR48 (eRVR+)	87%
T12PR48 (eRVR-)	64%
< 20 wk	23%
Treatment-experienced patients - Treatment group	
RESPOND-2	
Prior relapsers	PR48 29%
	BOC/RGT 69%
	BOC44/PR48 75%
Prior partial responders	PR48 7%
	BOC/RGT 40%
	BOC44/PR48 52%
REALIZE	
Prior relapsers	PR48 24%
	LIT12PR48 88%
	T12PR48 83%
Prior partial responders	PR48 15%
	LIT12PR48 54%
	T12PR48 59%
Prior null responders	PR48 5%
	LIT12PR48 33%
	T12PR48 29%

BOC: Boceprevir; RGT: Response-guided therapy; P: Pegylated interferon- α ; R: Ribavirin; T: Telaprevir; eRVR: Extended rapid virological response; LI: Lead-in; SVR: Sustained virological response.

for the first 12 wk (PR arm). PEG-IFN-2a was administered subcutaneously at a standard weekly dose of 180 μ g, RBV orally at a total daily dose of 1000-1200 mg according to body weight and telaprevir orally with food at a dose of 750 mg every 8 h. In both telaprevir arms, treatment was stopped at 24 wk in patients who achieved an eRVR defined as undetectable HCV RNA at weeks 4 and 12, and continued up to 48 wk in patients who did not achieve such an eRVR.

Significantly more patients in the telaprevir arms achieved an SVR compared with controls (75% and 69% *vs* 44%, $P < 0.001$), while patients of the T12PR arm showed a trend for a higher SVR rate compared with patients of the T8PR arm, but this did not reach statistical significance (T12PR: 75% *vs* T8PR: 69%, $P = 0.088$). The rates of eRVR were 57% and 58% in the T12PR and T8PR arms, respectively, compared with 8% in the control arm. Among those patients with an eRVR who received only 24 wk of therapy, an SVR was achieved in 89% and 83% of cases in the T12PR and T8PR arms, respectively. Among patients who did not achieve an eRVR and continued therapy up to 48 wk,

the SVR rates were 54% and 50% in the T12PR and T8PR arms, respectively (Table 2). The relapse rate was 9% in both telaprevir arms compared with 28% in the control arm.

Based on these data, we can conclude that telaprevir-based regimens, compared with the PEG-IFN/RBV combination, offer a 25%-31% benefit in the likelihood of SVR in treatment-naïve genotype 1 patients despite more than 50% of patients in the telaprevir arms receiving only 24 wk instead of 48 wk of therapy. Because of the numerically higher response rates in the T12PR than in the T8PR arm, the 12-wk telaprevir triple combination regimens were considered to be optimal for the treatment of genotype 1 patients.

ILLUMINATE was another phase III trial which included 440 treatment-naïve genotype 1 patients to assess whether 24 wk of a telaprevir-based regimen was sufficient for patients with an eRVR^[22]. All patients received telaprevir plus PEG-IFN-2a/RBV in the same doses used in the ADVANCE trial for the first 12 wk followed by PEG-IFN-2a/RBV for 12 or 36 wk^[3]. In particular, patients with an eRVR (undetectable HCV RNA at weeks 4 and 12) were randomized at week 20 to continue PEG-IFN-2a and RBV until 24 or 48 wk, while all patients without an eRVR were maintained on PEG-IFN-2a/RBV until 48 wk. Among the 60% of patients who achieved an eRVR and continued treatment after 20 wk, SVR rates were comparable between those treated for a total duration of 24 or 48 wk (92% *vs* 88%, respectively) (Table 2). In the 22% of patients who did not achieve an eRVR but continued treatment after 20 wk, the SVR rate was 64%, while treatment was discontinued prematurely before the randomization at week 20 in 18% of cases. The conclusion of the ILLUMINATE trial was that 24 wk of a telaprevir-based regimen is enough for the treatment-naïve genotype 1 patients who achieve an eRVR.

Efficacy data from phase III clinical trials with boceprevir- or telaprevir-based regimens in treatment-experienced patients with genotype 1

RESPOND-2 was a randomized, placebo-controlled trial designed to evaluate the efficacy and safety of boceprevir-based regimens compared with previous SOC for the retreatment of treatment-experienced genotype 1 patients. In total, 403 patients (259 relapsers: HCV RNA undetectable at the end but detectable at 6 mo after the end of previous therapy; 144 partial responders: HCV RNA decline $> 2 \log_{10}$ IU/mL at 12 wk but detectable during previous therapy) were randomly assigned to one of three treatment arms (1:2:2) similar to those used in the SPRINT-2 trial (control, boceprevir RGT and boceprevir fixed 48-wk arm)^[23]. The only difference was in the RGT arm, which included the initial 4-wk lead-in phase with only PEG-IFN-2b and RBV followed by boceprevir plus PEG-IFN-2b/RBV for 32 wk (week 4 to 36) with or without the addition of 12 wk of PEG-IFN-

2b/RBV in patients with or without detectable HCV RNA at 8 wk of therapy.

SVR rates were higher in the two boceprevir arms (RGT: 59%; fixed: 66%) than in the control arm (21%, $P < 0.001$) (Table 2). SVR rates were also higher in the boceprevir arms in both relapsers (RGT: 69%, fixed: 75%, control: 29%) and partial responders (RGT: 40%, fixed: 52%, control: 7%). Among patients with undetectable HCV RNA at week 8, SVR was 86% after 36 wk of therapy in the boceprevir RGT arm and 88% after 48 wk of therapy in the boceprevir fixed arm. The overall SVR rates were found to be lower in the boceprevir RGT than the fixed arm in patients with advanced fibrosis (metavir F3-F4) (44% *vs* 68%) or mostly in patients with cirrhosis (35% *vs* 77%), but similar between these two arms in patients with milder fibrosis. The negative effect of cirrhosis on SVR was observed in both prior relapsers and prior partial responders. The probability of an SVR was also significantly higher in patients with than without a $> 1 \log_{10}$ IU/mL HCV RNA drop at the end of the 4-wk lead-in phase (76% *vs* 33%). These data showed that boceprevir-based regimens compared with the PEG-IFN/RBV combination can improve the SVR rates by 40%-46% in previous relapsers and by 33%-45% in previous partial responders with genotype 1. Moreover, it was shown that a boceprevir-based RGT might be applied in treatment-experienced non-cirrhotic patients who achieve early (at 8 wk) HCV RNA undetectability. It should be noted, however, that the probability of an SVR is not very high in patients without a $> 1 \log_{10}$ IU/mL HCV RNA drop at the end of the 4-wk lead-in phase of a boceprevir-based regimen.

Since null responders ($< 2 \log_{10}$ IU/mL decline in HCV RNA at 12 wk) were not included in the RESPOND-2 trial, a fixed 48-wk boceprevir-based regimen (4-wk lead-in with PEG-IFN-2b/RBV followed by 44 wk of triple combination with boceprevir plus PEG-IFN-2b/RBV) was subsequently evaluated in a rollover, single arm, prospective study^[24]. Preliminary results reported an SVR rate of 38% in 42 previous null responders.

REALIZE was a randomized, placebo-controlled trial designed to evaluate the efficacy and safety of telaprevir-based regimens compared with the previous SOC in treatment-experienced genotype 1 patients as well as to determine whether a 4-wk lead-in therapy with only PEG-IFN/RBV can affect the probability of SVR in telaprevir-based regimens^[1]. In total, 663 patients (354 relapsers, 124 partial responders, 184 null responders) were randomly assigned to one of three arms (1:2:2): (1) telaprevir plus PEG-IFN-2a/RBV for the first 12 wk followed by PEG-IFN-2a/RBV for another 36 wk (T12PR48 arm); (2) 4-wk lead-in phase with PEG-IFN-2a/RBV and then telaprevir plus PEG-IFN-2a/RBV for 12 wk followed by PEG-IFN-2a/RBV for another 32 wk (lead-in T12PR48 arm); or (3) PEG-IFN-2a/RBV for 48 wk (control PR48 arm).

SVR rates were similar in the two telaprevir arms

(64% and 66%) and significantly higher compared with the control arm (17%, $P < 0.001$). In particular, SVR rates were higher in the telaprevir arms in prior relapsers (83% and 88% *vs* 24%, $P < 0.001$), prior partial responders (59% and 54% *vs* 15%, $P < 0.001$) and prior null responders (29% and 33% *vs* 5%, $P < 0.001$) (Table 2). The presence of cirrhosis was found to negatively affect the SVR rates in the telaprevir arms in prior partial responders (mild-moderate fibrosis: 72%, bridging fibrosis: 56%, cirrhosis: 34%) and mostly in prior null responders (mild-moderate fibrosis: 41%, bridging fibrosis: 39% cirrhosis: 14%), but not in prior relapsers (mild-moderate fibrosis: 86%, bridging fibrosis: 85% cirrhosis: 84%). Among the patients of the lead-in telaprevir arm, the probability of an SVR was significantly higher in patients with than without a $> 1 \log_{10}$ IU/mL HCV RNA drop at the end of the 4-wk lead-in phase (82% *vs* 33%), but this effect was more clinically relevant in prior null responders (54% *vs* 15%) than in prior partial responders (59% *vs* 56%) or in prior relapsers (94% *vs* 62%). Thus, according to these data, telaprevir-based regimens compared with PEG-IFN/RBV improve the SVR rates by 59%-64% in prior relapsers, by 39%-45% in prior partial responders and by 24%-28% in prior null responders, while a 4-wk lead-in phase with only PEG-IFN/RBV does not offer any advantage to telaprevir-based regimens.

Safety issues with boceprevir- or telaprevir-based regimens

The most common and clinically important adverse event in the boceprevir trials was anemia, which developed in approximately 50% of patients treated with boceprevir-based regimens, compared with 30% of patients treated with only PEG-IFN/RBV^[20,23]. Erythropoietin was administered by the investigators in 41%-46% of boceprevir-treated patients and in 21%-24% of the controls, while discontinuation due to anemia was necessary in 2% of patients in the boceprevir arms and in 1% of patients in the control arms. It should be noted that the SVR rates in the boceprevir arms were similar in patients with or without anemia, with or without erythropoietin use and with or without RBV dose reduction. Dysgeusia was another clinically important adverse event that was reported more frequently in the boceprevir than in the control arms (37%-43% *vs* 18%)^[20,23].

The most clinically important adverse events in the telaprevir trials were pruritus, rash and anemia. In particular, pruritus was reported by 45%-50% of telaprevir-treated patients compared with 36% of controls, and rash developed in 35%-56% and 19%-37% of cases, respectively^[21,22,25]. The rash during telaprevir therapy was typically eczematous and mild-to-moderate in $> 90\%$ of patients, while it was severe (involving $> 50\%$ of the body surface area) in 6%, leading to discontinuation of telaprevir in 5%-7% of patients (1% of controls) and of all drugs in 0.5%-1.4% of patients treated with telaprevir-based regimens (0% in controls)^[21,22,25].

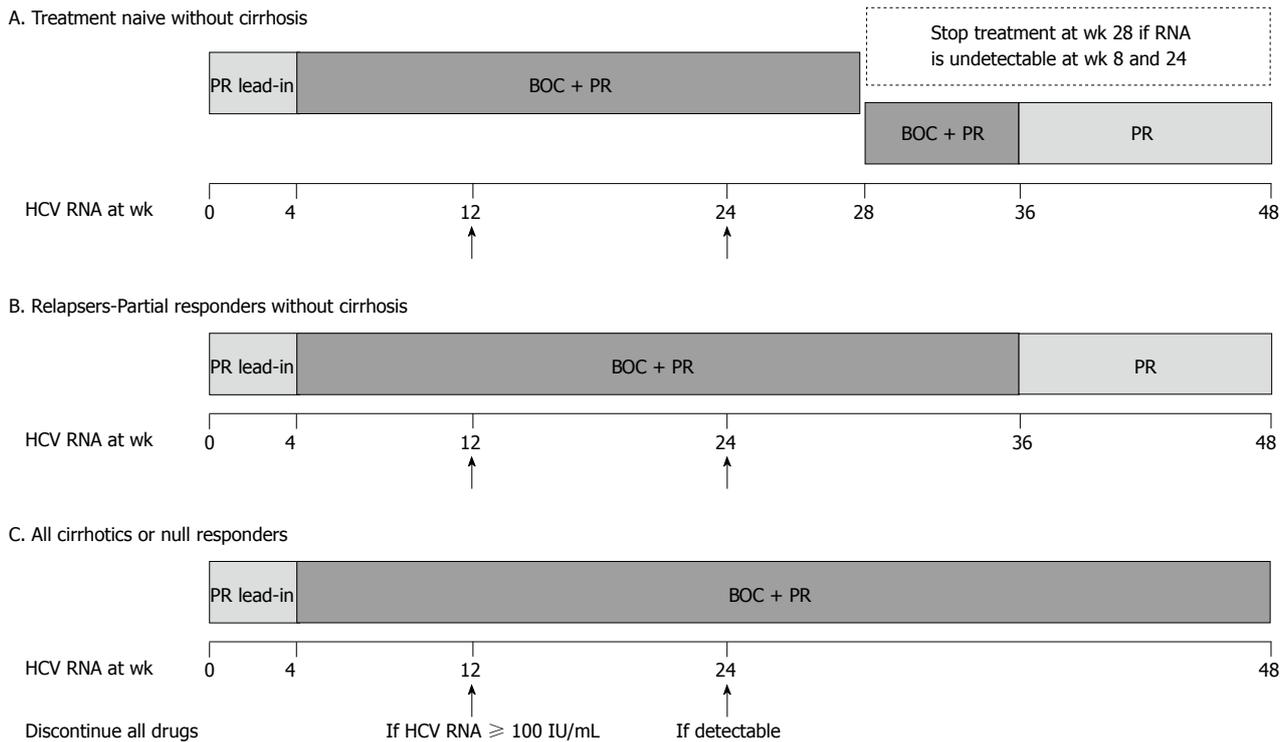


Figure 1 Treatment algorithms of boceprevir-based regimens for genotype 1 chronic hepatitis C virus patients recommended by the European Medicines Agency. All patients should start with a 4-wk lead-in phase with only pegylated interferon-alfa and ribavirin (PR). After 4 wk, boceprevir (BOC) is added. In treatment-naïve patients without cirrhosis who achieve an extended rapid virological response [eRVR: undetectable hepatitis C virus (HCV) RNA (< 10 IU/mL) at 8 and 24 wk], the triple therapy should last 24 wk and treatment end at 28 wk. In non-cirrhotic treatment-naïve patients who do not achieve such an eRVR and in all previous relapsers or partial responders without cirrhosis, the triple therapy should last 32 wk (until 36 wk of therapy) and should be followed by an additional 12 wk of PR. The United States Food and Drug Administration (FDA) recommendations suggest that prior relapsers or partial responders without cirrhosis who achieve an eRVR under BOC triple therapy can stop therapy at 36 wk without an additional 12-wk course of PR that is suggested by the European Medicines Agency. Finally, in all cirrhotics (treatment-naïve and experienced) and null responders, the triple therapy should last 44 wk (up to 48 wk of treatment). All patients should be tested for HCV RNA levels at 12 and 24 wk of total therapy and treatment should be discontinued for inefficacy if HCV RNA levels are > 100 IU/mL at 12 wk or HCV RNA is still detectable at 24 wk of therapy.

The mean time for the occurrence of rash was 22 d and the majority of rashes occurred during the first 4 wk of therapy. Anemia also developed more commonly in telaprevir-treated patients than in controls (37%-39% *vs* 19%)^[21,22,25]. Anemia was managed with RBV dose reduction, which did not affect the SVR rate^[21,22,25].

Another problem that may arise with the use of the protease inhibitors is the interactions with concomitant medications. Both boceprevir and telaprevir use hepatic drug metabolizing enzymes such as cytochrome P450 2C (CYP2C), CYP3A4, or CYP1A^[15]. Therefore, caution is definitely required for the use of these agents in patients taking other drugs metabolized by the same pathways, such as statins, calcineurin inhibitors, antiretroviral agents, methadone, *etc.*^[15]. Updated information on the possible drug-drug interactions with boceprevir and telaprevir can be found at the Food and Drug Administration (FDA) website (www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm) or other relevant sites (e.g., www.hep-druginteractions.org).

Use of boceprevir or telaprevir in current clinical practice

Both boceprevir and telaprevir have now been approved

for the treatment of genotype 1 patients in several countries around the world. However, the approved treatment algorithms differ between the two drugs and according to the type of patients (Figures 1 and 2). In addition, there are some differences between the treatment algorithms of boceprevir-based regimens recommended by the United States and European regulatory authorities (Figure 1).

All patients who receive boceprevir should start with a 4-wk lead-in phase with only PEG-IFN/RBV. PEG-IFN-2b or PEG-IFN-2a may be used, while RBV should be administered at a weight-based dosage. After 4 wk, boceprevir is added, given with food at a dose of 800 mg (4 capsules of 200 mg each) every 8 h. In treatment-naïve patients without cirrhosis who achieve an eRVR [undetectable HCV RNA by a sensitive polymerase chain reaction (PCR) assay (HCV RNA < 10 IU/mL) at 8 and 24 wk], the triple therapy should last 24 wk and treatment finish at 28 wk. In non-cirrhotic treatment-naïve patients who do not achieve an eRVR and in all previous relapsers or partial responders without cirrhosis, triple therapy should last 32 wk (until 36 wk of therapy) and should be followed by an additional 12 wk of PEG-IFN/RBV. Finally, in all cirrhotics (treatment-naïve and experienced)

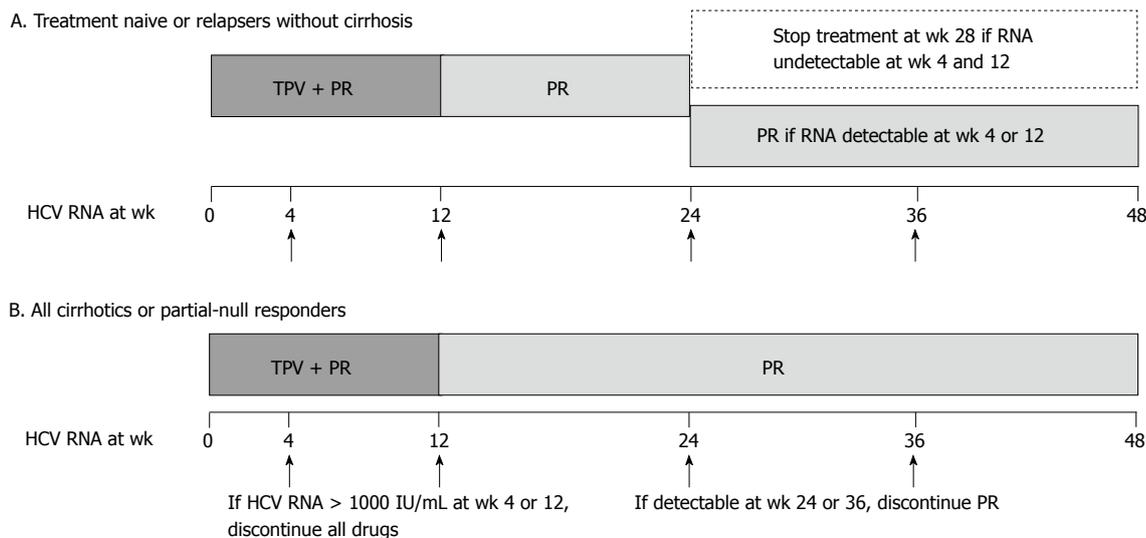


Figure 2 Treatment algorithms of telaprevir-based regimens for genotype 1 chronic hepatitis C virus patients recommended by both the European Medicines Agency and the Food and Drug Administration. All patients should start directly with the triple combination of telaprevir (TPV) plus pegylated interferon- α and ribavirin (PR). The triple combination should always last 12 wk. In treatment-naïve or previous relapser patients without cirrhosis who achieve an extended rapid virological response (eRVR) [undetectable hepatitis C virus (HCV) RNA (< 10 IU/mL) at 4 and 12 wk], triple therapy is followed by 12 wk of PR. In contrast, in non-cirrhotic treatment-naïve or relapser patients without an eRVR as well as in all cirrhotics or previous partial and null responders, triple therapy is followed by 36 wk of PR. Treatment should be discontinued for inefficacy if HCV RNA levels are > 1000 IU/mL at 4 or 12 wk or if HCV RNA is detectable at 24 or 36 wk of therapy.

and null responders, triple therapy should last 44 wk (up to 48 wk of treatment). All patients should be tested for HCV RNA levels at 12 and 24 wk of total therapy and treatment should be discontinued for inefficacy if HCV RNA levels are > 100 IU/mL at 12 wk or HCV RNA is still detectable at 24 wk of therapy.

All patients who receive telaprevir should start directly with the triple combination of telaprevir plus PEG-IFN/RBV. Theoretically, PEG-IFN-2a or PEG-IFN2b may be used, although PEG-IFN-2a has been used in all telaprevir trials. RBV is administered at a weight-based dosage, while telaprevir should be administered orally with a fatty meal at a dose of 750 mg (2 capsules of 375 mg each) every 8 h. The triple combination should always last 12 wk. In treatment-naïve or previous relapser patients without cirrhosis who achieve an eRVR [undetectable HCV RNA by a sensitive PCR assay (HCV RNA < 10 IU/mL) at 4 and 12 wk], triple therapy is followed by 12 wk of PEG-IFN and RBV. In contrast, in non-cirrhotic treatment-naïve or relapser patients without an eRVR as well as in all cirrhotics or previous partial and null responders, triple therapy is followed by 36 wk of PEG-IFN/RBV. Treatment should be discontinued for inefficacy if HCV RNA levels are > 1000 IU/mL at 4 or 12 wk or if HCV RNA is detectable at 24 or 36 wk of therapy.

OTHER DIRECT-ACTING ANTIVIRALS

NS5B polymerase inhibitors

There are 2 categories of NS5B polymerase inhibitors: nucleos(t)ide inhibitors (NIs) and non-nucleoside inhibitors (NNIs) (Table 1). NIs mimic the naturally occurring nucleotides and thus are incorporated into the nascent

RNA chain causing chain termination^[26]. NIs are considered to have a high genetic barrier to resistance, although single amino acid substitutions are able to confer drug resistance *in vitro*. Nevertheless, because the active site of NS5B is highly conserved and amino acid substitutions in any position of the active site can result in loss of function, such resistant variants fit poorly, requiring weeks or months to grow to detectable levels in the presence of the drug. NIs have antiviral activity against all HCV genotypes (pan-genotype activity) as the active site of NS5B is well conserved across genotypes^[27]. GS-7977 seems to be a promising representative of the NIs, as it appears to be rather safe and effective, achieving very high SVR rates (100%) in genotype 2 and 3 patients even when the drug is given for 12 wk only in combination with RBV^[28].

NNIs bind to a distant site of the HCV polymerase and cause a conformational change rendering the enzyme ineffective. In particular, NNIs bind to one of 4 allosteric sites at the surface of HCV polymerase (“thumb” domain I, “thumb” domain II, “palm” domain I, “palm” domain II). NNIs have a more limited spectrum of activity being specifically against genotype 1. Because NNIs bind more distantly from the active site, resistant variants can fit in the presence of the drug, and therefore NNIs have a low barrier to resistance.

NS5A inhibitors

NS5A protein is a regulator of replication. NS5A inhibitors have high antiviral activity against different genotypes, but they have a low genetic barrier to resistance. Daclatasvir, a representative of this group (Table 1), is under evaluation in several combinations with promising results^[29].

Cyclophilin A inhibitors

Cyclophilins are host proteins involved in protein folding. They play an important role in the HCV life cycle as a regulator of replication. The cyclophilin inhibitor, alisporivir (DEB-025) (Table 1), is a cyclosporine analogue without immunosuppressive properties that has shown pan-genotype antiviral activity and has been used either alone or in combination with PEG-IFN/RBV with promising results^[30-32]. Phase III trials with alisporivir were ongoing, but very recently the development of this drug was put on hold by the FDA due to safety concerns (a few cases of pancreatitis, one of which was fatal). SCY-635 (Scynexis) is another cyclophilin inhibitor under development.

NEW COMBINATIONS

Numerous trials of many combinations of the above drugs from different classes are currently ongoing. Much effort and interest has been given to the development of PEG-IFN-free regimens. Protease inhibitors have been combined with NNIs (telaprevir plus VX-222^[33], BI201335 plus BI207127^[34,35], GS-9256 plus tegobuvir^[36]), NIs (danoprevir plus mericitabine)^[37] or NS5A inhibitors as double or triple combinations including RBV^[28]. Double combinations of a NI with RBV (GS-7977 and RBV) or with NS5 inhibitors are also being evaluated. Promising examples of PEG-IFN-free trials include the combination of the NI GS-7977 with RBV which has been shown to achieve an SVR in 10 out of 10 genotype 2 or 3 treatment-naïve chronic HCV patients treated for 12 wk^[28,38] or the combination of the NS5A inhibitor daclatasvir and the NS3 protease inhibitor asunaprevir, which has shown interesting results in difficult to treat, genotype 1, prior null responders treated for 24 wk. The latter combination showed that an SVR can be achieved in 36% of 11 genotype 1 (mostly 1a) prior null responders from the United States^[29] and in $\geq 90\%$ of 21 genotype 1b prior null responders coming from Japan^[39]. Similarly, encouraging preliminary results have been reported by a 12-wk course of the NS3 protease inhibitor ABT-450 given with ritonavir boosting the combination of NNI ABT-072 and RBV, which achieved an SVR in $> 90\%$ of treatment-naïve genotype 1, IL28B rs12979860 genotype CC, non-cirrhotic chronic HCV patients^[40]. Thus, it is clear that an SVR can be achieved with interferon-free regimens even in difficult to treat chronic HCV patients.

CONCLUSION

The recently approved boceprevir and telaprevir used in combination with PEG-IFN/RBV substantially improves the SVR rates in both treatment-naïve and treatment-experienced genotype 1 patients, while the treatment duration can be reduced to only 24 wk in a large proportion of mainly treatment-naïve patients. There are, however,

several challenges with the use of the new triple combinations. There is a need for immediate results of HCV RNA testing using sensitive quantitative assays, there are new and more frequent adverse events and drug-drug interactions, and there will be increasing difficulties in compliance. In addition, the SVR rates are still poor in very difficult to treat subgroups of genotype 1 patients, such as null responders with cirrhosis, while there is no benefit in patients who cannot tolerate PEG-IFN/RBV and in patients infected with a non-1 HCV genotype. Many new drugs and combinations are currently under evaluation with encouraging results. Although it is yet early, preliminary data suggest that the treatment of chronic HCV patients with well tolerated combinations of oral agents without PEG-IFN is feasible and may lead to a universal HCV cure over the next 5-10 years.

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High densities of serotonin and peptide YY cells in the colon of patients with lymphocytic colitis

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Abstract

AIM: To investigate colonic endocrine cells in lymphocytic colitis (LC) patients.

METHODS: Fifty-seven patients with LC were included. These patients were 41 females and 16 males, with an average age of 49 years (range 19-84 years). Twenty-seven subjects that underwent colonoscopy with biopsies were used as controls. These subjects underwent colonoscopy because of gastrointestinal bleeding or health worries, where the source of bleeding was identified as haemorrhoids or angiodysplasia. They were 19 females and 8 males with an average age of 49 years (range 18-67 years). Biopsies from the right and left colon were obtained from both patients and controls during colonoscopy. Biopsies were fixed in 4% buffered paraformaldehyde, embedded in paraffin and cut into 5 μ m-thick sections. The sections immu-

nostained by the avidin-biotin-complex method for serotonin, peptide YY (PYY), pancreatic polypeptide (PP) enteroglucagon and somatostatin cells. The cell densities were quantified by computerised image analysis using Olympus software.

RESULTS: The colon of both the patient and the control subjects were macroscopically normal. Histopathological examination of colon biopsies from controls revealed normal histology. All patients fulfilled the diagnosis criteria required for of LC: an increase in intraepithelial lymphocytes (> 20 lymphocytes/100 epithelial cells) and surface epithelial damage with increased lamina propria plasma cells and absent or minimal crypt architectural distribution. In the colon of both patients and control subjects, serotonin-, PYY-, PP-, enteroglucagon- and somatostatin-immunoreactive cells were primarily located in the upper part of the crypts of Lieberkühn. These cells were basket- or flask-shaped. There was no statistically significant difference between the right and left colon in controls with regards to the densities of serotonin- and PYY-immunoreactive cells ($P = 0.9$ and 0.1 , respectively). Serotonin cell density in the right colon in controls was 28.9 ± 1.8 and in LC patients 41.6 ± 2.6 ($P = 0.008$). In the left colon, the corresponding figures were 28.5 ± 1.9 and 42.4 ± 2.9 , respectively ($P = 0.009$). PYY cell density in the right colon of the controls was 10.1 ± 1 and of LC patients 41 ± 4 ($P = 0.00006$). In the left colon, PYY cell density in controls was 6.6 ± 1.2 and in LC patients 53.3 ± 4.6 ($P = 0.00007$).

CONCLUSION: The change in serotonin cells could be caused by an interaction between immune cells and serotonin cells, and that of PYY density might be secondary.

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Key words: Colon; Computer image analysis; Immunohistochemistry; Lymphocytic colitis; Peptide YY; Serotonin

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INTRODUCTION

Lymphocytic colitis (LC), which was first described in 1989^[1], belongs to a group of conditions known as microscopic colitides (MC). This disorder is characterised mainly by chronic or intermittent watery diarrhoea^[2], which can be severe. Up to 25% of patients present with more than 10 bowel movements per day in addition to nocturnal diarrhoea^[3]. Other symptoms such as cramping, abdominal pain and weight loss may occur^[4]. The aetiology of LC is not yet clear, although several hypotheses have been suggested, such as association to various autoimmune conditions and drug induction^[2]. LC has an incidence of 3.1 to 9.8 and prevalence of 14.2 per 100 000 people^[5-12].

Sequential treatment for LC is recommended, in which a “therapeutic ladder” is followed. The suggested drugs in this ladder are: loperamide, bismuth salicylate, budesonide, cholestyramine 5-aminosalicylic acid preparations, prednisolone, azathioprine, 6-mercaptopurine methotrexate or octreotide^[2].

In a previous study, a high density of colonic chromogranin A immunoreactive cells were reported in patients with LC^[13]. Chromogranin A is a general marker for all endocrine cells^[14-16], but it is not clear which types of colonic endocrine cells are responsible for the increase in the density of chromogranin A cells in patients with LC. Therefore, the current study was performed to identify the endocrine cell types involved.

MATERIALS AND METHODS

Patients and controls

Fifty-seven patients with a diagnosis of lymphocytic colitis during the period 2007-2010 were included in this study. The patients were diagnosed in all 3 hospitals of Helse-Fonna region in Western Norway, namely Stord, Haugesund and Odda. They were 41 females and 16 males, with an average age of 49 years (range 19-84 years). These patients did not show any clinical signs of other autoimmune disorders. They did not had coeliac disease tested either by serology or duodenal biopsies.

Twenty-seven subjects that underwent colonoscopy with biopsies were used as controls. Twenty of these subjects underwent colonoscopy because of gastrointestinal bleeding, where the source of bleeding was identified as haemorrhoids (18), or angiodysplasia (2), and seven were examined because of health worries caused by a relative being diagnosed with colon carcinoma. They were 19 females and 8 males with an average age of 49 years (range 18-67 years). All these subjects had no other gastrointestinal complaints than those mentioned above.

The study was performed in accordance with the Declaration of Helsinki and was approved by the local Committee for Medical Research Ethics. All subjects gave oral and written consent.

Colonoscopy

Colonoscopies were performed for all patients and controls, and two biopsies were taken from the caecum, from the ascending colon and from the right half of the transverse colon. These biopsies were pooled together and were labelled as right colon. In addition, two biopsies were taken from the left half of the transverse colon, from the descending colon and from the sigmoid colon. These 6 biopsies were pooled together and labelled as left colon.

Histopathology and immunohistochemistry

Biopsies were fixed in 4% buffered paraformaldehyde overnight, embedded in paraffin and cut into 5 µm-thick sections. The sections were stained with haematoxylin-eosin and immunostained by the avidin-biotin-complex (ABC) method using the Vectastain ABC-kit (Vector laboratories) as described earlier in detail^[17]. The primary antibodies used were: monoclonal anti-human leucocytes common antigen (Dako, CD 45, clone 2B11), monoclonal anti-human CD8 lymphocytes (Dako, CD 57, clone 2B01), monoclonal mouse anti-serotonin (Dako, code no. M869), polyclonal anti-porcine peptide YY (PYY) (Eurodiagnostica, code B52-1), polyclonal rabbit anti-synthetic human pancreatic polypeptide (PP) (Dako, code no. A619), polyclonal rabbit anti-synthetic human somatostatin, and polyclonal rabbit anti-porcine glucagon (Eurodiagnostica, code B31). The antibodies were used at dilutions of 1:100, 1:200, 1:1500, 1:1500, 1:1000, 1:1600 and 1:200, respectively. The anti-PYY cross reacts with PYY in all vertebrates including humans. It does not cross react with PP or neuropeptide Y in immunohistochemical system. Anti-glucagon is directed to N-Terminus of glicentin (enteroglucagon) and does not cross react with glucagon, vasoactive intestinal polypeptide or gastric inhibitory polypeptide. The second layer biotinylated mouse anti-immunoglobulin G (IgG) and rabbit anti-IgG were obtained from Dako. Negative and positive controls were the same as those described previously^[17].

Computerised image analysis

The number of immunoreactive cells and the area of the epithelial cells were measured using Olympus software:

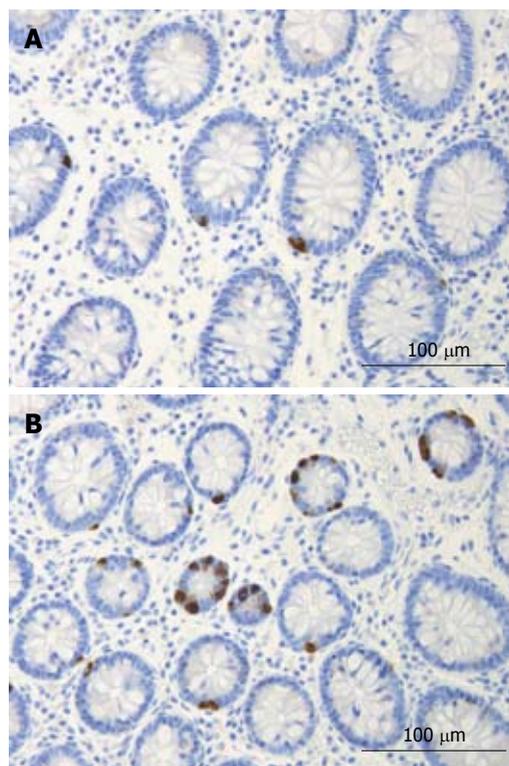


Figure 1 Serotonin-immunoreactive cells in the colon of a control (A) and of a patient with lymphocytic colitis (B).

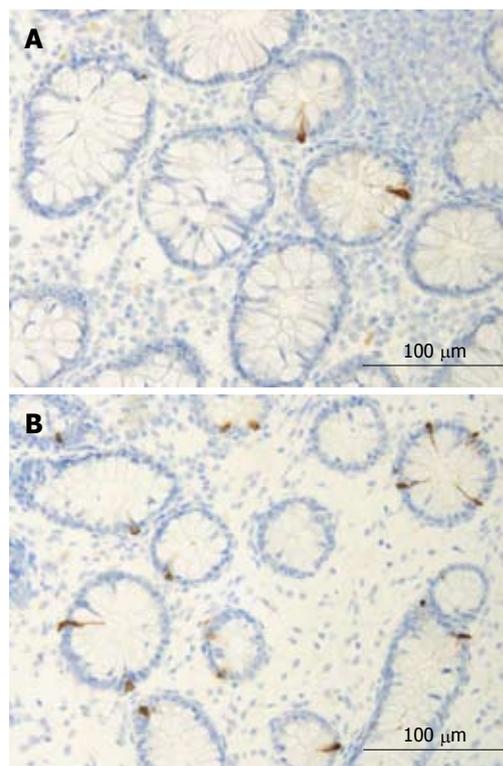


Figure 2 Colonic peptide YY-immunoreactive cells in a control (A) and in a patient with lymphocytic colitis (B).

Cell ^D. When using $\times 40$ objectives, the frame (field) on the monitor represents an area of 0.14 mm^2 of the tissue. Measurements were performed in 10 randomly chosen fields for each individual and hormone. The X40 objective was used. The data from fields were tabulated, and the number of cells/ mm^2 of the epithelium was computed and statistically analysed. The immunostained sections of IBS patients and controls were coded and mixed, and measurements were made without the knowledge of sections identity.

Statistical analysis

The non-parametric Mann-Whitney test was performed. $P < 0.05$ was considered to be statistically significant.

RESULTS

Endoscopy, histopathology and immunohistochemistry

The colon of both the patient and the control subjects were macroscopically normal. Histopathological examination of colon biopsies from controls revealed normal histology. All patients fulfilled the diagnosis criteria required for of LC: an increase in intraepithelial lymphocytes (> 20 lymphocytes/100 epithelial cells) and surface epithelial damage with increased lamina propria plasma cells and absent or minimal crypt architectural distribution. In the colon of both patients and control subjects, serotonin-, PYY-, PP-, enteroglucagon- and somatostatin-immunoreactive cells were primarily located in the upper part of the crypts of Lieberkühn. These

cells were basket- or flask-shaped (Figures 1 and 2).

Computerised image analysis

PP-, enteroglucagon- and somatostatin-immunoreactive cells were sparse in the biopsy material examined. This made it difficult to perform a reliable quantification of these cell types.

There was no statistically significant difference between the right and left colon in controls with regards to the densities of serotonin- and PYY-immunoreactive cells ($P = 0.9$ and 0.1 , respectively).

Serotonin cell density in the right colon in controls was 28.9 ± 1.8 (mean \pm SE) and in LC patients 41.6 ± 2.6 ($P = 0.008$). In the left colon, the corresponding figures were 28.5 ± 1.9 and 42.4 ± 2.9 , respectively ($P = 0.009$) (Figures 1 and 3). PYY cell density in the right colon of the controls was 10.1 ± 1 and of LC patients 41 ± 4 ($P = 0.00006$). In the left colon, PYY cell density in controls was 6.6 ± 1.2 and in LC patients 53.3 ± 4.6 ($P = 0.00007$) (Figures 2 and 4).

DISCUSSION

MC is a common cause of diarrhoea and 10% to 30% of older adults investigated for chronic diarrhoea have MC^[18]. LC seems to be associated with several autoimmune diseases^[19,20]. Furthermore, the prevalence of coeliac disease is high in patients with LC^[21]. The information available on the gut endocrine cells in coeliac disease is restricted to the duodenum^[22]. It is therefore difficult

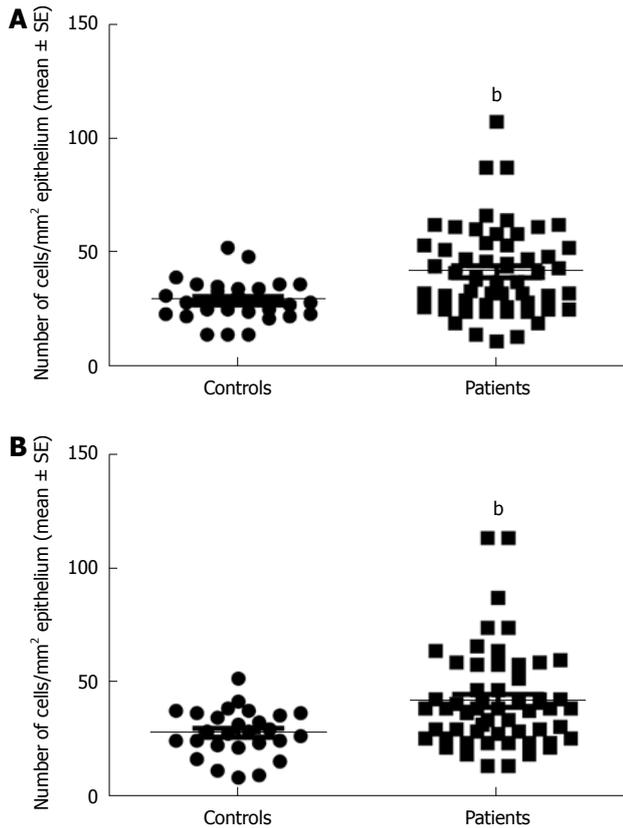


Figure 3 Serotonin cell density in the controls and patients with lymphocytic colitis in the right (A) and left colon (B). ^bP < 0.01 vs controls.

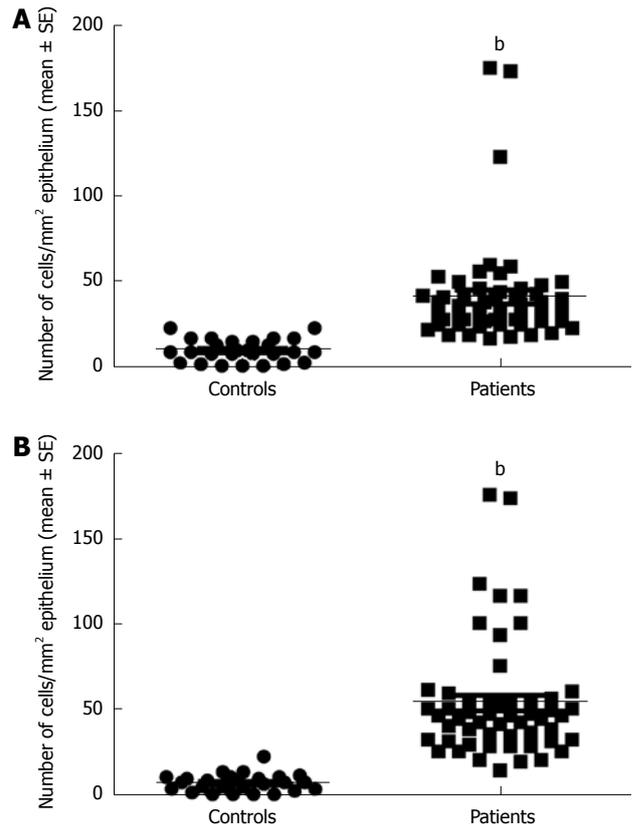


Figure 4 Peptide YY cell density in controls and patients with lymphocytic colitis in the right (A) and left colon (B). ^bP < 0.001 vs controls.

to compare the outcome of the present study with findings in coeliac disease.

The current study showed that serotonin and PYY cell densities were increased in both the right and left colon of patients with LC. Serotonin activates the submucosal sensory branch of the enteric nervous system, and controls gastrointestinal motility and chloride secretion *via* inter-neurons and motor neurons^[23,24]. PYY stimulates the absorption of water and electrolytes and is a major regulator of the “ileal brake”^[24]. There are several studies showing that inflammation and immune cells affect the neuroendocrine system of the gut^[25]. Thus, serotonin secreted by enterochromaffin (EC) cells can be enhanced or attenuated by secretory products of immune cells, such as CD4+T^[26]. Furthermore, serotonin modulates the immune response^[26]. The EC are in contact with or very close to CD3+ and CD20+ lymphocytes, and several serotonergic receptors have been characterised in lymphocytes, monocytes, macrophages and dendritic cells^[27].

One may speculate that the high density of serotonin cells in LC patients is caused by the interaction between the immune cells and serotonin cells in the epithelium and submucosa of LC patients. The increase in serotonin would cause accelerated colonic motility and visceral hypersensitivity. Accelerated colonic motility and visceral hypersensitivity can cause diarrhoea and abdominal pain, symptoms that occur in LC. It is probable

that the increase in PYY cell density is secondary to the increase in serotonin cell density in order to compensate for accelerated motility by activating the ileal brake and by increasing the absorption of water. In support for this assumption are the findings that large intestinal serotonin and PYY cells as well plasma levels are affected in patients with ulcerative colitis and Crohn’s disease^[28-32]. Similarly, these endocrine cells have been found to be affected in experimental animal model of colitis^[33,34].

LC and post-infectious IBS (PI-IBS) show a striking similarity. They have the same clinical presentation and both can regress spontaneously^[35,36]. Both LC and PI-IBS show intra-epithelial and submucosal infiltration of lymphocytes and mast cells, and exhibit a high density of colonic serotonin and PYY cells^[36-41]. This raises the question as to whether LC and PI-IBS are actually the same disorder. If this is proven to be true, it would have an important clinical implication. Thus, PI-IBS can be treated by the same “therapeutic ladder” which is proven to be effective in LC.

COMMENTS

Background

Microscopic colitis is a chronic condition with watery diarrhoea as the cardinal symptom, but other symptoms such as cramping, abdominal pain and weight loss may occur. Radiologic and endoscopic findings in these patients are normal. However, histopathological examinations of the colon reveal abnormal histology, which is of two distinctive types: lymphocytic colitis (LC) and collagenous

colitis. LC exhibits an increased number of colonic intra-epithelial lymphocytes (> 20/100 epithelial cells), increased inflammatory cells within the lamina propria and preserved crypt architecture. LC and irritable bowel syndrome (IBS) have similar symptoms and both are without radiologic or endoscopic abnormalities. Thus, LC can be mistakenly diagnosed as IBS. In a study on the colonic chromogranin A cell density in IBS patients, nine patients out of 50 showed extremely high colonic chromogranin A cell density. This high density was in contrast to the low density of chromogranin cells in the rest of the IBS patients studied. Re-examination of these nine patients revealed that they suffered from LC. This unexpected finding was confirmed on a larger patient's material. As chromogranin A is a common marker for endocrine cells, the present investigation was performed to identify the colonic endocrine cell-types that are affected.

Research frontiers

The current study is a further investigation of the unexpected observation that LC patients have high colonic chromogranin A cell density, which has been shown to be an excellent biomarker for the diagnosis of LC. This unexpected observation led to a novel approach toward LC, where the colonic hormones role in the symptom development in patients and their role in the pathogenesis of the disease come under focus. The current study showed that the endocrine cell-types that are affected in the colon of LC patients were peptide YY (PYY) and serotonin cells. This underlines the importance of these two hormones in LC.

Innovations and breakthroughs

The present findings underline the importance of the interaction between the gut hormones and the local immune system in the gut (the endocrine/immune axis) and its role in the pathogenesis and symptom development in disease. Such interactions should be put under the spotlight in several gastrointestinal diseases, especially those with known inflammation such as inflammatory bowel disease and coeliac disease. The similarity in the endocrine changes between LC and post-infectious irritable bowel syndrome (PI-IBS) has drawn attention to other histopathological and clinical similarities such as: both LC and PI-IBS show intra-epithelial and submucosal infiltration of lymphocytes and mast cells; both have the same clinical presentation; and both can regress spontaneously. This lead to the notion that LC and PI-IBS may be the same disorder.

Applications

Understanding the interaction between gut hormones and the local immune system of the gut would result in better understanding of the pathogenesis of gut inflammatory diseases and possibly open a new avenue for treatment. If LC and PI-IBS are proven to be the same disorder, PI-IBS can be treated by the same "therapeutic ladder" that has been proven to be effective in LC. PI-IBS constitutes a large subset of IBS without any effective treatment.

Terminology

Chromogranin A: Chromogranin A is a 68-kDa protein comprising 439 amino-acid residues. Chromogranin is co-stored and co-released with monoamines and peptide hormones of the adrenal medulla, pituitary gland, parathyroid, thyroid C-cells, pancreatic islets, endocrine cells of the gastrointestinal tract and sympathetic nerves; PYY: PYY is localised in endocrine cells in the ileum and large intestine; Serotonin: Serotonin is a monoamine that is localised in the enterochromaffin cells throughout the entire gastrointestinal tract. It also occurs also in the enteric nervous system and acts as a hormone and a neurotransmitter.

Peer review

This is an excellent paper which shows new light on lymphocytic colitis. The study was performed to identify the endocrine cell types in the colonic epithelium of LC by immunostaining using representative 5 antibodies against serotonin, PYY, pancreatic polypeptide, somatostatin, and glucagon. The results were that serotonin and PYY cell densities were increased in both the right and left colon of patients with LC, when compared with controls. They concluded that the high density of serotonin cells in LC patients were caused by the interaction between immune cells and serotonin cells, which occurs in the epithelium and submucosa of LC patients, and that the increase in PYY density is secondary to the increase in serotonin cell density.

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Galactosylated chitosan/5-fluorouracil nanoparticles inhibit mouse hepatic cancer growth and its side effects

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Abstract

AIM: To observe the curative effect of galactosylated chitosan (GC)/5-fluorouracil (5-FU) nanoparticles in liver cancer mice and its side effects.

METHODS: The GC/5-FU nanoparticle is a nanomaterial made by coupling GC and 5-FU. The release ex-

periment was performed *in vitro*. The orthotopic liver cancer mouse models were established and divided into control, GC, 5-FU and GC/5-FU groups. Mice in the control and GC group received an intravenous injection of 200 μ L saline and GC, respectively. Mice in the 5-FU and GC/5-FU groups received 200 μ L (containing 0.371 mg 5-FU) 5-FU and GC/5-FU, respectively. The tumor weight and survival time were observed. The cell cycle and apoptosis in tumor tissues were monitored by flow cytometry. The expression of p53, Bax, Bcl-2, caspase-3 and poly adenosine 50-diphosphate-ribose polymerase 1 (PARP-1) was detected by immunohistochemistry, reverse transcription-polymerase chain reaction and Western blot. The serum blood biochemical parameters and cytotoxic activity of natural killer (NK) cell and cytotoxicity T lymphocyte (CTL) were measured.

RESULTS: The GC/5-FU nanoparticle is a sustained release system. The drug loading was $6.12\% \pm 1.36\%$, the encapsulation efficiency was $81.82\% \pm 5.32\%$, and the Zeta potential was 10.34 ± 1.43 mV. The tumor weight in the GC/5-FU group (0.4361 ± 0.1153 g vs 1.5801 ± 0.2821 g, $P < 0.001$) and the 5-FU (0.7932 ± 0.1283 g vs 1.5801 ± 0.2821 g, $P < 0.001$) was significantly lower than that in the control group; GC/5-FU treatment can significantly lower the tumor weight (0.4361 ± 0.1153 g vs 0.7932 ± 0.1283 g, $P < 0.001$), and the longest median survival time was seen in the GC/5-FU group, compared with the control (12 d vs 30 d, $P < 0.001$), GC (13 d vs 30 d, $P < 0.001$) and 5-FU groups (17 d vs 30 d, $P < 0.001$). Flow cytometry revealed that compared with the control, GC/5-FU caused a higher rate of G0-G1 arrest ($52.79\% \pm 13.42\%$ vs $23.92\% \pm 9.09\%$, $P = 0.014$) and apoptosis ($2.55\% \pm 1.10\%$ vs $11.13\% \pm 11.73\%$, $P < 0.001$) in hepatic cancer cells. Analysis of the apoptosis pathways showed that GC/5-FU upregulated the expression of p53 at both the protein and the mRNA levels, which in turn lowered the ratio of Bcl-2/Bax expression; this led to the release of cytochrome C into the cytosol

from the mitochondria and the subsequent activation of caspase-3. Upregulation of caspase-3 expression decreased the PARP-1 at both the mRNA and the protein levels, which contributed to apoptosis. 5-FU increased the levels of aspartate aminotransferase and alanine aminotransferase, and decreased the numbers of platelet, white blood cell and lymphocyte and cytotoxic activities of CTL and NK cells, however, there were no such side effects in the GC/5-FU group.

CONCLUSION: GC/5-FU nanoparticles can significantly inhibit the growth of liver cancer in mice *via* the p53 apoptosis pathway, and relieve the side effects and immunosuppression of 5-FU.

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Key words: Galactosylated chitosan; Nanoparticles; 5-fluorouracil; Hepatocellular cancer; Targeted therapy; Apoptosis

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INTRODUCTION

Hepatocellular cancer (HCC) is one the most prevalent malignancies^[1,2]. Liver transplantation remains the most effective therapeutic option for HCC; however, due to the lack of donors and the relatively high cost, a substantial number of patients die while waiting for a donor liver^[3,4]. The disadvantages of most anti-cancer drugs that are currently available include low bioavailability, poor selectivity because they can act on both tumor cells and healthy cells, and immunosuppression that can cause complications and even patient death^[5]. However, targeted therapy for HCC may be useful because it is relatively less expensive compared with the current therapies and it also produces fewer side effects^[6,7]. 5-fluorouracil (5-FU) is a pyrimidine anticancer drug. Since its development by Heidelberg in 1957, 5-FU has occupied an important position in the cancer chemotherapy field. Because 5-FU is highly effective against a broad spectrum of malignancies, it is widely used in chemotherapy regimens against cancers such as hepatocellular, gastric,

pancreatic and breast cancers; 5-FU is very important in the management of liver cancer. 5-FU belongs to the cell cycle specific drugs and can be converted into fluorouracil deoxynucleotide to bind with thymidine synthase, which leads to the disruption of RNA, DNA and protein biosynthesis. However, because of the similarity of the nucleic acid metabolism pathways between tumor and normal tissues, 5-FU can also target normally proliferating tissues, leading to bone marrow suppression and gastrointestinal reactions. Other disadvantages of 5-FU include irregular absorption, a short half-life, and rapid turnover, which require lengthy, high-dose intravenous administration to maintain its effective *in vivo* concentration for a suitable period^[8,9]; these disadvantages can significantly restrict the clinical application of 5-FU. The emergence of a novel, sustained-release formulation of 5-FU is of clinical significance because it has fewer side effects compared to the regular 5-FU formulation^[10-12]. Galactosylated chitosan (GC) is a galactose ligand, with chitosan modifications on the molecular structure^[13-15]. Asialoglycoprotein receptor (ASGPR) is a receptor found on the membrane of hepatocytes facing the sinusoids, with specificity for glycoproteins with galactose or acetyl galactosamine at the end. Each hepatocyte contains approximately two million binding sites for ASGPR^[16]. The binding of the galactose ligand with ASGPR induces liver-targeted gene transfer. Our lab previously synthesized a GC nanoparticle as a gene carrier and showed that the GC nanoparticle can successfully transfer genes into the liver *in vitro* and *in vivo*. We also confirmed that this nanoparticle material has a high selectivity to the liver and a low cytotoxicity^[17]. In the present study, we synthesized GC/5-FU nanoparticles by combining the GC material with 5-FU, and tested its effect on liver cancer *in vitro* and *in vivo*. We found that the GC/5-FU nanoparticles can specifically target the liver and that the addition of GC increases the cytotoxicity of 5-FU and apoptosis mediated by the p53 pathway. GC/5-FU nanoparticles can ameliorate the side effects and immunosuppressive action of 5-FU.

MATERIALS AND METHODS

Reagents

Chitosan (deacetylation degree > 85%) GC was synthesized and stored by our group. HCl was from Shanghai Medpep, AR. LC-10A HPLC (Shimadzu, Japan), flow cytometry (FACS Calibur, United States). The immunohistochemistry kit was from GBI, United States. Caspase-3 and poly ADP-ribose polymerase 1 (PARP-1) antibodies were from Santa Cruz, CA, United States; Bax and Bcl-2 antibodies were from Temecula, CA, United States; and p53 antibody was from Beverly, MA, United States.

Mice and cell lines

The mouse liver cancer cell lines (H22) were purchased from the Cancer Institute of Fudan University, China. Female BALB/c mice, 7 wk of age and weighing 25 g, were

obtained from the Department of Experimental Animals of Fudan University, China. All mice were housed in specific pathogen-free level B animal facility and animal experiments were conducted following the guidelines of the Animal Research Ethics Board of Fudan University.

Synthesis of GC/5-FU

The 5-FU/GC was mixed at a mass ratio of 10:1 in solution, using vortex oscillator (2500 r/min) for 30 s; the final concentration of 5-FU was 1.857 g/L. The product was kept at room temperature for 30 min to assess for further particle formation. The final product was kept at 4 °C. The drug loading and encapsulation efficiency were calculated according to the following equations: drug loading = the amount of 5-FU within nanoparticle/nanoparticle mass × 100%; encapsulation efficiency = the amount of 5-FU within nanoparticle/total amount of 5-FU added × 100%.

In vitro release experiment

GC/5-FU nanoparticles (20 mg) were mixed with 30 mL of simulated body fluid (pH 7.4) in dialysis bags and incubated at 37 °C using a shaker with a fixed speed of 60 r/min. Samples were taken at 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 d after mixing. The optical density (A) was measured at 265 nm by an automated microplate reader (Bio-Rad Inc, California, United States). The amount of 5-FU released at different time points was calculated according to a standard absorbance curve. The concentration and cumulative release rate were calculated according to the standard curve equation. Each experiment was performed in triplicate.

Animal model

The subcutaneous liver cancer mouse model was established using the mouse HCC cell line H22. After euthanasia and dissection, fresh fast-growing tumor tissues were minced and made into a tumor cell suspension at a density of 6×10^4 /L. Recipient mice were anesthetized by 20% urethane, followed by an injection of 50 μ L of tumor cell suspension into the left liver lobe capsule. Approximately two min after completion of the procedure, when there was no leaking, the abdomen was sutured and the orthotopic liver cancer mouse model was established successfully^[18].

Curative effect of GC/5-FU in the orthotopic liver cancer mouse model

Five days after the establishment of the orthotopic liver cancer mouse model, the tumor reached a size of about 4-6 mm in diameter (Figure 1). The mouse models were randomly assigned into 4 groups labeled as control, GC, 5-FU and GC/5-FU. Mice in the control group received an intravenous injection of 200 μ L saline. GC group received 200 μ L GC nano-material. 5-FU and GC/5-FU groups received 200 μ L (containing 0.371 mg 5-FU) 5-FU and GC/5-FU, respectively. The drugs were given continuously for 5 d starting from day 5 after the tumor

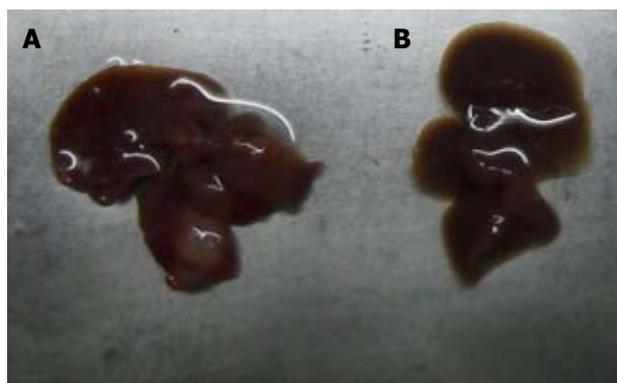


Figure 1 Establishment of the hepatic cancer mouse model. A: Liver cancer; B: Normal mouse liver.

was established. At day 15, 10 mice were sacrificed and the tumor growth was monitored. The remaining 15 mice in each group were kept for survival analysis.

Cell cycle and apoptosis analysis by flow cytometry

The cell suspension was made of 1-2 mL tumor tissues from each individual group. Cells were washed three times by 0.1 mol/L phosphate buffered solution (PBS) and fixed by 70% ethanol. Cells were then incubated with 50 mg/L propidium iodide (Zhengzhou Sigma Chemical, Zhengzhou, China), 1.0% Triton X-100 and 10 mg/L RNaseA for 30 min at 4 °C in dark. Cell cycle distribution was analyzed by flow cytometry. Proliferation index (PI) = $(S + G2/M)/(G0/G1 + S + G2/M)$. Apoptosis was determined by staining cells with annexin V-FITC (Pharmingen, San Diego, CA, United States) and propidium iodide because annexin V can identify the externalization of phosphatidylserine during the progression of apoptosis and therefore can detect early apoptotic cells. To quantify apoptosis, cells were washed twice with cold PBS and resuspended in binding buffer at 1×10^3 cells/L. A quantity of 100 μ L of this suspension was transferred to a 5 mL culture tube with 5 mL of annexin V-FITC and 10 mL of 20 mL/L propidium iodide, and analyzed using the flow cytometry.

Immunohistochemistry

The 4 μ m sections were deparaffinized by incubation at 65 °C. Sections were soaked in 3% H₂O₂ for 10 min at room temperature to deactivate endogenous peroxidases. Antigen retrieval was performed using a microwave. The primary antibody was incubated at 37 °C for 1 h in a humidified chamber; the secondary antibody was incubated at 37 °C for 30 min. After washing, the sections were developed using diaminobenzidine, and counter stained with hematoxylin. After dehydration, the sections were analyzed under a light microscope^[19]. p53 staining was mainly observed in the nucleus, which appeared brown and granular with little background. Caspase-3 staining was mainly present in cytoplasm, showing a brown granular staining pattern. PBS was used instead of primary antibody for a negative control. The Image-pro plus 6.0

system was used to analyze five fields randomly chosen from each slide. The images were amplified 200-fold, converted into gray-scale so as to distinguish the positive staining area from background. The positive-stained area and the total area of the field were measured by the system and the area ratio was calculated using the following equation: staining area/total area \times 100%. The stained area of each individual slide was determined by averaging the area ratio.

Reverse transcription-polymerase chain reaction

Primers were purchased from Shanghai R and S Biotechnology Co., Ltd. The oligonucleotide primers used were: Bcl-2: 5'-CGGGCTGGGGATGACTTCTCT-3' (sense), 5'-GCATCCCAGCCTCCGTTATCC-3' (antisense); Bax: 5'-AGACACCTGAGCTGACCTTGGAG-3' (sense), 5'-AGACACCTGAGCTGACCTTGGAG-3' (antisense); PARP-1: 5'-TCCCAAGGACTCCCTCCGCATGG-3' (sense), 5'-CTTTGCCTGCCACGCCTCCAGCC-3' (antisense); Caspase-3: 5'-TTGGAACAAATGGACCTG-3' (sense), 5'-ACAAAGCGACTGGATGAA-3' (antisense); P53: 5'-GTGGCCTCTGTCATCTTCCG-3' (sense), 5'-CCGTCACCATCAGAGCAACG-3' (antisense); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control: 5'-ACCGCAAAGACTGTGGATGC-3' (sense), 5'-TGAGCTTGACAAAGTGGTCG-3' (antisense). Tissue total RNA was extracted by TRIZOL (Invitrogen, California, United States). Total RNA (1 μ L) was used to reverse transcribe into cDNA using 0.5 μ L AMV reverse transcriptase. Polymerase chain reaction (PCR) was performed using 2.5 μ L cDNA, 0.1 μ L Ex Taq HS, 0.1 μ L forward primer and 0.1 μ L reverse primer. The PCR reaction conditions were as follows: 94 °C for 2 min, 35 cycles of 94 °C for 40 s, 50 °C to 6 °C for 40 s and 72 °C for 1 min, followed by 72 °C for 5 min. PCR products were kept at -20 °C^[20]. GAPDH was used as internal control. The PCR product (6 μ L) was resolved in 2% agarose gel for 30 min at 120 V, 100 mA, stained with ethidium bromide solution for 5 min, imaged by a ultraviolet gel imaging system, and analyzed by Quantity One software (Bio-Rad Inc, California, United States). The expression of target genes was presented as the ratio of target to internal control GAPDH.

Western blotting analysis

After the concentration was determined, the samples were loaded onto the 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and resolved at 80 V followed by 120 V. Methanol-pretreated polyvinylidene difluoride (PVDF) membrane was then soaked in transfer buffer (pH 8.3, 25 mmol/L Tris-HCl, 192 mmol/L glycine, 20% methanol) for 10 min. Proteins on the SDS-PAGE gel were then transferred onto the PVDF membrane under 100 volts (V) for 70 min. The membrane was blocked by 5% FBS/PBS at 4 °C overnight. Primary antibodies were diluted at 1:2000 and incubated with a membrane for 3 min at room temperature. The membrane was then washed three times for 10

min each with PBS containing 0.05% Tween 20. Goat-anti-mouse immunoglobulin G secondary antibody (1:8000) was added to incubate with the membrane for 3 h at room temperature, followed by washing three times using the same washing solution. The membrane was then developed for 1 min using an enhanced chemiluminescence kit with equal volumes of A and B solution^[21]. After imaging, Image J version 1.44 software (National Institutes of Health) was used to analyze the average density values.

Analysis of blood biochemical parameters

The animals were sacrificed by day 10 after treatment. Blood chemistry including the measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen and creatinine was examined by a Fuji Drychem 3500 automated analyzer (Fuji Medical System Co. Ltd., Tokyo), the blood routine such as hemoglobin (Hbg), platelet (PLT), white blood cell (WBC), lymphocyte and neutrophil was detected by a Sysmex XS-800i automated analyzer (Sysmex Shanghai Ltd, Shanghai, China) .

Cytotoxic assay for natural killer cell and cytotoxicity T lymphocyte

Natural killer cell (NK) and cytotoxicity T lymphocyte (CTL) cytotoxic activity was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetry assay (Sigma, United States) as reported previously^[17]. 1×10^4 YAC-1 as target cells were seeded in a 96-well plastic plates. Spleen cells used as effector cells were prepared from the mice and were simultaneously seeded in a 96-well plate at a 50:1 ratio of effector to target (E:T) in CTL assay. The effector cells from spleen cells were incubated with H22 target cells at a 50:1 ratio of E:T. All cytotoxic activity assays were performed in triplicate.

The activities of CTL and NK were calculated using the following formulas: CTL activity (%) = $[1 - (A_{E+T} - A_E)/A_T] \times 100\%$; NK activity (%) = $[1 - (A_{E+T} - A_E)/A_T] \times 100\%$, where A_E indicates the mean A value of effector cells, A_T indicates the mean A value of target cells, and A_{E+T} indicates the mean A value of effector cells + target cells.

Statistical analysis

All data was collected and expressed as mean \pm SD. Analysis of variance (ANOVA) was used to analyze data within the same group, one-way ANOVA was used to analyze data between groups, while the least significant digit method was used for pairwise comparison between groups. A value of $\alpha = 0.05$ and $P < 0.05$ was considered statistically significant.

RESULTS

Synthesis and characterization of GC/5-FU nanoparticles

5-FU/GC nanoparticles were successfully synthesized,

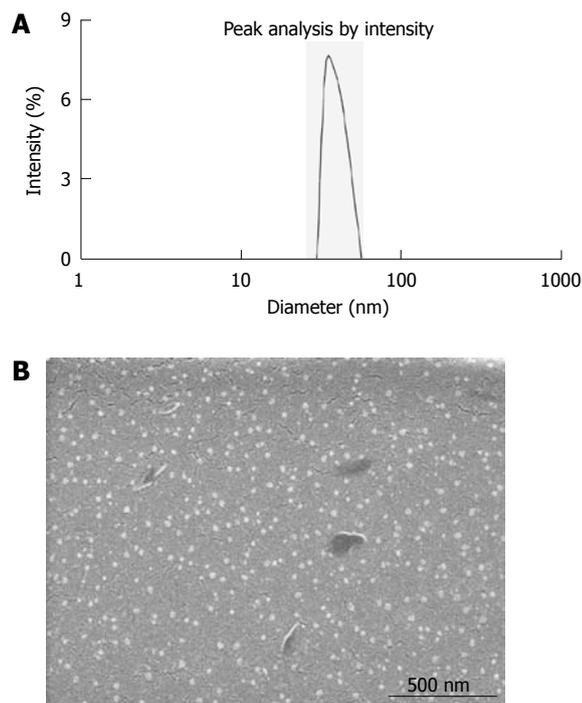


Figure 2 Particle size and scanning electron microscope image of galactosylated chitosan/5-fluorouracil. A: Particle size graph showing the diameter of galactosylated chitosan/5-fluorouracil (GC/5-FU) (35.19 ± 9.50 nm); B: Scanning electron microscope image of GC/5-FU. The particles show spherical structure with a smooth surface and no adhesion between nanoparticles.

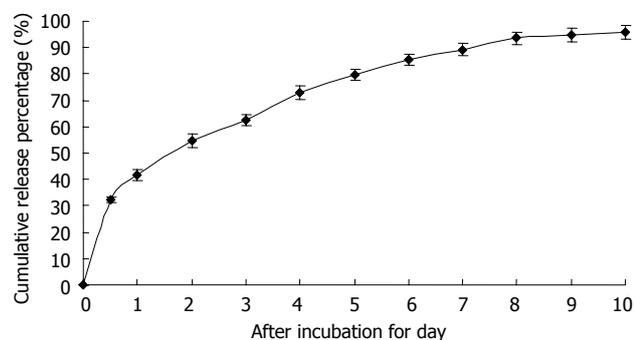


Figure 3 The *in vitro* release curve of nanoparticles in simulated body fluid (37 °C, pH 7.4, n = 3). A rapid release was observed from time 0 h to 12 h, with a cumulative release percentage of 32.4%; a smooth slow-release occurred between day 1 and day 8, with a cumulative release percentage of 93.50%. During days 8 to 10, the release reached a plateau, with a cumulative release percentage of 95.70% at day 10.

and the radius of the nanoparticles was 35.19 ± 9.50 nm, which had a normal distribution (Figure 2A). Electron microscopy showed that the particles were in regular spherical shape, with a smooth surface, a uniform size, and no adhesion between nanoparticles (Figure 2B). The drug loading was $6.12\% \pm 1.36\%$, the encapsulation efficiency was $81.82\% \pm 5.32\%$, and the Zeta potential was 10.34 ± 1.43 mV. Figure 3 shows the *in vitro* release curve of nanoparticles in simulated body fluid (37 °C, pH 7.4). A rapid release was observed from 0 h to 12 h, with a cumulative release percentage of 32.4%, presum-

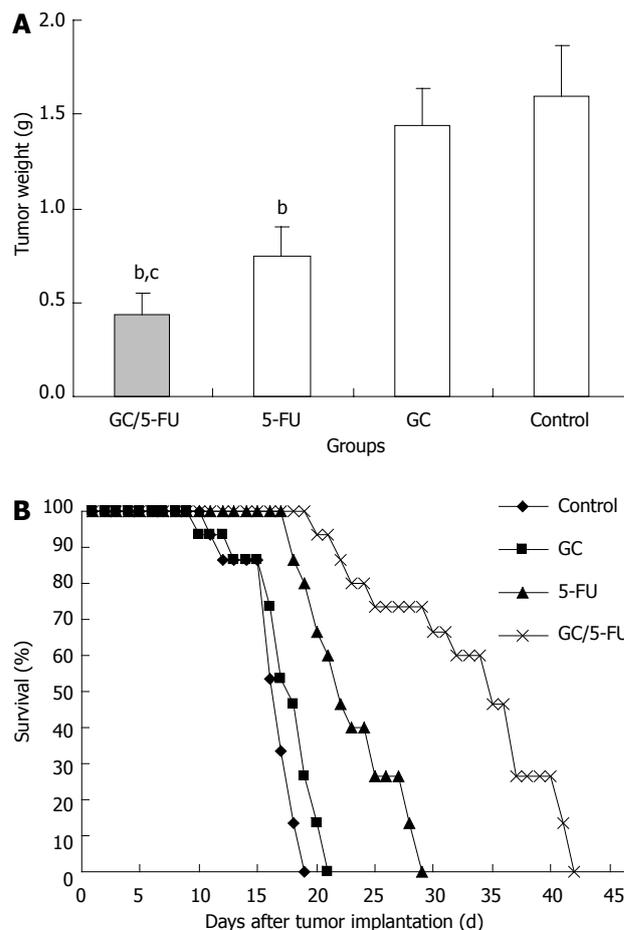


Figure 4 The curative effect of galactosylated chitosan/5-fluorouracil on liver cancer in the orthotopic transplant model of hepatocellular carcinoma. A: Five days after the tumor was established, galactosylated chitosan/5-fluorouracil (GC/5-FU), 5-FU, GC or phosphate buffered solution was given to the mice. Tumor weight was measured at day 15; B: Treatment was given as described previously and the survival was monitored. The median survival for control, GC, 5-FU and GC/5-FU groups were 12, 13, 17 and 30 d, respectively. ^b*P* < 0.01 vs control group; ^c*P* < 0.05 vs 5-FU group.

ably due to the diffusion of surface 5-FU into the solution; a smooth slow-release occurred between day 1 and day 8, with a cumulative release percentage of 93.50%, indicating that the GC/5-FU nanoparticles have a sustained release effect from days 1 to 8. From days 8 to 10, the release reached a plateau, with a cumulative release percentage of 95.70% at day 10.

Effect of GC/5-FU on tumor mass and survival in the mouse model

The tumor samples were harvested and weighted 15 d after treatment (Figure 4A). The weight of the tumor was 0.4361 ± 0.1153 g in GC/5-FU group, 0.7932 ± 0.1283 g in 5-FU group, 1.3989 ± 0.2125 g in GC group and 1.5801 ± 0.2821 g in control group. The differences between the groups was statistically significant (*P* < 0.01). The tumor weight in the GC/5-FU and the 5-FU groups were significantly lower than in the GC group and control group (*P* < 0.01) while tumor weight in the GC/5-FU group was significantly lower than in the 5-FU group (*P* < 0.01);

however, the tumor weight in the GC group and the control group was not different ($P > 0.05$). After the model was developed, the mice were randomly assigned to four groups with 15 mice each, and treated as described above. The survival of the mice was monitored, and there was a 100% mortality in all groups. The Kaplan-Meier survival curve (Figure 4B) showed that mice all the mice in the control group died between day 6 and day 14, with a median survival time of 12 d. In the GC group, all mice died between day 5 and day 16, the median survival time being 13 d. There was no statistical difference in the survival time between the control and the GC groups ($P > 0.05$). Mice treated with 5-FU also all died between day 13 and day 24, with a median survival of 17 d. All mice in the GC/5-FU group died between day 15 and day 37, with a median survival time of 30 d. The median survival time of mice treated with either 5-FU or GC/5-FU was significantly longer than that of mice in the GC or control groups; the longest median survival time was seen in the GC/5-FU group ($P < 0.01$ compared with the control, GC and 5-FU groups).

Effect of GC/5-FU on cell cycle, proliferation and apoptosis of H22 cells

Flow cytometry was used to analyze the liver cancer samples harvested 15 d after beginning the treatment. As shown in Figure 5A and B, the percentage of cells in the G0-G1 phases was significantly higher in the GC/5-FU- and 5-FU-treated tumors ($P < 0.01$), while the PI was lower than that in the GC and control groups ($P < 0.01$), suggesting that GC/5-FU and 5-FU had an overt anti-proliferative effect and arrested the tumor cells in the G0-G1 phases. The percentage of apoptotic cells in the GC/5-FU and 5-FU groups was significantly increased when compared with that in the control and GC groups ($P < 0.01$). Also, the percentage of apoptotic cells of GC/5-FU group was higher than that in the 5-FU group ($P < 0.01$), suggesting that GC is able to enhance the cellular influx of 5-FU, thereby improving the pro-apoptotic effect of 5-FU (Figure 5C).

GC/5-FU induced hepatic cancer cell apoptosis via activating the p53 pathway

To understand which pathway(s) mediated the GC/5-FU-induced apoptosis, we examined the expression of p53 at both protein and mRNA levels. Compared with the control and GC groups, the expression of p53 was increased in the 5-FU and GC/5-FU groups, with the highest increase seen in the GC/5-FU group ($P < 0.01$, Figures 6, 7A and B). The ratio of Bcl-2/Bax showed a decreasing tendency from control to GC to 5-FU to GC/5-FU groups ($P < 0.01$, Figure 7D); specifically, the ratio in 5-FU and GC/5-FU was significantly lower than that in the control and GC groups, with a lowest ratio observed in the GC/5-FU group ($P < 0.01$). GC/5-FU can also significantly induce the expression of caspase-3 in the tumor tissues ($P < 0.01$, Figures 6, 7A and B). The expression of PARP-1 also displayed a decreasing ten-

dency from control to GC to 5-FU to GC/5-FU groups ($P < 0.01$, Figure 7C), with the most significant reduction seen in the GC/5-FU group. Therefore, it is likely that GC/5-FU was involved in upregulating the genes in the p53 pathway.

Side effects of GC/5-FU

In order to understand the side effect of liver and kidney function and blood cells, we examined the blood of the mouse model by day 10 after treatment. The levels of AST and ALT in 5-FU group were obviously higher than those in control group ($P < 0.01$), while those in GC/5-FU group were lower compared with 5-FU group ($P < 0.01$), there were no differences among control, GC and GC/5-FU groups ($P > 0.05$). The numbers of PLT, WBC and lymphocyte in 5-FU group were decreased more obviously as compared with the control, GC and GC/5-FU groups ($P < 0.05$ or < 0.01), however, there were no differences among these three groups ($P > 0.05$). The levels of blood urea nitrogen, creatinine, Hbg and neutrophil were approximate in different groups ($P > 0.05$), as shown in Table 1.

Cytotoxic activities of CTL and NK in mice

We evaluated whether the GC/5-FU could affect the activity of CTL and NK *in vivo* in mouse model. The harvested splenocytes were washed with PBS. The activity of CTL and NK was detected by MTT colorimetry. Figure 8 shows that the cytotoxic activities of CTL and NK cells were significantly decreased in 5-FU group compared with other three groups ($P < 0.01$), while the crosscurrent was found in GC group compared with 5-FU group ($P < 0.05$). There were no differences between control and GC/5-FU groups. These results thus demonstrate that the GC/5-FU nanoparticles could ameliorate the decreased cytotoxic activities of CTL and NK in 5-FU group.

DISCUSSION

The utilization of nanotechnology and nano-materials in the pharmaceutical field gave rise to the drug-nanoparticle carrier-release system, which is a drug delivery system using nanoparticles as the drug carriers. A particle ranging from 0.1 nm to 100 nm is considered to be a nanoparticle^[22]. The size of a nanoparticle is very important for drug delivery, as the spaces between the cells in various tissues are different: it is now known that the aperture of vascular endothelial within most normal tissues is 2 nm, the aperture of the postcapillary venule is 6 nm, while that of non-continuous tumor blood vessels ranges from 100 nm to 780 nm^[23,24]. The size of the nanoparticles used in this study was approximately 35.19 nm (Figure 2A), which is smaller than most nanoparticles reported^[25], allowing them to enter the space within tumor cells but restricting them from penetrating the normal tissues. Scanning electron microscopy revealed a spherical structure with a smooth surface and no adhe-

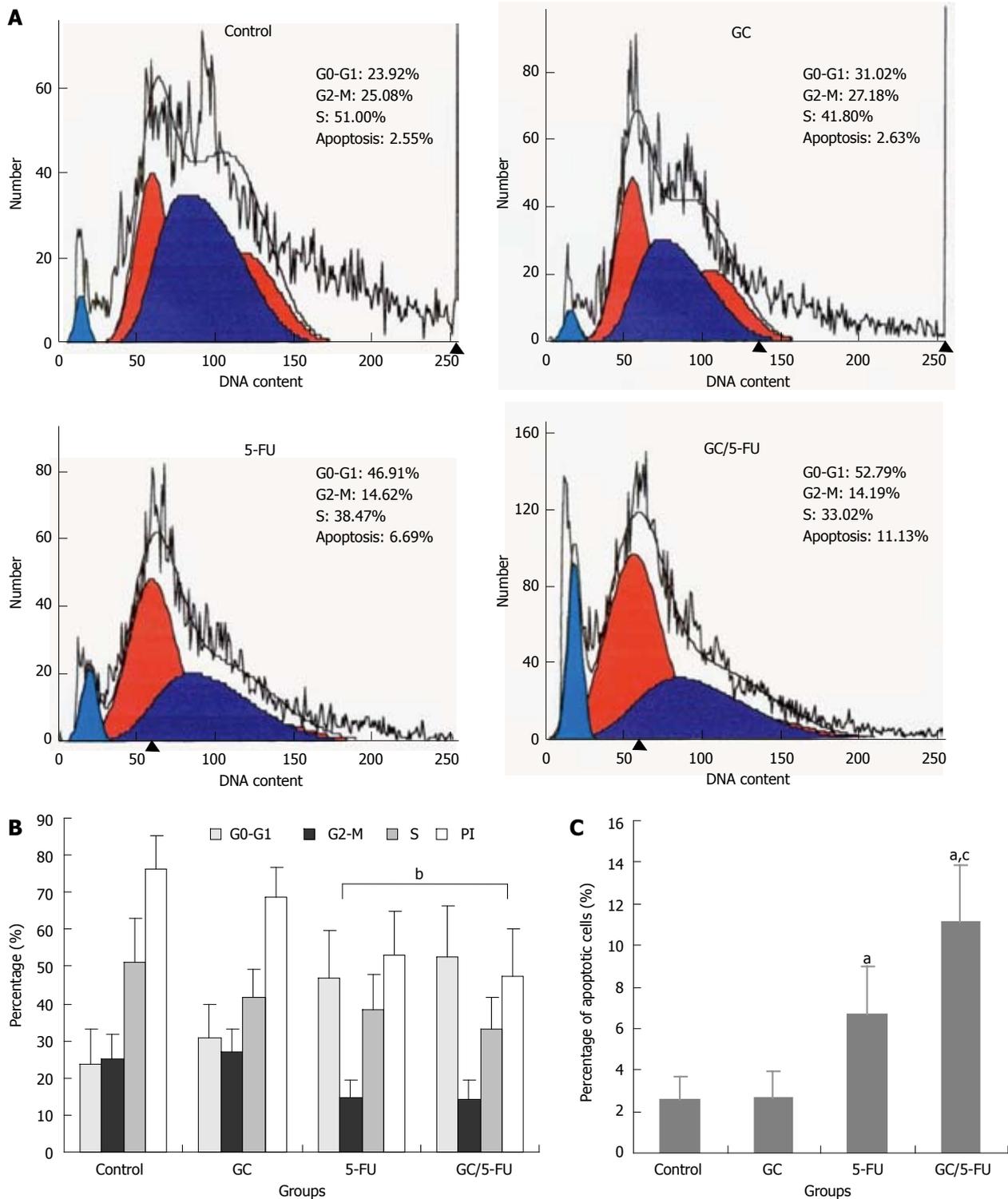


Figure 5 The effects of different treatments on cell cycle, proliferation index and apoptosis index. A: Flow cytometry analysis of cell cycle distribution of mouse liver cancer cell line (H22) cells; B: Quantification of cell cycle distribution and proliferation index of H22 cells. Percentage of cells in G0-G1 in the galactosylated chitosan/5-fluorouracil (GC/5-FU) and 5-FU groups was higher than that in control and GC groups, while the proliferation index (PI) decreased significantly ($P < 0.01$); C: Quantification of apoptosis of H22 in different treatment groups. ^a $P < 0.05$, ^b $P < 0.01$ vs control group; ^c $P < 0.05$ vs 5-FU group.

sion between nanoparticles (Figure 2B), which is consistent with previous reports^[25,26]. In order to confirm the sustained release effect, we performed an experiment on GC/5-FU. The *in vitro* release curve of GC/5-FU in simulated body fluid showed that the sustained release of the nanoparticle lasted 1-8 d. Such sustained release

effect makes the drug evenly distribute in the body, thereby increasing the half-life of GC/5-FU in the circulation system and decreasing the toxic effects of 5-FU on normal tissues^[27].

In order to evaluate the curative efficiency of intravenously injected GC/5-FU in a liver cancer mouse model,

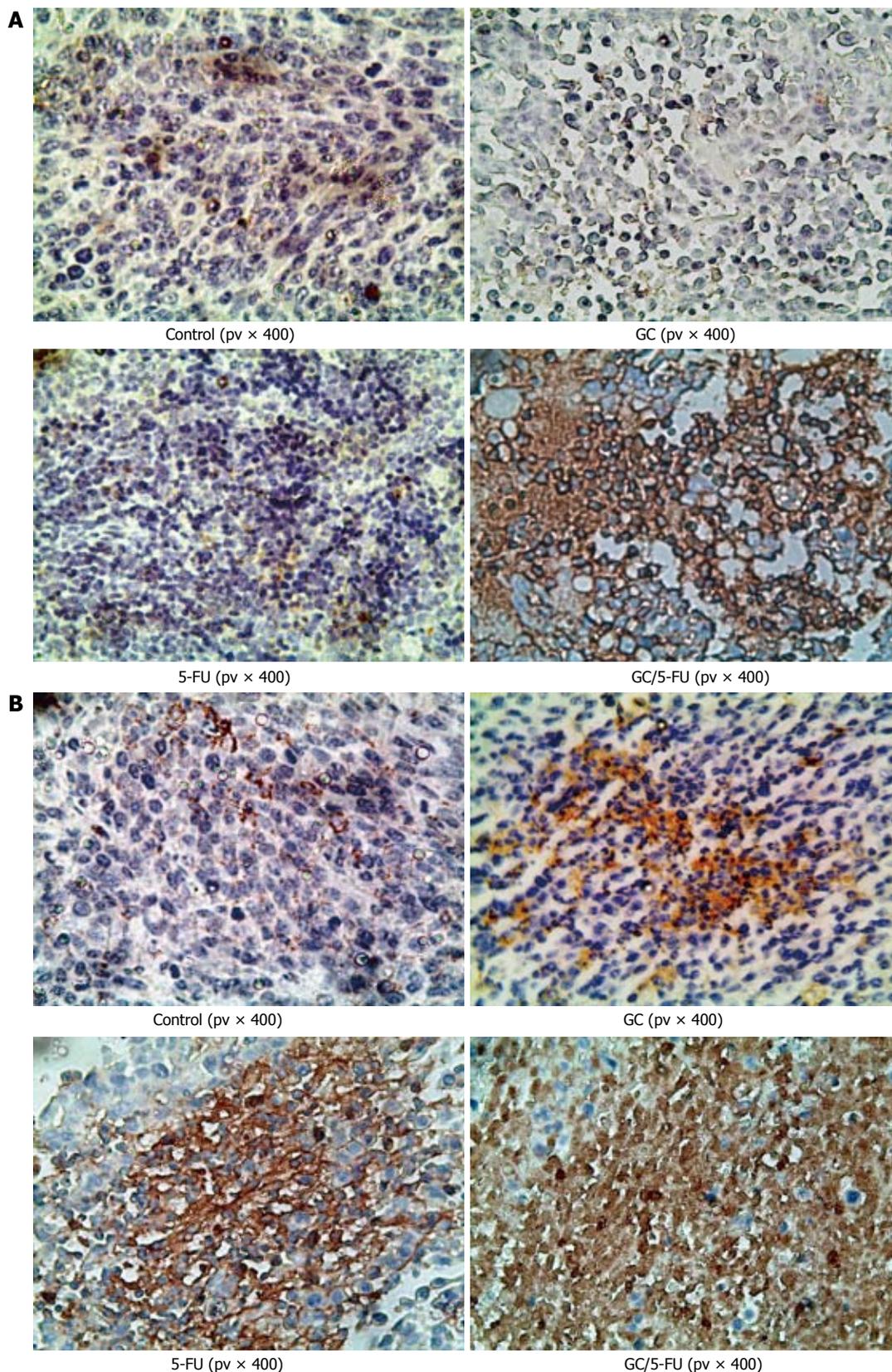


Figure 6 Immunohistochemistry of p53 and caspase-3 in the tumor sections from mice with different treatments. A: p53 staining in the control and galactosylated chitosan (GC) groups showed a scattered nuclear distribution pattern, in dark yellow or dark brown; while in 5-fluorouracil (5-FU) and GC/5-FU groups, p53 showed a sheet staining pattern, which was more dramatic; B: Caspase-3 staining in the control and GC groups showed a scattered cytoplasmic distribution pattern, in dark yellow or dark brown; while in 5-FU and GC/5-FU groups, caspase-3 showed a sheet staining pattern, which was more dramatic in the GC/5-FU group.

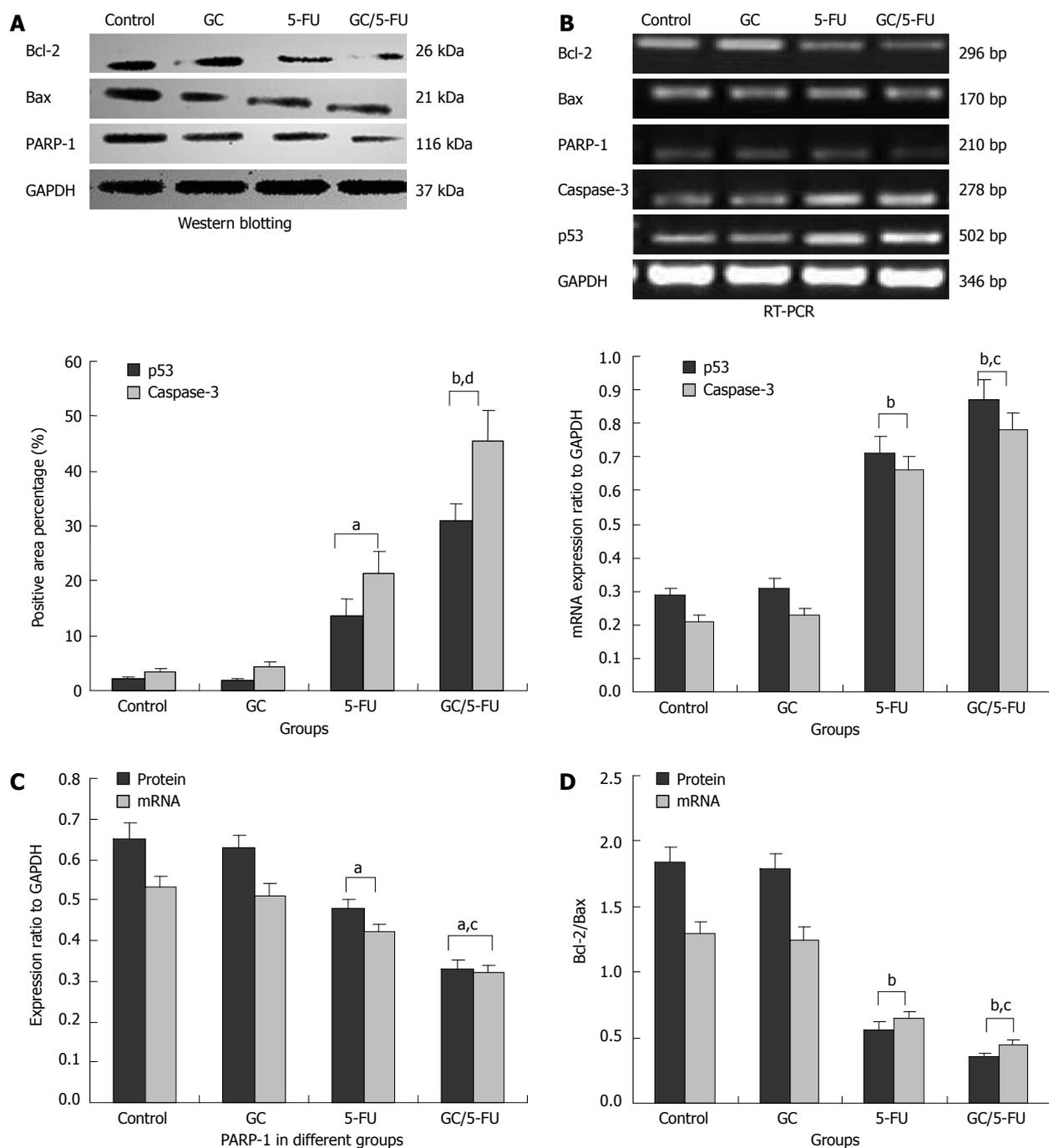


Figure 7 Expression of p53, caspase-3, Bax, Bcl-2 and poly adenosine 50-diphosphate-ribose polymerase 1 in the tumor tissues from mice with different treatments. A: Quantification of p53 and caspase-3 expression as detected by immunohistochemistry and shown pictorially in Figures 6 and 7; B: mRNA levels of p53 and caspase-3 in individual tissues was measured by reverse transcription-polymerase chain reaction (RT-PCR), and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH); C: Poly adenosine 50-diphosphate-ribose polymerase 1 (PARP-1) expression in individual tumor samples was determined by both RT-PCR and western blot analysis; results were normalized to GAPDH; D: Expression of Bcl-2 and Bax was quantified by both RT-PCR and Western blotting; the ratio of Bcl-2/Bax was shown. ^a*P* < 0.05, ^b*P* < 0.01 vs control group; ^c*P* < 0.05, ^d*P* < 0.01 vs 5-fluorouracil (5-FU) group. GC: Galactosylated chitosan.

some of the mice were sacrificed and analyzed at day 15. As shown in Figure 4A, the tumor weight in mice treated with GC/5-FU and 5-FU was significantly less than that in the mice treated with GC or in the control; the weight of GC/5-FU-treated tumors was even lower than the 5-FU-treated tumors, while the GC- or control-treated tumors did not show any statistically significant difference. All mice died after treatment. In the control group,

mice died between day 6 and day 14, with a median survival of 12 d; in the GC group, mice died between day 5 and day 16, with a median survival of 13 d, showing no difference from the control group (Figure 4B, *P* > 0.05). Mice in the 5-FU group succumbed to a tumor-associated death between day 13 and day 24, with a median survival time of 17 d, while mice in the GC/5-FU group died between day 15 and day 37, with a median survival

Table 1 Serum levels of blood biochemical parameters in different groups by day 10

Groups	AST (U/L)	ALT (U/L)	BUN (mmol/L)	Creatinine (μ mol/L)	Hbg (g/L)	PLT ($\times 10^9/L$)	WBC ($\times 10^9/L$)	Lymphocyte ($\times 10^9/L$)	Neutrophil ($\times 10^9/L$)
Control	92.79 \pm 8.74	49.73 \pm 4.83	23.74 \pm 5.84	0.23 \pm 0.09	117.32 \pm 9.87	69.43 \pm 8.94	6.32 \pm 1.24	3.86 \pm 1.34	2.18 \pm 0.73
GC	92.34 \pm 7.65 ^d	49.89 \pm 5.13 ^d	23.25 \pm 6.54	0.24 \pm 0.08	118.823 \pm 10.85	71.43 \pm 6.54 ^d	6.53 \pm 1.32 ^d	3.95 \pm 1.35 ^d	2.17 \pm 0.68
5-FU	113.25 \pm 7.65 ^b	81.48 \pm 6.81 ^b	23.64 \pm 5.45	0.25 \pm 0.07	109.41 \pm 10.73	55.63 \pm 7.43 ^a	3.83 \pm 1.18 ^b	1.57 \pm 1.20 ^a	2.75 \pm 0.87
GC/5-FU	93.42 \pm 8.32 ^d	48.97 \pm 4.93 ^d	22.94 \pm 5.26	0.24 \pm 0.05	116.38 \pm 8.53	68.64 \pm 7.38 ^c	6.21 \pm 1.04 ^d	3.81 \pm 1.17 ^c	2.17 \pm 0.71
F value	8.227	33.222	0.020	0.058	0.868	4.349	5.498	4.249	0.712
P value	0.002	0.000	1.012	0.941	0.482	0.018	0.008	0.024	0.526

Data were expressed as mean \pm SD. $n = 5$ in each group. ^a $P < 0.05$, ^b $P < 0.01$ vs control group; ^c $P < 0.05$, ^d $P < 0.01$ vs 5-fluorouracil group. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; BUN: Blood urea nitrogen; Hbg: Hemoglobin; PLT: Platelet; WBC: White blood cell; GC: Galactosylated chitosan; 5-FU: 5-fluorouracil.

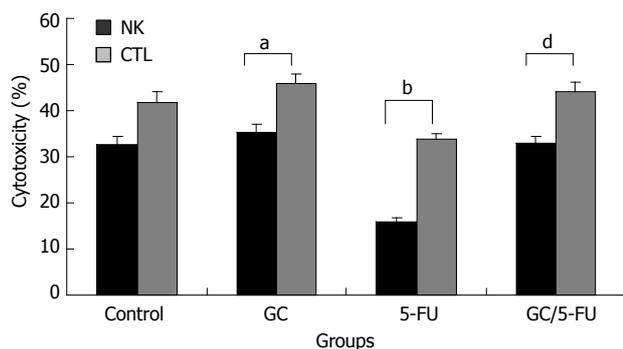


Figure 8 The activity of cytotoxicity T lymphocyte and natural killer cell was detected by 3-(4, 5-dimethylthiazid-2-yl)-2,5-diphenyltetrazolium bromide in the orthotopic transplant model of hepatocellular carcinoma. The cytotoxic activities of cytotoxicity T lymphocyte (CTL) and natural killer (NK) cells were significantly decreased in 5-fluorouracil (5-FU) group compared with other three groups ($P < 0.01$), while, the crosscurrent was found in galactosylated chitosan (GC) group compared with 5-FU group ($P < 0.05$). ^a $P < 0.05$, ^b $P < 0.01$ vs control group; ^d $P < 0.01$ vs 5-FU group.

time of 30 d. The survival time of mice treated with either 5-FU or GC/5-FU was significantly longer than that of mice in the control and GC groups, with the longest survival time seen in the GC/5-FU group. This result suggested that although GC alone cannot affect tumor growth, the conjugation of GC to 5-FU improved the tumor suppressive effect of 5-FU. To determine the mechanism of effect of GC/5-FU nanoparticles on the hepatic cancer, we used flow cytometry to examine tumor cell apoptosis. The results revealed that both 5-FU and GC/5-FU enhanced apoptosis when compared with either control or GC, and compared with 5-FU alone, GC/5-FU further increased the apoptosis index, suggesting that GC improves the pro-apoptotic effect of 5-FU by promoting its entry into the cells. In addition, as shown in Figure 5A and B, compared with control and GC treatment, 5-FU and GC/5-FU can increase the percentage of cells in the G0-G1 phases, but lower the PI, suggesting 5-FU and GC/5-FU are cytotoxic to the proliferating cells by arresting them in the G0-G1 phases; this is consistent with previously reported research^[28,29]. Therefore, GC facilitates intracellular transport of 5-FU, improving the effects of 5-FU on tumor cell apoptosis and on inhibition of the cell cycle.

To further study whether the apoptosis induced by the GC/5-FU nanoparticles was mediated by the p53 pathway, we used immunohistochemistry, reverse transcription-PCR and Western blotting analysis to examine the expression of p53, Bax, Bcl-2, caspase-3 and PARP-1. We found that the addition of GC/5-FU and 5-FU induced p53 expression at both the protein and the RNA levels; the strongest induction of p53 was noted in the GC/5-FU group, and a moderate to strong induction seen in the 5-FU group (Figure 7A and B). The change of Bcl-2/Bax ratio also showed a similar pattern. Administration of GC/5-FU and 5-FU decreased the Bcl-2/Bax ratio, with the most dramatic reduction observed in the GC/5-FU group (Figure 7D). It is now known that Bax, belonging to the Bcl-2 family, is able to promote apoptosis. Although both Bax and Bcl-2 coexist in cells as dimers, each suppresses the function of the other. Physiologically, both Bax and Bcl-2 are present in cells in the same amounts, ensuring the normal growth of the cells. If Bcl-2 is overexpressed, the heterodimer Bcl-2/Bax is induced to suppress apoptosis, while if the level of Bax increases, the formation of Bax/Bax homodimer promotes apoptosis by antagonizing the anti-apoptotic effect of Bcl-2^[30]. Wild-type p53 induces Bax synthesis to mediate apoptosis, while mutant p53 can inhibit apoptosis leading to uncontrolled proliferation^[31]. We also found that GC/5-FU was able to significantly enhance the expression of caspase-3 ($P < 0.01$), which is known to be an important promoter of apoptosis. Caspase-3 can be activated by cytochrome c in the cytosol, which is released from mitochondria under the control of Bax and Bcl-2. Therefore, the ratio of Bax and Bcl-2 determines the activation of caspase-3^[32,33]. Figure 7C shows a tendency toward a decrease in PARP-1 expression from the control to the GC to the 5-FU to the GC/5-FU groups, with a most significant reduction in the GC/5-FU group. It has been reported that caspase-3 is a pivotal effector in apoptosis. Activation of PARP-1 after severe DNA damages results in depletion of cellular energy. In order to prevent the consumption of NAD⁺ and adenosine triphosphate, activated caspase-3 cleaves and inactivates PARP-1, leading to apoptosis^[34]. Taking into consideration all the above results, GC improved the apoptotic effect of 5-FU in hepatic cancer cells. The mechanism underlying GC/5-FU nanoparticle-induced apoptosis was

inducing the expression of p53 at the protein and mRNA levels. The elevated p53 level can significantly lower the Bcl-2/Bax ratio which in turn promotes the release of cytochrome c from the mitochondria into the cytosol, leading to the activation of caspase-3. Upregulation of the caspase-3 gene and protein contributed to the reduction of PARP-1 at both the protein and mRNA levels, thus triggering apoptosis. Therefore, GC/5-FU-induced apoptosis is p53 dependent.

5-FU is a common chemotherapy drug, and its common side effects are the suppression of bone marrow^[35], dysfunction of liver and kidney and suppression of immune function^[36-38], leading to a decreased efficacy and survival time of the patients with cancer. In this experiment, 5-FU increased significantly the levels of AST and ALT, decreased obviously the numbers of PLT, WBC and lymphocyte in tumor-bearing mice compared with the control group. The levels of ALT and AST, the numbers of PLT, WBC and lymphocyte, remained stable in GC/5-FU group compared with control group. It is indicated that GC nanoparticles can improve the damage of liver function caused by 5-FU and the suppression state of bone marrow. We found that the cytotoxic activities of CTL and NK cells by 5-FU were significantly inhibited, and the GC nanoparticles could relieve the suppression state of NK and CTL cells by 5-FU, which is consistent with our previously reported experiments which verified that GC nanoparticles can stimulate the cytotoxic activities of CTL and NK cells in tumor-bearing mice^[17]. So the GC/5-FU nanoparticles can alleviate the inhibition of 5-FU on the body's immunity.

In conclusion, we demonstrated that GC is a good carrier for nano-material, especially 5-FU. GC/5-FU nanoparticles had a sustained release effect. GC/5-FU nanoparticles can also significantly inhibit the tumor growth in the orthotopic liver cancer mouse model, and this *in vivo* effect was stronger than that of 5-FU alone. The mechanism underlying GC/5-FU nanoparticles may be the elevated G0-G1 arrest and apoptosis mediated by the p53 pathway. GC/5-FU nanoparticles can ameliorate the side effects and immunosuppressive action of 5-FU.

COMMENTS

Background

Biodegradable polymer nanoparticle drug delivery systems are characterized by targeted drug delivery, improved pharmacokinetic and biodistribution, enhanced drug stability and reduced side effects. These drug delivery systems are widely used for delivery of cytotoxic agents. The galactosylated chitosan (GC)/5-fluorouracil (5-FU) nanoparticle is a nanomaterial made by coupling GC, a polymer known to have the advantages described above, and 5-FU.

Research frontiers

5-FU can target normal, proliferating tissues, but with side effects of bone marrow suppression and gastrointestinal reactions. Targeted therapy for hepatocellular cancer may be useful because it is relatively less expensive compared with the current therapies and it also has fewer side effects. The emergence of a novel, sustained-release formulation of 5-FU is of clinical significance because it has fewer side effects compared with the regular 5-FU formulation.

Innovations and breakthroughs

In this paper, the authors examined the effects of GC/5-FU nanoparticle on liver cancer mouse model. As a result, GC/5-FU treatment could significantly lower

the tumor weight and increase the survival time of mice when compared with 5-FU treatment alone. In addition, it was suggested that the abovementioned effects of GC/5-FU was associated with G0-G1 arrest and apoptosis of tumor cells mediated by p53 pathway. GC/5-FU nanoparticles can relieve the side effects and immune-suppressive action of 5-FU.

Terminology

The effects of GC/5-FU nanoparticle on liver cancer mouse model. GC is a galactose ligand, with chitosan modifications on the molecular structure. Asialoglycoprotein receptor (ASGPR) is a receptor found on the membrane of hepatocytes facing the sinusoids, with specificity for glycoproteins with galactose or acetyl galactosamine at the end. The galactose ligand with ASGPR in GC/5-FU nanoparticle induces liver-targeted 5-FU transfer.

Peer review

The significance of this study is evident because the improvement of chemotherapy for liver cancer is an important subject in the clinical setting. In addition, the animal experiments were generally performed appropriately.

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Small intestine contrast ultrasonography vs computed tomography enteroclysis for assessing ileal Crohn's disease

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Abstract

AIM: To compare computed tomography enteroclysis (CTE) vs small intestine contrast ultrasonography (SICUS) for assessing small bowel lesions in Crohn's disease (CD), when using surgical pathology as gold standard.

METHODS: From January 2007 to July 2008, 15 eligible patients undergoing elective resection of the distal

ileum and caecum (or right colon) were prospectively enrolled. All patients were under follow-up. The study population included 6 males and 9 females, with a median age of 44 years (range: 18-80 years). Inclusion criteria: (1) certain diagnosis of small bowel requiring elective ileo-colonic resection; (2) age between 18-80 years; (3) elective surgery in our Surgical Unit; and (4) written informed consent. SICUS and CTE were performed \leq 3 mo before surgery, followed by surgical pathology. The following small bowel lesions were blindly reported by one sonologist, radiologist, surgeon and histopathologist: disease site, extent, strictures, abscesses, fistulae, small bowel dilation. Comparison between findings at SICUS, CTE, surgical specimens and histological examination was made by assessing the specificity, sensitivity and accuracy of each technique, when using surgical findings as gold standard.

RESULTS: Among the 15 patients enrolled, CTE was not feasible in 2 patients, due to urgent surgery in one patient and to low compliance in the second patient, refusing to perform CTE due to the discomfort related to the naso-jejunal tube. The analysis for comparing CTE vs SICUS findings was therefore performed in 13 out of the 15 CD patients enrolled. Differently from CTE, SICUS was feasible in all the 15 patients enrolled. No complications were observed when using SICUS or CTE. Surgical pathology findings in the tested population included: small bowel stricture in 13 patients, small bowel dilation above ileal stricture in 10 patients, abdominal abscesses in 2 patients, enteric fistulae in 5 patients, lymphnodes enlargement ($>$ 1 cm) in 7 patients and mesenteric enlargement in 9 patients. In order to compare findings by using SICUS, CTE, histology and surgery, characteristics of the small bowel lesions observed in CD each patient were blindly reported in the same form by one gastroenterologist-sonologist, radiologist, surgeon and anatomopathologist. At surgery, lesions related to CD were detected

in the distal ileum in all 13 patients, also visualized by both SICUS and CTE in all 13 patients. Ileal lesions > 10 cm length were detected at surgery in all the 13 CD patients, confirmed by SICUS and CTE in the same 12 out of the 13 patients. When using surgical findings as a gold standard, SICUS and CTE showed the exactly same sensitivity, specificity and accuracy for detecting the presence of small bowel fistulae (accuracy 77% for both) and abscesses (accuracy 85% for both). In the tested CD population, SICUS and CTE were also quite comparable in terms of accuracy for detecting the presence of small bowel strictures (92% vs 100%), small bowel fistulae (77% for both) and small bowel dilation (85% vs 82%).

CONCLUSION: In our study population, CTE and the non-invasive and radiation-free SICUS showed a comparable high accuracy for assessing small bowel lesions in CD.

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Key words: Crohn's disease; Ileal lesions; Computed tomography enteroclysis; Small intestine contrast ultrasonography; Surgical findings

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INTRODUCTION

Accurate assessment of the lesions is mandatory for a proper pre-operative evaluation in Crohn's disease (CD). Diagnostic imaging of the small bowel traditionally included the small bowel follow through (SBFT) or small bowel enteroclysis^[1]. A comparable accuracy for assessing small bowel lesions in CD has been shown in referral centres^[2]. More recently, magnetic resonance enterography (MRE) and computed tomography enterography or enteroclysis (CTE) showed to accurately assess the presence, site and extent of small bowel lesions in CD, including stenosis, bowel dilation above stenosis and accurate measurement of the lumen diameter^[3-6]. These techniques also provide detailed extraluminal findings, including the bowel wall thickness (BWT), fis-

tulas, abscesses and phlegmons not detected by barium studies^[7-9]. For these reasons, MRE and CTE currently represent the standard techniques for assessing small bowel lesions in CD^[10]. The major limit of CTE is represented by the high radiation exposure for the patient^[5-11]. However, CTE has a greater availability and is less time-consuming than MRE^[10]. Therefore, as CTE and MRE show a comparable sensitivity for assessing small bowel lesions in CD, their use is also related to the local feasibility and availability of an experienced radiologist^[10].

Transabdominal ultrasonography also has been proposed for detecting small bowel lesions in patients with suspected or known CD, showing a sensitivity and specificity of 67%-84% and 81%-95%, respectively^[12-14]. The use of oral contrast significantly increases the sensitivity of ultrasonography for assessing small bowel lesions in CD (by more than 95%)^[10,15-19]. In particular, small intestine contrast ultrasonography (SICUS) performed by an experienced sonographer may visualize both established CD lesions (i.e., stenosis with possible pre-stenotic dilation) and minor changes of the small bowel^[10,15-20]. In experienced hands, SICUS may detect lesions in suspected small bowel diseases with a high (> 95%) sensitivity and specificity, when compared with SBFT and enema^[10,15-19]. The use of SICUS has also been proposed in the follow-up of CD patients after ileo-colonic resection, in order to avoid radiation exposure or the more invasive ileocolonoscopy^[19].

To our knowledge, only one retrospective study compared CTE and surgical pathology findings in patients with small bowel CD^[21]. A detailed information of the small bowel lesions is mandatory before elective surgery in CD^[10]. Moreover, surgical indication in subgroup of patients with small bowel CD may also be related to characteristics of the lesions, including abscesses, marked lumen narrowing and/or strictures with prestenotic dilation. On the basis of these observations, in a prospective longitudinal study, we aimed to compare the sensitivity, specificity and accuracy of SICUS vs CTE for assessing the presence of small bowel lesions in patients with CD undergoing elective ileo-colonic resection, when using surgical pathology findings as a gold standard.

MATERIALS AND METHODS

Patients

From January 2007 to July 2008, 18 eligible patients undergoing elective resection of the distal ileum and cecum (or right colon) with ileo-colonic anastomosis were enrolled. Among these 18 patients, there were 15 patients with ileal CD (8 males, median age 44 years, range: 19-73 years) and, as a control group, 3 patients (2 males, mean age 69 years, range: 60-77 years), requiring ileal resection due to small bowel duplication, carcinoid or ischemic enteritis. All patients were under regular follow-up in our unit.

Inclusion criteria included: (1) Patients with a certain

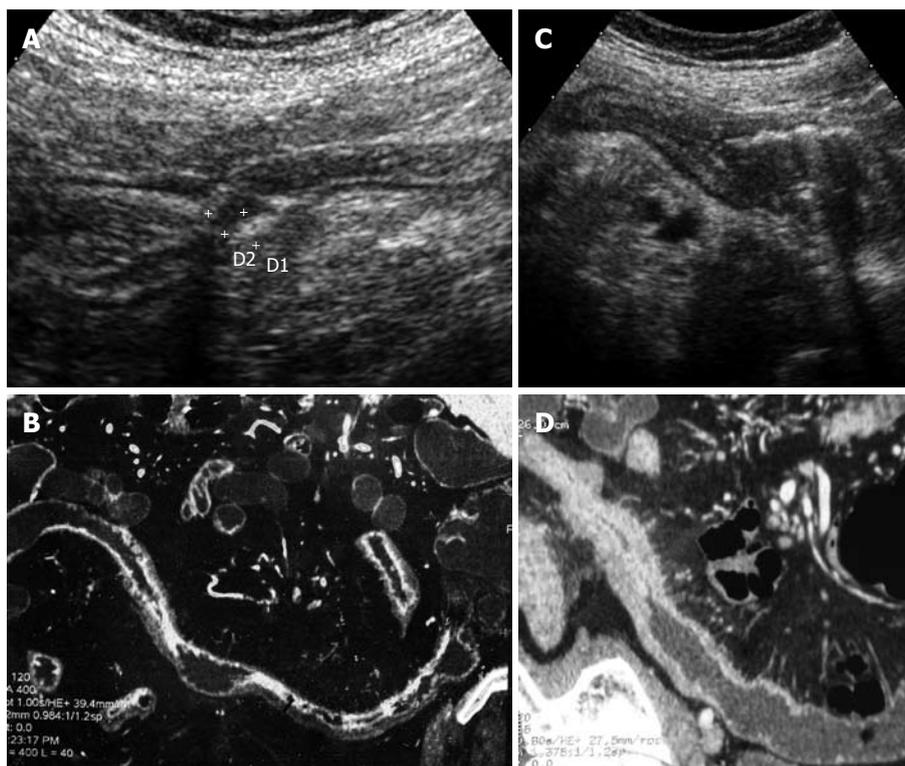


Figure 1 The figure shows images from the distal ileum from one patient with Crohn's disease of the distal and neo-terminal ileum undergoing ileo-colonic resection, as assessed by small intestine contrast ultrasonography and computed tomography-enteroclysis. A: Small intestine contrast ultrasonography (SICUS) showed a stenosis of the terminal ileum, with a thickened bowel wall, with lumen narrowing. The lumen diameter did not change after the ingestion of poly-ethylen glycole; B: Computed tomography-enteroclysis (CTE) showed findings comparable with SICUS, including a marked narrowing of the distal ileum, associated with an increased bowel wall thickness; C: SICUS showed a stricture of the neo-terminal ileum presenting as a thickened bowel wall, with lumen narrowing associated with pre-stenotic dilation; D: CTE showed findings comparable with SICUS, including a marked narrowing of the distal ileum, associated with an increased bowel wall thickness, but no bowel dilation).

diagnosis of small bowel diseases including CD or other non-inflammatory bowel disease (IBD) related conditions, requiring elective ileo-colonic resection; (2) age between 18-80 years; (3) elective surgery in our Referral Surgical Unit; and (4) written informed consent.

Exclusion criteria included: (1) Low compliance to perform both SICUS and CTE, including the introduction of a naso-gastric tube; (2) patients requiring urgent surgery; (3) obesity (body mass index > 30) not allowing a proper assessment by SICUS; and (4) allergy to contrast agents. In patients with CD, the diagnosis was made according to standard clinical, endoscopic and radiological criteria^[10]. Clinical characteristics of each of the 16 patients studied are summarized in Table 1.

Study protocol

From January 2007 to July 2008, all patients fulfilling the inclusion criteria and requiring elective ileo-colonic resection in our Unit due to small bowel CD were prospectively enrolled. In all patients, ileal lesions were assessed by using both SICUS and CTE within 3 mo before surgery, followed by surgical pathology findings used as a gold standard. Histological assessment of the surgical specimen was performed. In order to compare findings by using SICUS, CTE, histology and surgery,

characteristics of the small bowel lesions were blindly reported in the same form by one gastroenterologist-sonologist (Calabrese E), radiologist (Fiori R), surgeon (Simonetti G) and anatomopathologist (Palmieri G). The following parameters detailing the characteristics of the small bowel lesions were blindly reported by each specialist: site of the lesions (ileum, ileum-colon, colon, others), extent of the lesions (< 10 cm *vs* > 10 cm), strictures (yes/no, number), fistulae (yes/no, number), abscesses (yes/no, number), bowel dilation above strictures (yes/no), lymphnodes enlargement > 1 cm (yes/no, number), mesenteric enlargement (yes/no) (Figure 1).

CTE

CTE was performed by one experienced radiologist unaware of SICUS findings, from the Department of Diagnostic Imaging from our university, as previously described^[22]. Colonic cleaning was performed the day before CTE by using polyethylen glycole (PEG) 4000 solution. A 20G needle was placed in the antecubital vein and an 8-F naso-jejunal catheter with a Teflon-covered guide wire was positioned under fluoroscopic guidance (Guerbetm Guerbet GmbH D65838, Sulzbach/Ts) and the distal tip was located in the distal duodenum. The patient was then taken into CT room and contrast material (1500 mL of PEG) was administered manually with

Table 1 Clinical characteristics of the 16 patients considered in the analysis

Disease	Sex	Age (yr)	Surgical indication	Lesions extent (cm)	CD pattern
CD	M	39	Sub-obstructions	30	Fibrostricturing
CD	F	19	Abscess	20	Fistulizing
CD	F	49	Sub-obstructions	30	Fibrostricturing
CD	M	38	Abscess	21	Fibrostricturing
CD	F	73	Sub-obstructions	40	Fibrostricturing
CD	F	33	Sub-obstructions	25	Fibrostricturing
CD	F	57	Sub-obstructions	30	Fibrostricturing
CD	M	31	Abscess	15	Fistulizing
CD	F	49	Sub-obstructions	35	Fibrostricturing
CD	M	41	Sub-obstructions	20	Fibrostricturing
CD	M	45	Sub-obstructions	15	Fibrostricturing
CD	M	30	Sub-obstructions	40	Fibrostricturing
CD	M	30	Sub-obstructions	35	Fibrostricturing
Small bowel duplication	M	60	Sub-obstructions	20	NA
Ischemic enteritis	M	69	Abdominal pain	10	NA
Small bowel carcinoid	F	77	Diarrhoea, weight loss	35	NA

CD: Crohn's disease; M: Male; F: Female; NA: Not applicable (non-CD).

60-mL syringes, with a continuous injection rate of 150 mL/min followed by a flow rate of 200 mL/min until the maximum tolerance of the patient. Before the examination, a smooth muscle relaxant (N butyl-scopolamine) was administered. CTE was performed by a 64-slice multidetector (LightSpeed VCT, General Electric Medical System, Milwaukee, WI, United States). After PEG infusion, CT scan was performed before and after the administration of *iv.*, iodinated contrast material. The contrast-enhanced study was acquired 70 s after the administration of contrast material (Ultravist 370, Schering AG, Berlin, Germany) with a "double-bolus" technique (a first bolus of 60 cc at a flow rate of 1.5 mL/s and a second bolus of 80 cc at a flow rate of 2.0 mL/s).

SICUS

SICUS was performed as previously described^[15,18,19]. In particular, SICUS was performed after the ingestion of 375 mL (range: 250-500 mL) of oral contrast solution consisting of PEG (Promefarm, Milano, Italy), by using a convex transducer (frequency 3.5-5 MHz) and then with a high frequency linear-array transducer (5-12 MHz) (Hitachi, EUB 6500, Japan). All procedures were performed by the same expert EC (> 2000 examinations).

The following findings were considered compatible with CD^[16,18,19]: (1) increased BWT (> 3 mm); (2) "stiff loop", identified by the presence of small bowel loop, with increased BWT not distended by contrast solution; (3) small bowel dilation, defined as a lumen diameter > 2.5 cm; (4) bowel stricture defined as lumen diameter < 1 cm, measured at the level of maximally distended loop, independently of the presence of pre-stenotic dilation; (5) fistulae defined as hypoechoic tract with or without hyperechoic content; (6) mesenteric enlargement and/or

masses; (7) lymphnodes enlargement (> 1 cm); and (8) abscesses identified as roundish anechoic lesions, with an irregular wall, often presenting internal echoes and posterior echo enhancement.

Surgical assessment

At time of intestinal resection, one single GS filled up the above reported form in order to assess the small bowel lesions. Findings at surgery were considered as the gold standard for assessing the small bowel lesions described by SICUS and CTE. The surgical specimen was fixed in formalin for histological examination.

Histological assessment

The surgical specimen was examined by the one single GP unaware of previous findings at SICUS, CTE and surgery. At this purpose, routine hematoxylin and eosin staining was performed and the GP filled up the same form used by the sonographer, radiologist and surgeon.

Statistical analysis

All results were expressed as median and range. Comparison between findings at SICUS, CTE, surgical specimens and histological examination was made by assessing the specificity, sensitivity and accuracy of each technique, when using surgical findings as a gold standard.

RESULTS

From January 2007 to July 2008, 15 CD patients undergoing elective ileo-colonic resection and fulfilling the inclusion criteria were prospectively enrolled. Among these 15 patients, 2 CD patients performed SICUS but were not able to perform CTE, as one patient required "urgent surgery", and the second patient refused to perform CTE due to the discomfort related to the naso-jejunal tube. Therefore, among the initial 15 patients eligible for the study, only 13 patients were studied by both SICUS and CTE and were therefore considered for the analysis. No side effects were reported after SICUS and CTE procedures.

Among the 13 CD patients considered in the analysis, surgical pathology findings included: small bowel stricture in 13, small bowel dilation above stricture in 10, abdominal abscesses in 2, fistulae in 5 (associated with abscess in 2), lymphnodes enlargement (> 1 cm) in 7 and mesenteric enlargement in 9 patients (Table 2).

Small bowel assessment by CTE and SICUS vs findings at surgery

Site of the lesions: At surgery, lesions were detected in the distal ileum in all 13 patients, and also in the right colon in 5. Both SICUS and CTE also visualized ileal lesions in all 13 patients, while concomitant lesions in the right colon were detected in 3 out of the 5 patients by both SICUS and CTE (including the same patients in 2 cases) (Table 2). Histological findings were comparable to surgery in all CD patients.

Table 2 Characteristics of the lesions in the 13 Crohn's disease patients, as assessed by surgical pathology (considered as a gold standard), small intestine contrast ultrasonography, computed tomography-enterography and histology

Characteristics	Surgery	SICUS	CTE	Histology
CD site				
Ileum	8/13	10/13 ¹	10/13	8/13
Ileum-colon	5/13	3/13	3/13	5/13
CD extent (cm)				
< 10	0/13	12/13	12/13	9/13
≥ 10	13/13	1/13	1/13	4/13
Strictures				
Yes	13/13	12/13	13/13	13/13
No	0/13	1/13	0/13	0/13
Dilation				
Yes	10/13	10/13	11/13	5/13
No	0/13	0/13	2/13	8/13
Fistulae				
Yes	5/13	6/13	4/13	6/13
No	8/13	7/13	9/13	7/13
Abscesses				
Yes	3/13	5/13	3/13	4/13
No	11/13	8/13	10/13	9/13

¹Including the jejunum in 1 patient. CD: Crohn's disease; SICUS: Small intestine contrast ultrasonography; CTE: Computed tomography-enterography.

Extent of the small bowel lesions: In CD group (Table 2), histology detected ileal lesions of > 10 cm length in only 9 patients.

Small bowel strictures: Ileal strictures were detected at surgery in all 13 CD patients. Comparable findings were detected by using CTE and histology, while no strictures were detected in one CD patient by using SICUS (Table 2).

Bowel dilation above strictures: Dilation above ileal strictures was detected by both surgery and SICUS in 10/13 CD patients, by CTE in 11/13 patients and by histology in 5/13 patients (Table 2). However, discrepant findings vs surgery were observed by using SICUS in 4/13 CD (dilation not detected in 2; dilation detected only by SICUS in 2), by using CTE in 5/13 CD (dilation not detected in 2; dilation detected only by CTE in 3) and by histology in 5/13 patients (dilation not detected in 5, discrepant findings vs surgery but comparable with SICUS in 2 and with CTE in 1) (Table 2). In the same 2 CD patients, both SICUS and CTE concordantly reported dilation above strictures not detected at surgery. Findings at CTE and SICUS were comparable in only 8/13 patients, as dilation was detected only by SICUS in 2 (confirmed at surgery) and only by CTE in 3 CD patients (confirmed at surgery in 2).

Fistulae: The presence of enteric fistulae were detected in 5 out of the 13 CD patients at surgery, in 6 patients when using SICUS, in 4 patients by using CTE, while histology reported the presence of fistulae in 6 CD patients (Table 2). Findings different from surgery were detected by SICUS in 3/13 patients (fistulae detected by SICUS and not at surgery in 2 patients; fistulae de-

Table 3 Sensitivity, specificity and accuracy of small intestine contrast ultrasonography and computed tomography-enterography for detecting the presence of small bowel abscesses, strictures, fistulae and bowel dilation in Crohn's disease

Parameter	SICUS			CTE		
	Sens.	Spec.	Accuracy	Sens.	Spec.	Accuracy
Strictures	92	0	92	100	0	100
	(TN 0; TP 12; FN 1; FP 0)			(TN 0; TP 13; FN 0; FP 0)		
Dilation	100	50	85	78	0	82
	(TN2; TP 9; FN 0; FP2)			(TN 0; TP 7; FN 2; FP 4)		
Fistulae	60	88	77	60	88	77
	(TN 7; TP 3; FN 2; FP 1)			(TN 7; TP 3; FN 2; FP 1)		
Abscesses	100	80	85	67	100	85
	(TN 8; TP 3; FN 0; FP 2)			(TN 9; TP 2; FN 1; FP 1)		

SICUS: Small intestine contrast ultrasonography; CTE: Computed tomography-enterography; TN: True negative; TP: True positive; FN: False negative; FP: False positive; Sens.: Sensitivity; Spec.: Specificity.

tected at surgery and not by SICUS in 1 patient) by CTE in 4/13 patients (fistulae detected only by CTE in 2; detected only at surgery in 2); and by histology in 3/13 patients (fistulae detected by histology and not at surgery in 2, and detected at surgery and not by histology in 1). In 3 patients, SICUS and CTE concordantly reported enteric fistulae not confirmed at surgery (detected histologically in 2). When comparing SICUS vs CTE, the presence of fistulae was concordantly detected in 9/13 patients, while fistulae were detected only by SICUS in 1 CD patient.

Abscesses: Abdominal abscesses were detected at surgery in 3/13 CD patients (surgically drained in 1), by using SICUS in 5, by CTE in 3 and by histology in 4 patients (Table 2). Findings different from surgery were reported by SICUS in 2 patients (abscess detected only by SICUS in both), by CTE in 2 patients (abscess detected only by CTE in 1, only at surgery in 1), and by histology in 3 patients (abscess detected only by histology in 2 and only at surgery in 1 patient performing surgical drainage). In one patient, abdominal abscess was detected by both SICUS and CTE but not by surgical pathology and histology. When comparing SICUS and CTE, the presence of abscesses was concordantly detected in 11 out of the 13 patients, while in 2 patients SICUS only reported the presence of an intestinal abscess (confirmed at surgery in one of them).

Sensitivity and specificity of SICUS vs CTE

When using surgical findings as a gold standard, sensitivity, specificity and accuracy of SICUS and CTE for assessing the presence of stenosis, dilation above stenosis and fistulae are reported in Table 3. As indicated, the two techniques showed the same sensitivity, specificity and accuracy for detecting the presence of small bowel fistulae (accuracy 77% for both) and abscesses (accuracy 85% for both). SICUS and CTE were also quite comparable for detecting the presence of small bowel strictures, fistulae and abscesses. Nevertheless, there was a

not significant trend for a higher sensitivity and accuracy of CTE vs SICUS for assessing small bowel strictures (accuracy 100% vs 92%; the observed 0% specificity related to the absence of true negative findings), while SICUS showed a not significantly higher accuracy vs CTE for detecting small bowel dilation (85% vs 82%).

DISCUSSION

Appropriate surgical treatment of CD involves an accurate knowledge of the characteristics of the lesions, including the site, extent and possible presence of complications (strictures, dilation above strictures, fistulae, abscesses). The development of a marked bowel dilation above stricture or abscesses may represent indication for surgery^[10]. Colonoscopy represents the gold standard technique for assessing colonic lesions, while small bowel lesions were previously assessed by SBFT or small bowel enteroclysis^[11,2]. More recently, CTE or MRE represent the gold standard techniques at this purpose^[10]. These techniques indeed provide not only an accurate assessment of the presence, site and extent of the lesions, but they also allow the visualization of extraluminal findings related to the disease (i.e., increased BWT, fistulae, abscesses, mesenteric enlargement)^[5-9]. The preferential use of CTE vs MRE is related to the feasibility and easy access to these techniques in each IBD referral centre. At this purpose, both appropriate radiologic instruments and an experienced radiologist with specific competence in the field are required^[10]. MRE shows the advantage of a radiation-free procedure.

SICUS also has also been recently suggested as a non-invasive technique able to assess, in experienced hands, the presence of small bowel lesions in CD, including the BWT, strictures, bowel dilation, fistulae and abscesses^[16,18,21]. Indication for surgery in CD may also be related to the characteristics of the small bowel lesions (i.e., marked dilation above strictures, abscesses). Whether CTE and SICUS provide a comparable definition of the small bowel lesions in CD is currently unknown. On the basis of these observations, in the present study we aimed to compare these 2 techniques in terms of assessment of the small bowel lesions in patients with CD undergoing elective ileo-colonic resection. The use of small bowel capsule endoscopy has also been shown to accurately visualize small bowel lesions in CD^[23-25]. However, the use of small bowel capsule endoscopy (SBCE) is limited by the impact risk in patients with intestinal stenosis^[23-25]. In our study, according to the inclusion criteria, all CD patients were undergoing elective ileo-colonic resection. Therefore, all the enrolled CD patients were by definition at high risk of SBCE impact, related to severe lesions requiring surgical resection. For this reason, this useful technique able to visualize the entire small bowel was not feasible in the present study aimed to compare findings using CTE vs SICUS. A comparative estimate of the costs of the current techniques able to assess small bowel lesions, including not only CTE and SICUS, but also MRE and SBCE, could

be of great interest. However, these cost may greatly differ in different hospitals, thus limiting the usefulness of this estimate. Nevertheless, among techniques tested in the present study, it appears conceivable to consider CTE more expensive than SICUS.

Limitations of the study include the limited number of tested patients ($n = 15$), related to difficulties to perform 2 consecutive small bowel examinations in patients with active CD undergoing surgical resection. Additional limitation include the absence of a control group, as the purpose of the study was to compare the accuracy of SICUS vs CTE for assessing small bowel lesions in patients with a certain diagnosis of CD. Results from our limited series suggest that SICUS and CTE are quite comparable techniques at this purpose. However, while the accuracy of these two procedures for assessing the presence of strictures was quite comparable, SICUS showed a slightly higher accuracy for detecting the presence of dilation above strictures. In our series, CTE and SICUS were absolutely comparable for assessing the presence of fistulae and abscesses. SICUS is not feasible in obese patients, due to inaccurate findings and may be less accurate than CTE for visualizing lesions in the deeper layer of the abdominal cavity^[12]. Nevertheless, it seems relevant to note that CTE could not be performed in 2 out of the 17 enrolled CD patients (11.7%) already studied by SICUS. These 2 patients were therefore excluded from the analysis, as CTE could not be performed due to low compliance in one patient refusing the naso-jejunal tube and to need of urgent surgery in the second patient. These observations therefore support that CTE may not be performed in a relatively high proportion of patients undergoing ileo-colonic resection for CD.

Nevertheless, differently from SICUS, CTE is an invasive procedure associated with a high radiation exposure for the patient^[11]. This issue assumes particular relevance when considering that small bowel assessment before surgery for CD is most often required in young patients already performing other diagnostic radiological procedures and treated with immunomodulatory drugs^[26-29]. These observations, together with findings from our study therefore suggest that in referral IBD centres with a feasible experienced ultrasonographer, SICUS may represent the procedure of choice when compared with CTE, for assessing small bowel lesions in patients undergoing elective ileo-colonic resection for CD.

COMMENTS

Background

Magnetic resonance enterography (MRE) and computed tomography enterography or enteroclysis (CTE) accurately assess small bowel lesions in Crohn's disease (CD), representing the standard techniques at this purpose. The major limit of CTE is represented by the high radiation exposure for the patient. Recently, small intestine contrast ultrasonography (SICUS) performed by an experienced sonographer has been shown to visualize CD lesions of the small bowel. These findings suggest that SICUS may be used for assessing CD lesions, although comparison with CTE when using surgical pathology as standard is unknown.

Research frontiers

Proper follow up of CD patients includes the assessment of the lesions in order to choose appropriate treatment strategies and the presence of complications. In this study, the authors compared the sensitivity, specificity and accuracy of SICUS vs CTE for assessing the presence of small bowel lesions in patients with CD undergoing elective ileo-colonic resection, when using surgical pathology findings as a gold standard.

Innovations and breakthroughs

Small bowel lesions in CD may be accurately detected by CTE or MRE. However, the use of CTE is associated with a high radiation exposure, while MRE shows a low availability. Moreover, the need of intestinal preparation, insertion of the naso-gastric tube may limit the use of both techniques. The authors performed a prospective longitudinal study in patients undergoing elective surgery, aimed to assess the accuracy of SICUS vs CTE for assessing small bowel lesions in CD, when using surgical pathology as gold standard.

Applications

This study provides the first evidence that SICUS and CTE show a comparable high accuracy for assessing small bowel lesions in CD. These results suggest that the radiation-free, non-invasive SICUS performed by an experienced sonographer may be used for assessing small bowel lesions in patients with CD.

Peer review

CTE and MRE may not be performed in patients with low compliance. Results from this study support that, differently from CTE, SICUS may be performed in all CD patients undergoing elective surgery. As SICUS and CTE showed a comparable high accuracy for assessing small bowel lesions in CD, the non-invasive SICUS should be used at this purpose in referral centres provided of an experienced and available sonologist.

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Quasispecies dynamics in main core epitopes of hepatitis B virus by ultra-deep-pyrosequencing

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Abstract

AIM: To investigate the variability of the main immunodominant motifs of hepatitis B virus (HBV) core gene by ultra-deep-pyrosequencing (UDPS).

METHODS: Four samples (2 genotype A and 2 genotype D) from 4 treatment-naïve patients were assessed for baseline variability. Two additional samples from

one patient (patient 4, genotype D) were selected for analysis: one sample corresponded to a 36-mo treatment-free period from baseline and the other to the time of viral breakthrough after 18 mo of lamivudine treatment. The HBV region analyzed covered amino acids 40 to 95 of the core gene, and included the two main epitopic regions, Th50-69 and B74-84. UDPS was carried out in the Genome Sequencer FLX system (454 Life Sciences, Roche). After computer filtering of UDPS data based on a Poisson statistical model, 122 813 sequences were analyzed. The most conserved position detected by UDPS was analyzed by site-directed mutagenesis and evaluated in cell culture.

RESULTS: Positions with highest variability rates were mainly located in the main core epitopes, confirming their role as immune-stimulating regions. In addition, the distribution of variability showed a relationship with HBV genotype. Patient 1 (genotype A) presented the lowest variability rates and patient 2 (genotype A) had 3 codons with variability higher than 1%. Patient 3 and 4 (both genotype D) presented 5 and 8 codons with variability higher than 1%, respectively. The median baseline frequencies showed that genotype A samples had higher variability in epitopic positions than in the other positions analyzed, approaching significance ($P = 0.07$, sample 1 and $P = 0.05$, sample 2). In contrast, there were no significant differences in variability between the epitopic and other positions in genotype D cases. Interestingly, patient 1 presented a completely mutated motif from amino acid 64 to 67 ($E_{64}LMT_{67}$), which is commonly recognized by T helper cells. Additionally, the variability observed in all 4 patients was particularly associated with the $E_{64}LMT_{67}$ motif. Codons 78 and 79 were highly conserved in all samples, in keeping with their involvement in the interaction between the HBV virion capsid and the surface antigens (HBsAg). Of note, codon 76 was even more conserved than codons 78 and 79, suggesting a possible role in HBsAg interactions or even in hepatitis B e antigen

conformation. Sequential analysis of samples from patient 4 (genotype D) illustrated the dynamism of the HBV quasiespecies, with strong selection of one minor baseline variant coinciding with a decrease in core variability during the treatment-free and lamivudine-treated period. The drop in variability seemed to result from a "steady state" situation of the HBV quasiespecies after selection of the variant with greatest fitness.

CONCLUSION: Host immune pressure seems to be the main cause of HBV core evolution. UDPS analysis is a useful technique for studying viral quasiespecies.

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Key words: Hepatitis B virus; Ultra-deep-pyrosequencing; Epitopes; Quasiespecies; Linkage analysis

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INTRODUCTION

Hepatitis B virus (HBV) infection is a global health problem. Around 350 million people are chronically affected with this pathogen, which confers a higher risk of developing liver disease, liver cirrhosis, and hepatocellular carcinoma. The course of HBV infection is closely related to the host immune response and genetic factors^[1], and disease progression is related to mutations in the HBV core gene^[2-4].

HBV core gene codes for two partially collinear proteins, the hepatitis B e antigen (HBeAg) and hepatitis B core antigen (HBcAg). These proteins, together with the surface antigen (HBsAg) are important targets for antiviral immunity, but HBcAg seems to be the most immunogenic^[5]. Several epitopes have been identified in the HBV core gene. Among them, two regions play a particularly important immunodominant role: the sequence from amino acid 50 to 69, which immunostimulates CD4+ T-helper lymphocytes (Th50-69)^[6] and the sequence from amino acid 74 to 84, which stimulates B lymphocytes (B74-84)^[7,8].

During chronic HBV infection, a large number of amino acid substitutions are seen in the core gene, mainly clustered in epitopic regions. These amino acid changes have been associated with viral persistence because of their impact on the host immune response and the

natural course of HBV infection^[7,9-14]. The largest number of core gene changes is associated with interferon (IFN) therapy^[10-12]. The effect of nucleoside/nucleotide analogues on the core gene has been little investigated, although some variability in a minor epitope (Th28-47) was recently reported^[11]. In another recent study, entecavir and adefovir were associated with an enhanced immune response^[13]. Selection of core gene amino acid changes might result in evasion of HBV from the host immune system, thereby lengthening the life of infected hepatocytes. For this reason, core gene baseline variability in chronic hepatitis B patients might be crucial for understanding the evolution of the viral quasiespecies in response to host immune pressure.

Next-generation sequencing technologies enable deep assessment of gene variability and are especially useful to study the dynamics of viral quasiespecies^[16-21]. Core gene variability can be studied with this technology, specifically, the 454 FLX platform, which analyzes fragments of 250- to 400-bp length. Although this length does not permit complete analysis of the gene, ultra-deep analysis of the main immunodominant regions of the core protein (Th50-69 and B74-84) is possible. The aim of this study was to analyze the variability of these main HBV core epitopes in chronic hepatitis B patients by ultra-deep pyrosequencing (UDPS).

MATERIALS AND METHODS

Patients and samples

Four chronic hepatitis B patients with complete clinical documentation were selected for the study; baseline characteristics are indicated in Table 1. All patients were diagnosed with active HBV replication and treated with lamivudine (LVD) 100 mg/d (Zeffix, Glaxo Wellcome, United Kingdom). After 18 to 24 mo, they all presented mutations conferring resistance to treatment. Owing to their similarities in LVD non-response, they were selected for inclusion in the study. To evaluate baseline variability, a sample taken at the time of the diagnosis (antiviral treatment naïve) was selected for each patient. HBV DNA was retested in all samples by TaqMan real-time polymerase chain reaction (PCR) (Roche) technology, and all presented values higher than 5 log₁₀ IU/mL.

Two sequential samples from patient 4 were additionally selected for UDPS analysis. HBV-DNA had been quantified using the branched-DNA (bDNA, limit of detection 5 logs IU/mL) technology available at that time, but the samples selected were retested with TaqMan technology for this study. At the time of the diagnosis (baseline sample), high HBV-DNA levels were detected by bDNA (retesting with TaqMan, 7 log₁₀ IU/mL). However, HBV-DNA spontaneously dropped below the limit of detection of bDNA technology and the patient remained untreated, according to the guidelines at that time. After 36 mo (treatment-free sample), bDNA significantly increased (> 8 logs₁₀ IU/mL on TaqMan retesting) and LVD was started. After an initial suboptimal response (HBV DNA decrease to 4 logs), viral breakthrough (7

Table 1 Baseline characteristics of the four patients included in the study

Patient	Sex	Age (yr)	ALT (IU/mL)	HBV DNA (log ₁₀ IU/mL)	Genotype	HBeAg status
1	Male	46	167	7.4	A	- ¹
2	Male	39	95	7.8	A	+
3	Male	31	392	8.3	D	-
4	Female	55	117	7.5	D	-

Age at the time of sample collection. ¹Wild type in main precore mutation. ALT: Alanine transaminase; HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus.

log₁₀ HBV-DNA) was observed after 18 mo, and the rtL180M and rtM204V HBV polymerase variants were selected. Ultimately, adefovir was added to LVD.

Epitopic region amplification and UDPS amplicon preparation

All the samples included in this study had HBV viral loads higher than 6 logs IU/mL. HBV-DNA was extracted from serum by QIAamp microspin columns (QIAamp DNA Mini Kit, QIAGEN, Hilden, Germany), according to the manufacturer's instructions. To obtain optimal amplification of HBV DNA, the process was optimized with two PCRs. To minimize the error rate of the PCR process (false nucleotide substitutions), high fidelity polymerase (Pfu Ultra-II, Stratagene, La Jolla, United States) was used. At the time the study was designed, the maximal amplicon length that could be analyzed by the FLX platform was 250 nucleotides; PCR primers were selected for amplification of a specific 210-bp HBV fragment, which included main epitopic regions (Th50-69 and B74-84). The first PCR primers were sense (position 1662-1681); 5'-^c/τATAAG AGGACTCTTGGACT-3' and anti-sense primers (position 2912-2931); 5'-TGTTCCCA^A/GGAATA^A/τGGTGA-3'. The nested primers included the recognition site for UDPS, in italics. The sequence of the sense primer (position 1997-2016) was 5'-GCCTCCCTCGC-GCCATCAGACCGCCTCAGCTCT^c/τTAT CG-3', and the anti-sense primer (position 2178-2206) was 5'-GCCTTGC CAGCCCGCTCAGCCACA^A/GAGTT-GCCTGA^A/GCTT-3'. PCR products were isolated from 0.9% agarose gel and quantified using Quan-iT Picogreen sDNA reagent (Invitrogen). Before the sequencing reaction, each amplicon was pooled to obtain a concentration of 4 × 10⁶ molecules of the HBV region. This working solution was enriched with the capture beads needed for sequencing. After optimal enrichment, clonal amplification in beads was performed in forward and reverse directions (emPCR kits II and III, 454 Life Sciences). UDPS was carried out in the Genome Sequencer FLX system (454 Life Sciences). The HBV region analyzed covered amino acids 40 to 95 of the core gene.

Bioinformatics filter

A total of 122 813 sequences was obtained. Reads were acquired with forward and reverse sequences and were

aligned according to the primer sequence (designed by our group). Initial raw data filtering was performed as previously reported^[16-18,20,22].

After applying the Poisson-based statistical filter validated in a study from our group^[21], the empirical distribution of mismatch errors determined by UDPS analysis of an HBV DNA clone from the same region yielded an average of 0.006%; however in 8 positions, errors were higher than 0.02% but lower than 0.05%. Therefore, the sensitivity of UDPS to detect mutations was primarily limited by the highest mismatch error rate in the HBV DNA clone of 0.05%, which is similar to the value recently obtained in UDPS amplicons including an internal sequence as a control^[20]. Thus, the measurements and biological conclusions in this study are based only on mutations present at a percentage above 0.05%.

Phenotyping, mutagenesis and cell culture

Cloning of a more than full-length HBV genome^[20] in pTriEx-mod vector was performed as described by Durrantel *et al.*^[23]. The influence on HBV viral replication of the most conserved position observed in UDPS analysis, codon 76, was analyzed by site-directed mutagenesis (Agilent Technologies, Stratagene, United States) according to the manufacturer's instructions. The wild-type clone had an L in codon 76, which was changed to V or P to test the effect of maintaining or deeply altering the physical-chemical properties of core codon 76. The introduction of mutations was confirmed by direct sequencing, as previously described^[20].

Huh7 human hepatoma cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% calf serum. Transfection of plasmids was performed as previously described^[20] using Fugene-HD (Roche, Germany). The supernatant was used to quantify HBsAg (Architect, Abbot), HBeAg (Vitros, Johnson and Johnson), and HBV DNA (CobasTaqman, Roche) production. The results were statistically analyzed with the Student *t* test. DNA was extracted from the supernatant (QiagenAMP DNA Mini Kit, Qiagen, Germany) following the manufacturer's instructions and used to evaluate HBV-DNA production. As has been previously described^[20], to confirm that HBV-DNA detected after transfection was the result of HBV replication and not due to contamination from the HBV genome in the pTriEx-mod vector of the transfection experiments, the supernatant extractions were used in 1/10, 1/10², 1/10³, and 1/10⁴ dilutions and PCR amplification of HBV-DNA and pTriEx-mod was performed.

Statistical analysis

To obtain the percentage of amino acid variability in each sample, the total number of amino acid substitutions was divided by the total number of amino acids analyzed. This value gave the theoretical variability for each position, and was used to estimate the expected variability for the regions studied (the theoretical variability was multiplied by the length of the epitope: 20

Table 2 Baseline variability of all the codons analyzed

Codon	Patient N sequences Master AA	Pt 1 33245 % var	Pt 2 28254 % var	Pt 3 27156 % var	Pt 4 19748 % var	Median baseline variability
40	E	0.17	0.00	0.27	36.27	9.18
41	<i>A134/S2</i>	0.04	7.54	1.58	0.03	2.30
42	L	0.20	0.08	0.14	0.09	0.13
43	E	0.06	0.07	0.08	0.12	0.08
44	S	0.03	0.01	0.01	0.05	0.03
45	<i>S1/P234</i>	0.24	0.07	0.10	0.09	0.12
46	E	0.06	0.05	0.06	0.08	0.06
47	H	0.06	0.06	0.04	0.03	0.05
48	C	0.09	0.18	0.07	0.27	0.15
49	S	0.09	0.07	0.03	0.01	0.05
50	P	0.07	0.04	0.08	0.02	0.05
51	H	0.14	0.11	0.06	0.01	0.08
52	H	0.03	0.04	0.02	0.02	0.03
53	T	0.11	0.14	0.07	0.04	0.09
54	A	0.14	0.18	0.17	0.19	0.17
55	L	0.03	0.05	0.06	18.58	4.68
56	R	0.21	0.22	0.14	0.10	0.17
57	Q	0.05	0.05	0.03	0.00	0.03
58	A	0.15	0.18	0.09	0.04	0.12
59	<i>I134/V2</i>	0.04	4.75	1.68	0.03	1.62
60	L	0.03	0.02	0.03	0.06	0.04
61	C	0.10	0.08	0.04	0.03	0.06
62	W	0.15	0.21	0.19	0.09	0.16
63	G	0.17	1.93	0.34	0.18	0.65
64	<i>D14/E23</i>	0.69	0.50	0.41	29.96	7.89
65	<i>V1/L234</i>	0.08	0.02	0.09	0.19	0.09
66	<i>T1/M234</i>	0.12	0.21	0.11	0.11	0.14
67	<i>N1/T234</i>	0.05	0.06	0.06	0.04	0.05
68	L	0.04	0.02	0.03	0.02	0.03
69	A	0.05	0.12	0.10	0.09	0.09
70	T	0.05	0.02	0.02	0.01	0.02
71	W	0.11	0.24	0.20	0.17	0.18
72	V	0.07	0.12	0.07	0.03	0.07
73	G	0.17	0.28	0.19	0.11	0.19
74	<i>N12/A3/V4</i>	0.46	0.04	2.06	13.72	4.07
75	N	0.01	0.02	0.02	0.02	0.02
76	L	0.00	0.01	0.02	0.02	0.01
77	<i>G134/Q2</i>	0.14	0.30	1.94	3.03	1.35
78	D	0.03	0.08	0.03	0.08	0.05
79	P	0.16	0.10	0.07	0.03	0.09
80	<i>A123/T4</i>	0.11	0.17	0.12	8.09	2.12
81	S	0.04	0.03	0.01	0.00	0.02
82	R	0.19	0.12	0.15	0.06	0.13
83	D	0.05	0.04	0.06	0.12	0.07
84	<i>Q1/L234</i>	0.21	0.52	0.12	0.05	0.23
85	V	0.02	0.01	0.03	0.01	0.02
86	V	0.02	0.03	0.01	0.04	0.03
87	<i>N1/S234</i>	0.06	0.03	1.93	0.02	0.51
88	Y	0.08	0.03	0.04	0.05	0.05
89	V	0.01	0.00	0.02	0.03	0.02
90	N	0.02	0.02	0.08	0.00	0.03
91	T	0.07	0.05	0.02	0.03	0.04
92	N	0.03	0.02	0.70	18.93	4.92
93	<i>M123/V4</i>	0.02	0.05	0.15	18.89	4.78
94	G	0.04	0.12	0.15	0.09	0.10
95	L	0.03	0.02	0.15	0.14	0.09

Italic numbers indicate the patient in whom the master amino acid (AA) was detected.

for Th50-69, 11 for B 74-84, and 25 for the remaining positions).

Fisher's exact test was used to evaluate possible rela-

tionships between the most variable codons (variability $\geq 1\%$) and their positions in the epitopic region or other regions. The Wilcoxon signed-rank test was used to compare the evolution of the codons in the sequential analysis.

RESULTS

Baseline variability of main epitopic regions of HBV core gene

The amplicon analyzed was limited to codons 40 to 95, which include the main Th50-69 and B74-84 epitopes. A total of 122 814 sequences corresponding to 4 baseline samples were analyzed, and 108 403 of them were validated by bioinformatics and Poisson filtering. A total of 61 499 sequences were from genotype A samples and the remaining from genotype D. Variability was analyzed attending to the percentage of changes in all codons of the amplicon, and the results obtained for each position are shown in Table 2.

In the two genotype A samples (patients 1 and 2), differences between the master sequences were found in ten codons (41, 45, 59, 64, 65, 66, 67, 77, 84 and 87, Table 2), seven of which were located in Th50-69 or B74-84. Of particular note, the master sequence of the motif delimited by codons 64 to 67, commonly defined by E₆₄LMT₆₇ and recognized by T-cells^[13], differed in patient 1. The sequence found, D₆₄VTN₆₇, was completely different from the consensus sequence of genotypes A and D. The amino acid variability detected in patient 1 (average, 0.1%), which ranged from 0.69% to values under the cut-off ($< 0.05\%$), was the lowest in all 4 samples. In this patient, the main epitopic regions contained 67.7% of the changes, a percentage 1.2 times higher than would be expected by the length of these regions, and the changes were equally distributed between the two epitopes. In contrast, patient 2 had higher variability (average, 0.35%), particularly in codons 41 (7.54%), 59 (4.75%) and 63 (1.93%). Only 53.1% of these changes were located in epitopic regions, a rate similar to the expected random percentage, but in the Th50-69 epitope the substitutions were 1.3 times higher than would be expected. Interestingly, two of the main substitutions detected in patient 2, S41 (A, 7.51%) and V59 (I, 4.21%), coincided with the master sequence of patient 1. The third main variant position was G63 (V, 1.62%).

The two genotype D baseline samples (patients 3 and 4) had the same master sequence, except in codons 64, 74, 80 and 93, which were also the most highly variable in patient 4. In patient 3, five codons with more than 1% variability were detected: A41 (1.58%), I59 (1.68%), A74 (2.1%), E77 (1.94%) and S87 (1.93%) (3 of them in epitopic regions). The average amino acid variability was 0.26%, and 57.8% of changes were located in the main epitopic regions. Overall, this percentage was not higher than expected; however, changes in B74-84 were 1.6 times higher than the expected random percentage (31.6% *vs* 19.7%). In patient 4, variability was higher

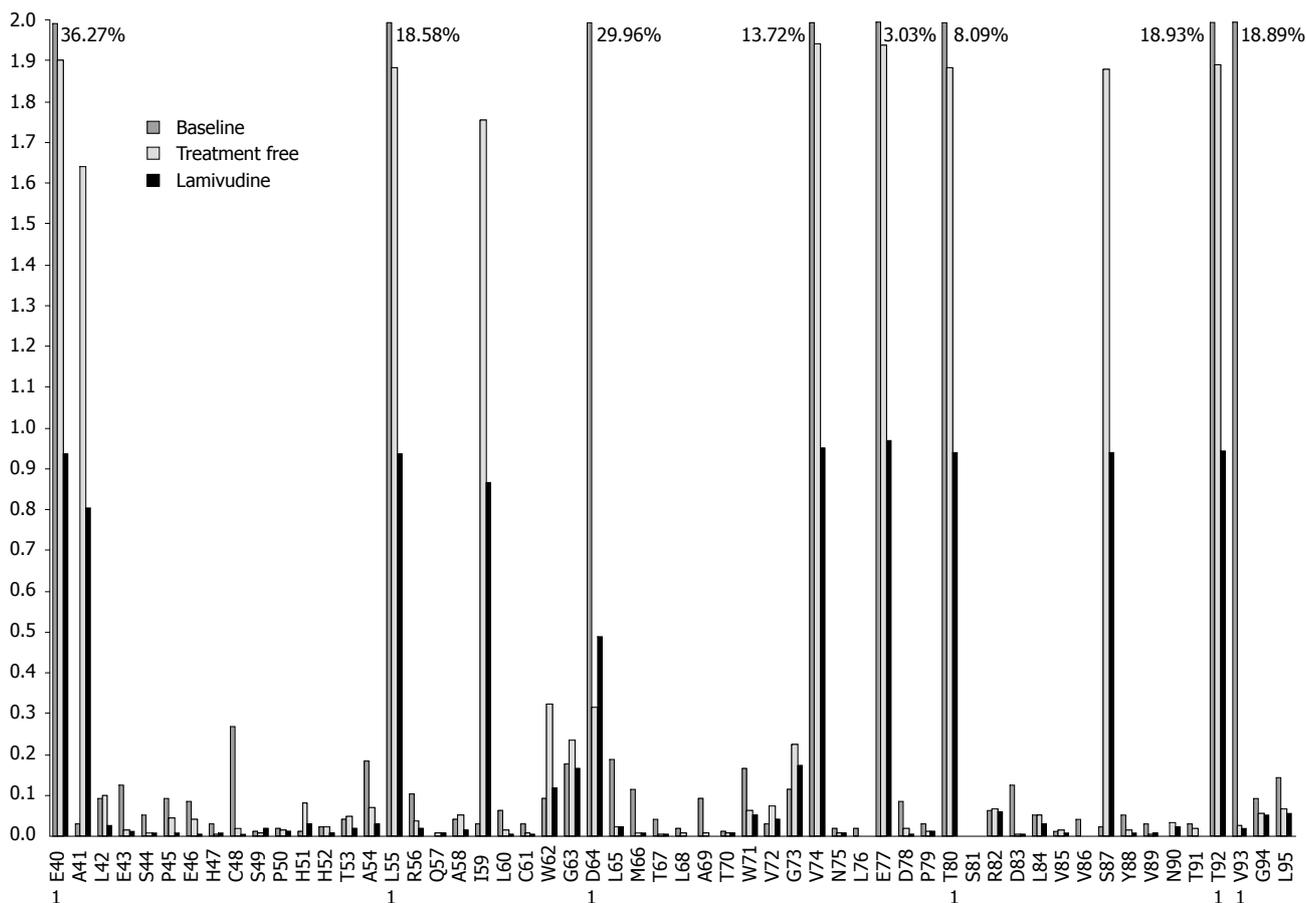


Figure 1 Variability of all codons analyzed from the 3 sequentially studied samples, corresponding to patient 4. Percentages higher than 2% are indicated at the top of the bars. 1: Master aminoacid different between treatment free and lamivudine samples.

than 1% in 8 codons - Q40 (36.27%), L55 (18.58%), D64 (29.96%), V74 (13.72%), E77 (3.03%), T80 (8.08%), T92 (18.93%) and V93 (18.89%) - 5 of which were in epitopic regions. Linkage analysis showed that some of the main variants seen in this patient (S at codon 41, V at 59, N at 74, E at 77 and N at codon 87) were located in the same viral strain (1.5% of quasispecies). This observation seems to indicate possible selection by the effect of immune pressure on the core gene. Surprisingly, despite the high total amino acid variability detected in patient 4 (2.69%), only 49.8% of changes affected positions located in main epitopic regions, a value lower than was expected in both Th50-69 and B74-84.

Median baseline frequencies were compared between the epitopic and other positions. Only genotype A samples showed high variability approaching significance ($P = 0.07$, sample 1 and $P = 0.05$, sample 2) in epitopic positions. Regarding the median baseline variability (Table 2), 6 of the highest values were located in positions within the main epitopic regions (codons 55, 59, 64, 74, 77 and 80), and 4 positions outside the main epitopes (codons 40, 41, 92 and 93) accumulated high percentages of changes. The variability in positions 40, 92 and 93 was due to changes in patient 4, whereas the variability of codon 41 (median 2.3% of changes) was due to changes in patients 2 and 3. Interestingly, positions

64 and 66, corresponding to the E₆₄LMT₆₇ motif of Th50-69, showed significant variability in all 4 samples (0.69%, 0.5%, 0.41% and 29.96% in position 64 and 0.12%, 0.21%, 0.11% and 0.11% in position 66).

Attending to the conserved positions, 12 codons showed variability lower than the system error rate ($< 0.05\%$): positions 44, 52, 57, 68, 70, 75, 76, 78, 81, 85, 86 and 89. The most highly conserved was leucine in codon 76, with frequencies clearly below the system error rate (0.003-0.02%) and a median baseline error of 0.013% (Table 2). Based on this finding, codon 76 was analyzed by site-directed mutagenesis analysis.

HBV quasispecies dynamics: The sequentially studied patient

Patient 4 was selected for sequential analysis, and 3 samples were processed (Figure 1): a baseline sample (also included in the *Baseline Study*), a sample following a treatment-free period of 36 mo, and a sample following 18 mo of LVD non-response. After application of the bioinformatic filter, 34 320 sequences from the treatment-free sample and 43 257 sequences from the LVD sample were obtained. The average amino acid variability of the baseline sample was higher than that of the treatment-free one (2.69% *vs* 0.34%, $P = 0.001$) and the average amino acid variability of the treatment-free sample was

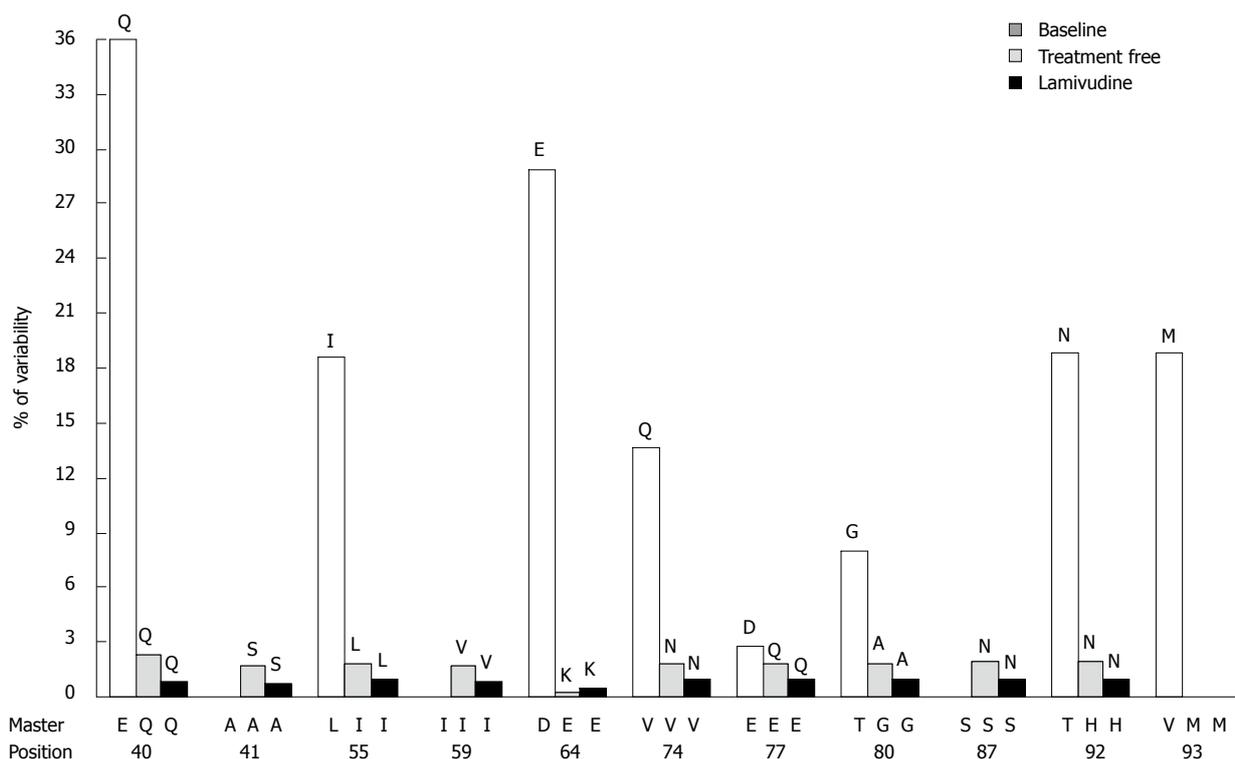


Figure 2 Evolution of the most variable codons of the core region analyzed in the sequentially studied patient. The variable position and its corresponding master amino acid are shown on the X-axis. The main mutated amino acids are indicated at the top of the bars.

higher than that of the LVD treatment sample (0.34% *vs* 0.18%, $P = 0.001$). The main variants detected by sequential analysis are represented in Figure 2. Of note, 6 of these 11 variable positions (codons 55, 57, 64, 74, 77, 80) were located in main core epitopic regions.

Linkage analysis was performed to determine whether the most frequent amino acid substitutions were simultaneously present in the same viral sequence (Figure 3A). At baseline, 50.15% of sequences in the positions studied were wild type, and 38.88% were mutated sequences in the most variable positions (15 different mutated variants, Figure 3A); 10.97% showed mutations in other positions. The mutated sequences in highly variable positions detected at baseline were found to be decreased in the treatment-free period (1.85%, Figure 3B) and after LVD breakthrough (1.24%, Figure 3B).

Attending to these variable positions, the most common strain at baseline (7.5%) had only one mutated codon (E40Q), followed by a strain (6.84%) with 5 mutated codons (E40Q, D64E, V74G, T92N and V93M) and another strain (5.45%) with 3 mutated codons (E40Q, L55I and D64E). Surprisingly, the baseline strain that had been selected as master after the treatment-free period and maintained during LVD was a low-frequency baseline mutant strain (1.31%) with the following substitutions: E40Q, L55I, D64E, T80G, T92H and V93M (variant 12, Figure 3A).

The time period with an absence of therapy (between baseline and treatment-free sampling) represented the complete time of HBV infection, and the HBV core

quasispecies showed a tendency to decreased variability. This was reflected by a drop in the percentage of accumulated variability from baseline (38.88%) to the treatment-free sample (1.85%) and coincided with the change in the master sequence between the two samples, which could indicate a possible alternative immune escape mechanism. No significant differences were observed between samples from the treatment-free and LVD periods, which showed similar composition and percentages of mutated variant strains (Figure 3).

Evaluation of the conserved position, leucine 76, by site-directed mutagenesis

As is described above, leucine (L) at codon 76 was the most highly conserved position in all the samples analyzed (Table 2). Although there were other conserved positions (Q57, T70, D78 and S81), codon 76 focused our interest because it was a leucine (one amino acid coded by 6 different codons), because of its location in the core gene, and because it has never been described as essential. L76 was even more conserved than D78 (0.05%) or P79 (0.09%), both of which are reported to be involved in the core-HBsAg interaction^[24]. Mutagenic studies of L76 were performed to evaluate a possible essential role of this amino acid. The experiments included a change to valine (V), whose hydrophobicity is similar to that of L, and a change to proline (P), whose physical-chemical properties differ from those of L.

After transfection, HBsAg, HBeAg and HBV DNA were quantified in cell culture supernatants. The presence

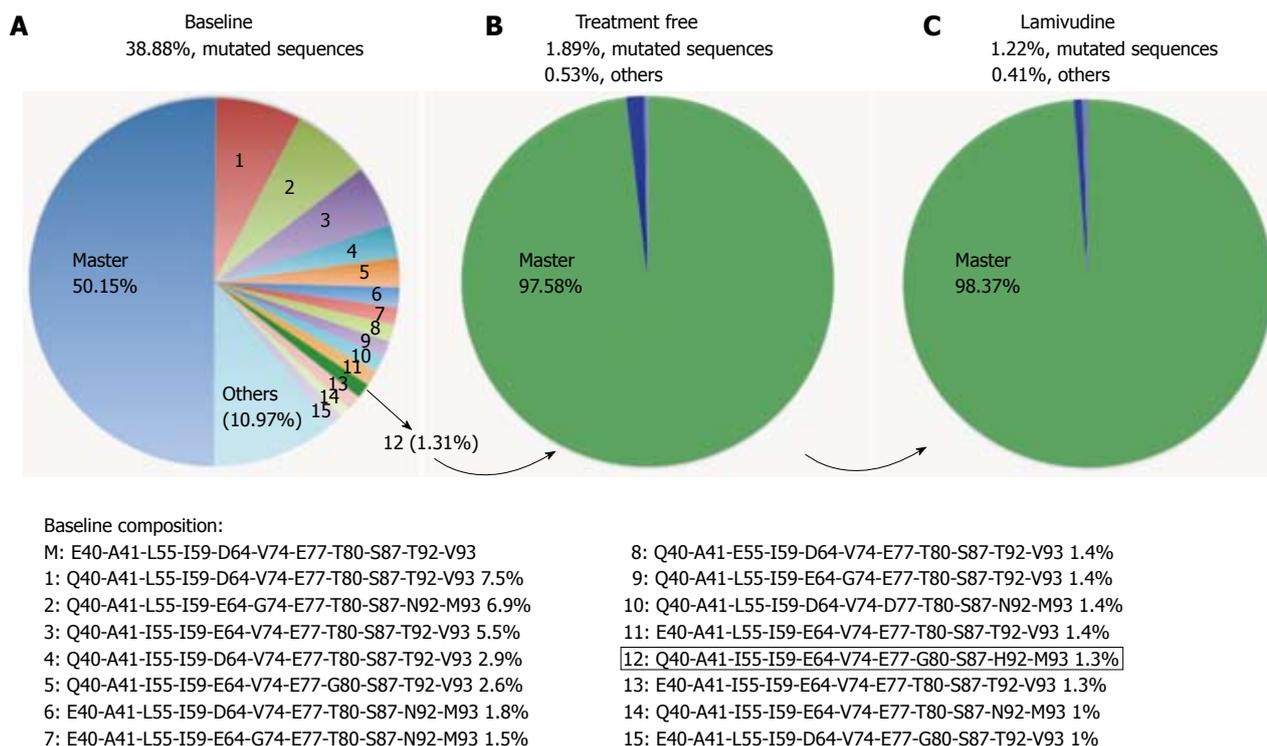


Figure 3 Quasispecies composition of the hepatitis B virus core region in the 3 sequentially analyzed samples. A: Baseline variants (1-15) in percentages \geq 1%; B: Treatment-free variability; C: Lamivudine sample variability of the most frequent amino acid substitutions defined in Figure 2. Linkage analysis was also performed attending these most variable codons.

of P in position 76 significantly decreased production of HBsAg, HBeAg and HBV-DNA in comparison with the wild type (L76) (both, $P < 0.001$). However, when V was in position 76, a reduction was observed in HBsAg levels ($P < 0.001$), but not in HBV DNA. Surprisingly, the V substitution resulted in a four-fold increase in HBeAg production in comparison with L76 ($P < 0.001$).

DISCUSSION

Several epitopic regions have been described in HBV core gene. Core region variability under antiviral treatment has been extensively studied, particularly in relation to IFN therapy^[10,12]. However, differing patterns of amino acid substitutions under the effect of nucleotide/nucleoside analogues and during periods without treatment have been recently described^[11]. Some studies have used the classical clonal method to analyze the evolution and composition of the entire HBV core gene^[25-27]. However, in all these reports, only a small number of clones were processed and minor populations could not be studied. Thus, high-resolution clonal studies are needed to deeply analyze the composition of the HBV core gene quasispecies. To this end, the recently developed UDPS technology provides an opportunity to bypass the restrictions of the classical clonal method to determine the composition of viral quasispecies^[16,17,19-21].

When this study was designed, UDPS technology based on the 454 Life Science Platform (GS-FLX, Roche Applied Science) only allowed analysis of around 200

nucleotide sequences. Because of this limitation, the present work was focused on analysis of the main immunodominant core motifs, Th50-69 and B74-84^[6,7]. The high economic cost and complex computing analysis of UDPS strongly restrict the number of samples to be processed. For this reason, we were only able to study six selected samples: four samples to evaluate the baseline variability and two more samples to sequentially analyze one patient.

To achieve the main aim of UDPS analysis, a parallel analysis was needed to define the cut-off for analyzing the UDPS data. In our previous report of UDPS analysis of the HBV quasispecies^[20], inclusion of an internal control sequence within the analyzed amplicon enabled establishment of a cut-off percentage to define the limit of viral variability, which was set at 0.03%. In the present study, a Poisson computational model recently validated by our group was applied by processing an HBV-DNA clone^[21], the percentage established to differentiate variability from UDPS error was set at 0.05%^[21].

In the study of baseline HBV quasispecies, the most variable codons (median baseline variability \geq 1%) were 40, 41, 55, 59, 64, 74, 77, 80, 92 and 93, all of which have been previously described in a study using clonal methodology to analyze acute exacerbations in HBeAg-negative patients^[25]. Furthermore, some of these highly variable codons (codons 64, 74 and 77) were previously described as common changes in untreated chronic hepatitis B^[11]. Genotype A sequences showed fewer codons with variability \geq 1% and, consequently, lower over-

all median variability than genotype D. The statistical analysis of the median baseline frequencies showed that genotype A samples had higher variability in epitopic positions, approaching significance ($P = 0.07$, sample 1 and $P = 0.05$, sample 2). In contrast, in genotype D cases, no significant differences in variability were observed between the epitopic and other positions. These findings could be related to the high frequencies of mutations in codon 40, 87, 92 and 93 in genotype D samples (patients 3 and 4), which might be involved in the minor epitopic regions Th28-47 and Th82-101^[6,11]. These differences in HBV core gene variability seem to indicate an influence of genotype on immune activation.

Patient 1 showed the lowest variability, but interestingly, the codon 64 to 67 master sequence was defined by the sequence D₆₄VTN₆₇, which is completely different from the well-characterized E₆₄LMT₆₇ motif. This motif is commonly recognized by T-cells, and the simultaneous E₆₄D and T₆₇N change has been reported to reduce T-cell proliferation *in vitro*^[13]. Hence, we suggest that the changes observed in patient 1 might be the result of an alternative mechanism to escape from immune pressure, over the E₆₄LMT₆₇ motif. In fact, the finding that positions 64 and 66 of this motif showed significant variability in all 4 samples suggests that the motif could be a central target for immune pressure in HBV infection^[11]. The low variability observed in sample 1 (0.1% of total amino acid variability) could reflect attainment of a kind of “steady state” in the viral quasispecies, resulting from strong selection of an escape variant with the mutated motif D₆₄VTN₆₇, similar to that seen in the longitudinal study of patient 4.

Evolution of the HBV quasispecies was evaluated in a single patient and involved a baseline sample and two additional samples, one taken after a period without treatment and one taken after LVD treatment. During the treatment-free period, a decrease in quasispecies variability was observed, with a reduction in the number of viral strains. This change may have been a consequence of host immune pressure^[25]. One of the baseline viral strains present in a small percentage (variant 12, 1.31%, Figure 3) was strongly selected and became the master in the treatment-free and LVD samples, a fact suggesting that this variant might be an escape mutant, whose selection could be related to better fitness, regardless of its initial frequency.

Based on these results, we postulated that the HBV quasispecies achieved a kind of “steady state” after the treatment-free period that did not change with LVD treatment, because immune pressure could also be decreased during treatment. The significant differences in average variability in the two periods suggest that the equilibrium is dynamic. The structure of the HBV quasispecies in the three samples represents a complex reservoir of different minor variants, resulting from natural HBV evolution and likely affected by antiviral treatment^[28].

Several codons in our region were found to be highly

conserved (positions 57, 70, 76, 78 and 81). Position 76, located at the tip of the spike was of special interest, being next to the major immunodominant region and part of the B74-84 main epitope. Indeed, this position has never been described as essential, in contrast to the nearby positions 78 and 79^[24]. The HBV core sequence is involved in the process of capsid conformation by interacting with the surface antigens in viral particle assembly. However, the interaction between HBsAg and HBcAg in virions is still unclear^[29-31]. The core region analyzed in the present study is part of the assembly domain (amino acids 1-149); thus, the changes in this region might potentially modify the shell conformation.

Electrostatic interactions between core and HBsAg take place at the tip of the spike of the core antigen (codons 74-84)^[32,33]. Cryo-electron microscopy studies have shown that codon 78 and 79 are within the contact area of core with envelope proteins^[24]. This essential role might explain the high level of conservation of these positions (especially codon 78, 0.05%) observed in our UDPS analysis. However, surprisingly, the most conserved codon of the HBV core region in baseline and sequential samples was leucine at position 76.

To our knowledge, few studies^[30,31] have analyzed the effect of single core amino acid mutations on HBV replication *in vitro*. Only one such study conducted by Ponsel *et al.*^[31] evaluated the leucine 76 position (among others) by inducing a change to alanine; no significant reduction in nucleocapsid or virion production was observed. In the present study, we induced changes in the hydrophobic characteristics of position 76 and determined their effect on HBsAg, HBeAg and HBV DNA production. In contrast to the results of Ponsel *et al.*^[31], we found a significant reduction in HBsAg production with both the V and P changes, suggesting possible involvement of L76 of HBcAg in the HBsAg interaction. A significant decrease in HBV replication in the presence of P76 was detected, leading us to speculate that the hydrophobic characteristics of position 76, conferred by the presence of L or V, are needed for HBV replication. The increase in HBeAg production in the presence of the V mutation and the absence of increasing HBV DNA were surprising, particularly because some authors have reported a correlation between HBeAg and HBV DNA levels^[34]. Nonetheless, this correlation was not found by other authors^[35] and currently remains controversial. We suggest that this unexpected HBeAg increase may indicate alternative pathways between HBeAg and HBV replication, as has been indicated previously^[36]. Based on our *in vitro* results, it can be postulated that the amino acid changes induced in the core sequence are not as important as the structure adopted by the capsid and HBeAg.

Our study is mainly limited by the high cost of the UDPS process, which restricted the number of samples studied and created a risk that some results could be due to random chance. A larger number of samples, additional sequential studies, and duplicate UDPS experiments would have given more information about the

HBV core sites involved in quasispecies evolution and potentially related to HBV chronic infection and to treatment non-response. In addition, at the time of the study, the available UDPS methodology only allowed analysis of sequences up to 250 bp. For this reason, we limited the study to the widely described main epitopic regions included in the HBV core, previously investigated by conventional methodologies^[6,7,11,25].

In conclusion, this study validates application of UDPS to study the variability of the main core epitopes, substantiates the significant richness of the HBV baseline quasispecies, and suggests a relationship between core variability and HBV genotype. The highest variability was mainly detected in Th50-69 and B74-84, supporting their role as the main immune-stimulating core regions. The significant variability associated with well-characterized Th-cell motifs, such as E₆₄LMT₆₇, seems to indicate that the host immune system may be the main factor responsible for HBV core evolution. The relevant conservation of codon 76 may be related to possible interactions with the viral envelope. In the single longitudinally analyzed patient, a minor variant present in the baseline quasispecies was selected as the main variant in the absence of treatment and was maintained after lamivudine. These findings indicate the utility of UDPS to describe the dynamic behavior of the HBV quasispecies. More extended analyses with a larger number of samples must be performed to confirm the findings. The expected spread of this technology will probably allow a significant decrease in the cost, enabling processing a large number of samples. The UDPS application for diagnostic and routine analysis might serve for the quantitative estimation of the viral quasispecies, for defining mutant variants or for establishing the quasispecies complexity. All these parameters would be useful as prognostic factors for disease outcome or therapy efficacy.

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COMMENTS

Background

Hepatitis B virus (HBV) infection is a global health problem, with more than 300 million HBV carriers. Viral distribution in individual infection is defined as a quasispecies, which means that variability can be accumulated along the HBV genome. The core gene is the least overlapped gene of the HBV genome and core antigen is the most immunogenic of all the viral peptides. Therefore, the region encoding the main core epitopes is useful to study the effect of the host immune response and to analyze the variability of HBV quasispecies composition.

Research frontiers

The distribution of viral infection as a quasispecies (in HBV, but also hepatitis C virus and human immunodeficiency virus) is an important advantage for the virus. It enables fast, easy establishment of infection and enables adaptation against changes in the viral environment. The study of viral quasispecies has been limited by the available methods, with which analysis of a significant number of clonal sequences was extremely difficult. The possibility of obtaining thousands of sequences in a single sample, provided by next-generation sequencing methods such as ultra-deep-pyrosequencing (UDPS), will allow

more in-depth study of the viral quasispecies. In the present study, the authors applied the UDPS to analyze HBV quasispecies variability and adaptability of the virus in single patient.

Innovations and breakthroughs

To date, few studies have used UDPS to study the HBV quasispecies, likely because of the current high cost of the technique for this purpose. However, the results presented in these recently published studies have shown clear advantages of UDPS for viral quasispecies analysis. Most of these studies have been mainly focused on the polymerase gene (treatment resistance variants). The present study is the first work analyzing the core gene by this method, to evaluate the effect of the host immune response and the variability of this little overlapped region of the HBV genome.

Applications

This study illustrates the value of deep quantitative analysis of the HBV quasispecies composition to investigate its clinical relevancy. The results provide an indication of the role of HBV core gene quasispecies structure in the natural host immune response and have prompted us to continue investigating HBV variability by UDPS, particularly the precore and core regions because of their relationship with the immune response.

Terminology

Viral quasispecies distribution in an infected patient refers to a group of viruses that are different, but highly related. This distribution results in competition between viruses from the same infection, but also confers plasticity and adaptability to environmental changes.

Peer review

The authors analyzed the quasispecies of HBV core epitopic regions by UDPS. They found that positions with highest variability rates, mainly clustered in the main core epitopes, showed some relationship with HBV genotype, and were particularly associated with the T-helper motif. The authors suggest that immune system pressure is the main cause of HBV core evolution.

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Cost of treating chronic hepatitis B: Comparison of current treatment guidelines

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Abstract

AIM: To compare program costs of chronic hepatitis B (CHB) screening and treatment using Australian and other published CHB treatment guidelines.

METHODS: Economic modeling demonstrated that in Australia a strategy of hepatocellular cancer (HCC) prevention in patients with CHB is more cost-effective than current standard care, or HCC screening. Based upon this model, we developed the B positive program to optimize CHB management of Australians born in countries of high CHB prevalence. We estimated CHB

program costs using the B positive program algorithm and compared them to estimated costs of using the CHB treatment guidelines published by the Asian-Pacific, American and European Associations for the Study of Liver Disease (APASL, AASLD, EASL) and those suggested by an independent United States hepatology panel. We used a Markov model that factored in the costs of CHB screening and treatment, individualized by viral load and alanine aminotransferase levels, and calculated the relative costs of program components. Costs were discounted by 5% and calculated in Australian dollars (AUD).

RESULTS: Using the B positive algorithm, total program costs amount to 13 979 224 AUD, or 9634 AUD per patient. The least costly strategy is based upon using the AASLD guidelines, which would cost 34% less than our B positive algorithm. Using the EASL and the United States Expert Group guidelines would increase program costs by 46%. The largest expenditure relates to the cost of drug treatment (66.9% of total program costs). The contribution of CHB surveillance (20.2%) and HCC screening and surveillance (6.6%) is small - and together they represent only approximately a quarter of the total program costs.

CONCLUSION: The significant cost variations in CHB screening and treatment using different guidelines are relevant for clinicians and policy makers involved in designing population-based disease control programs.

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Key words: Chronic hepatitis B; Markov model; Hepatocellular cancer; Treatment guidelines

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INTRODUCTION

Although hepatocellular cancer (HCC) remains relatively uncommon in Australia, its incidence has increased approximately fivefold since 1972, and based on current trends, a threefold increase is expected by 2020^[1]. HCC rates are highest in Southwestern Sydney, where its incidence (7.7 per 100 000 persons, 95% CI: 7.0-8.4) is significantly higher than the state average (5.2 per 100 000 persons, 95% CI: 5.0-5.5)^[1].

Nearly 90% of hepatitis-B-related HCC in NSW occurs in people born overseas, with approximately 70% affecting Australians born in countries of high hepatitis B prevalence^[2]. Migrants born in these countries are 6-12 times more likely to develop HCC than other Australians^[3], explained by the strong association between hepatitis B infection acquired early in life and the subsequent development of hepatic cirrhosis and HCC^[4]. In recent years, effective treatments for chronic hepatitis B (CHB) infection have achieved sustained suppression of viral replication and significant reductions in disease progression to cirrhosis, end-stage liver disease and HCC^[5-7]. This opens unique opportunities to reduce CHB-related morbidity and mortality among the 350 million chronically infected people worldwide^[8], provided that treatment costs are affordable at a population level. This is particularly relevant in the developing world, where the great majority of people with CHB reside^[4].

Our previous modeling work showed that in Asian populations with CHB residing in Australia (representing > 50% of people diagnosed with CHB in Australia^[9]), a strategy of HCC prevention is more cost-effective than HCC screening^[10]. For people with CHB, we defined HCC prevention as an intervention comprising regular (6-monthly) patient follow-up, the institution of antiviral therapy in those with active disease, and HCC surveillance. Following the confirmation of CHB diagnosis, general practitioners (GPs) order the relevant investigations and stratify participants into discrete risk categories, based upon hepatitis B virus (HBV) DNA and alanine aminotransferase (ALT) levels (Figure 1). Low-risk patients [those with low HBV DNA (defined as < 20 000 IU/mL for participants aged < 50 years and < 2000 IU/mL for those aged > 50 years) and normal ALT levels [defined as < 1.5 times upper limit of normal (ULN)]] are offered routine CHB surveillance, consisting of 6-monthly GP follow-up visits and testing for hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), viral load and ALT levels. Patients with normal

ALT levels, but elevated viral loads (as defined above) are followed up by their local medical practitioners under a program of enhanced CHB surveillance (which in addition to routine blood tests also includes 6-monthly HCC surveillance using α fetoprotein (AFP) measurements and liver ultrasound (US) examinations. Patients with elevated ALT and viral load levels are referred to tertiary care for assessment and consideration of antiviral therapy (Figure 1). The general assumptions of the Markov model are summarized in Table 1.

These treatment strategies are at slight variance with those described in hepatitis B practice management guidelines, because they factor in age as a consideration for treatment initiation (program participants are aged ≥ 35 years and treatment criteria change at age 50 years) and ALT cutoff levels are $\geq 1.5 \times$ ULN. Additionally, HCC screening is being offered to groups deemed at higher risk, rather than to all Asian males over the age of 40 years and Asian women over the age of 50 years, as it is recommended by the American Association for the Study of Liver Disease (AASLD) guidelines^[11].

The modeling work informed the development of a program of CHB management targeting the area of Sydney with the highest burden of hepatitis-B-related HCC in Australia, located in Southwest Sydney. As the Gastroenterology Society of Australia uses viral loads > 2000 IU/mL as a cut-off for treatment initiation irrespective of age^[12], we subsequently modified the viral load cut-off for the B positive program, to avoid confusion among primary care providers participating in the program.

In order to inform hepatitis B management and provide data to policy makers, we estimated B positive program costs using the original B positive screening and treatment algorithm and compared them with those incurred using the modified B positive algorithm (viral load cutoff of 2000 IU/mL for treatment initiation, irrespective of patient age) and to costs incurred when applying guidelines published by the American, European and Asia-Pacific Associations for the Study of Liver Disease (AASLD, EASL and APASL)^[6,13,14] as well as those developed by an independent panel of hepatologists from the United States (referred to here as the United States Expert Group)^[15]. We also determined the relative proportion of program costs attributable to CHB screening, drug treatment, CHB surveillance and HCC screening and surveillance incurred by applying each of these algorithms. Costs were calculated in Australian dollars (AUD).

MATERIALS AND METHODS

The B positive project targets Asian migrant communities in Southwest Sydney, but is inclusive of all individuals who meet eligibility criteria. Eligibility criteria include: confirmed CHB, age ≥ 35 years, and attending a general practice in the target local government areas. To estimate the size of the eligible population, we used data provided by the Australian Bureau of Statistics 2006 National Cen-

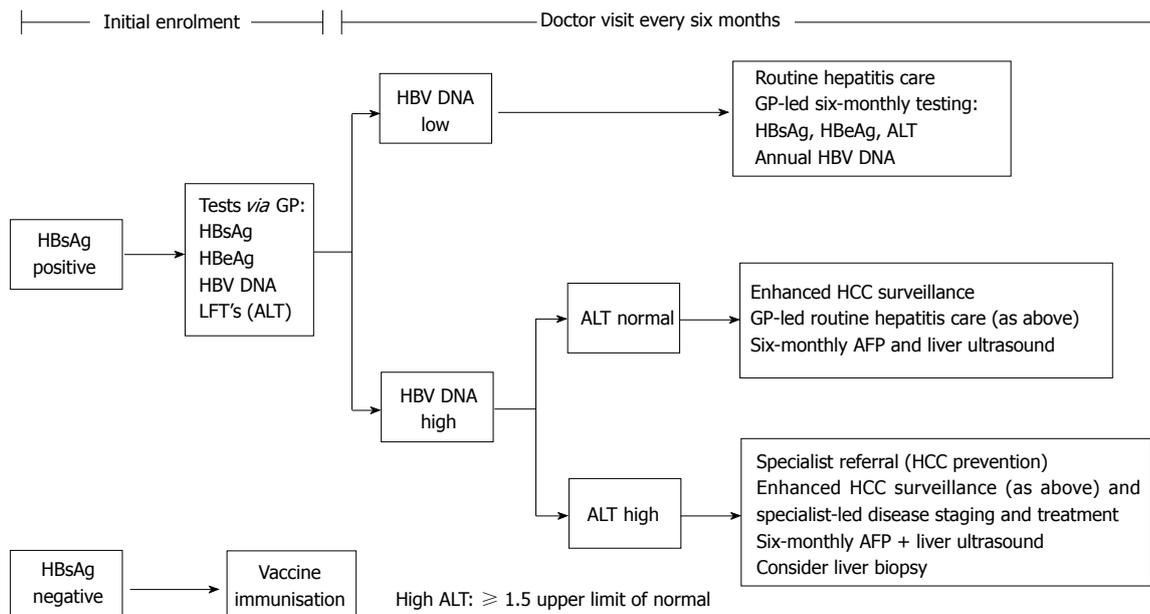


Figure 1 B positive program for chronic hepatitis B screening and treatment protocol. Depicts the algorithm used to stratify participants aged > 35 years into routine surveillance [for those with viral loads below 2000 IU/mL and normal alanine aminotransferase (ALT)], enhanced hepatocellular cancer (HCC) surveillance (for elevated viral loads and normal ALT), and those at highest HCC risk (referred for specialist opinion and antiviral therapy). CHB: Chronic hepatitis B; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; GP: General practitioner; HBV: Hepatitis B virus; AFP: α fetoprotein; LFTs: Liver function tests.

sus on the number of local residents born in China, Hong Kong and Vietnam aged ≥ 35 years. We applied HBV seroprevalence data on these numbers, based upon epidemiological estimates from the respective countries of birth^[16].

We estimated the proportion of people in different CHB-related disease stages over the 50-year timeframe [CHB without cirrhosis; CHB with cirrhosis; CHB with liver failure; CHB and HCC; spontaneous HBsAg clearance and death due to CHB-related causes (liver failure or HCC), or death from other causes], using the assumptions of our previously published Markov model^[10]. The model takes a health care funder perspective and discounts costs at 5% per annum. We estimated a program participation rate of 25%, informed by the experience of the New Zealand HBV screening program, which screened 27% of their eligible population^[17]. Table 1 summarizes the key assumptions of our model. We used an ALT level of $\geq 1.5 \times \text{ULN}$ to define high-risk patients and estimated that about 50% of the target population have ALT levels $< 1 \times \text{ULN}$, 12.5% have ALT levels of $1-1.5 \times \text{ULN}$, 12.5% between 1.5 and $2 \times \text{ULN}$ and 25% levels $> 2 \times \text{ULN}$, informed by a Hong Kong population-based study of CHB patients^[18].

We estimated the proportion of the target population receiving antiviral treatment based upon the cutoffs for ALT and viral load recommended by the different guidelines for patients who are HBeAg negative (Tables 2 and 3). Briefly, in this population group the AASLD guidelines recommend treatment to be initiated when viral load exceeds 20 000 IU/mL and ALT levels are $> 2 \times \text{ULN}$ ^[6]. The APASL guidelines use the same ALT level cutoff, but a lower threshold (2000 IU/mL) for viral load^[14], while the United States expert group and EASL

use the 2000 IU/mL cutoff for viral load, but recommend treatment for patients with ALT levels exceeding the normal range^[13,15].

The B positive program (unpublished) data suggested that 94% of those enrolled in the program were HBeAg negative at the time of enrolment (which corroborates the findings of Yuen *et al*^[19], that in an Asian population, HBeAg seroconversion occurs around 35 years of age); consequently we compared estimated CHB program costs using treatment guidelines developed for HBeAg-negative populations.

We factored in the costs of CHB screening and follow-up provided by specialists and/or primary care providers, the costs of HCC screening, and CHB and HCC treatment, but not recruitment costs, or the costs of immunization for those susceptible.

RESULTS

The CHB population targeted by the B positive program in Southwest Sydney was estimated at 5800 patients. Assuming a 25% enrolment rate, about 1500 patients (1451 patients) with CHB in Southwest Sydney would be enrolled in the program. Nearly two-thirds (63%) of these patients would be followed up by their GPs under a program of routine surveillance (Figure 1). Half of the patient population would receive this type of management if using the other guidelines, except for the AASLD guidelines, where 70% of all patients would be receiving routine surveillance. The proportion of patients under enhanced surveillance (which is 23% for B positive and 31% for the modified B positive) would range from a low of 23% using the AASLD guidelines to a high of 37% using

Table 1 General assumptions of the Markov model of the B positive hepatocellular cancer prevention program

Assumption	How addressed and rationale
Participant recruitment	Target population age ≥ 35 yr, HBsAg +ve for ≥ 6 mo, born in China, Hong Kong, Vietnam
Contact testing and immunisation	Not factored into the model
Seroprevalence in target populations	10.7% for people born in China 10.5% for people born in Vietnam 7.7% for people born in Hong Kong (Nguyen <i>et al</i> ^[16])
Initial testing to confirm chronic hepatitis B	Not factored in the GP consultation calculations
Program participation rates	Base case assumption: 25% of eligible people are enrolled
HCC screening	All participants have AFP and liver US at enrolment Participants receiving enhanced surveillance have 6-monthly AFP and US Participants receiving treatment also have liver biopsy
Follow up requirements	Routine surveillance arm: 2 GP appointments/yr Enhanced HCC surveillance arm: 2 GP appointments/yr Interferon treatment: 6 specialist appointments/yr Entecavir treatment: 4 specialist appointments/yr
Viral load distribution	Based upon Risk Evaluation of Viral Load Elevation and Associated Liver Disease study data (Chen <i>et al</i> ^[20])
ALT level distribution	Based upon Hong Kong data (Yuen <i>et al</i> ^[18])
Progression rates through different disease stages	Constant
Treatment protocol	30% receive first line interferon (weekly for 12 mo); 30% seroconvert and receive no further treatment; 70% commence entecavir the following year 70% receive entecavir as first-line treatment; 20% seroconvert in first year and receive no further treatment; 80% continue lifelong entecavir
Patients with liver failure	Receive lifelong entecavir

Lists the general assumptions used in building the model, including treatment protocols and follow up and other relevant elements and data sources. HBsAg: Hepatitis B surface antigen; GP: General practitioner; AFP: α fetoprotein; ALT: Alanine aminotransferase; HCC: Hepatocellular cancer; US: Ultrasound.

Table 2 Participant distribution by disease stage at initial enrolment and management pathways, according to the B positive algorithm and hepatitis B treatment published guidelines *n* (%)

Treatment guideline	B positive	Modified B positive	EASL, United States experts	APASL	AASLD
HBV DNA level to treat	> 2000 if > 50 > 20 000 if < 50	> 2000	> 2000	> 2000	> 20 000
ALT (ULN)	> 1.5	> 1.5	> 1	> 2	> 2
Number receiving interferon	61 (4)	81 (6)	108 (8)	54 (4)	33 (2)
Number receiving entecavir	143 (10)	190 (13)	253 (17)	126 (9)	76 (5)
Total on treatment	204 (14)	271 (19)	361 (25)	181 (12)	109 (8)
Total under enhanced surveillance	340 (23)	452 (31)	361 (25)	542 (37)	326 (23)
Total under routine surveillance	907 (63)	728 (50)	728 (50)	728 (50)	1016 (70)
Total	1451 (100)	1451 (100)	1451 (100)	1451 (100)	1451 (100)

Tabulates the estimated number of participants undergoing different monitoring strategies and treatment options, according to recommendations put forward by Australian, Asia-Pacific, United States and European chronic hepatitis B treatment guidelines. ULN: Upper limit of normal; HBV: Hepatitis B virus; EASL: European Associations for the Study of Liver Disease; APASL: Asia-Pacific Associations for the Study of Liver Disease; AASLD: American Associations for the Study of Liver Disease.

the EASL and United States Expert Panel guidelines. The proportion of patients receiving antiviral treatment (14% for B positive) ranges from a low of 8% under AASLD to a high of 25% under the more stringent EASL and United States Expert Panel Group guidelines (Table 2).

Overall, the lowest program costs are associated with the application of AASLD guidelines, because the 20 000 IU/mL viral load cutoff for treatment initiation and ALT levels $\geq 2 \times$ ULN make fewer patients eligible for treatment. Treatment costs are highest when applying EASL and United States Expert Group guidelines, because they recommend treatment for all patients with viral loads > 2000 IU/ mL and ALT levels > 1 \times ULN.

The total B positive program costs would amount

to 13 979 224 AUD, or 9634 AUD per patient using the original B positive algorithm, ranging from a low of 6344 AUD for the AASLD guidelines to a high of 14 039 for the EASL and United States Expert Group recommendations (Table 3). The largest component of the cost structure relates to antiviral treatment, which represents over three-quarters (75.8%) of program costs if using EASL and United States Expert Group recommendations, approximately 70% (70.2%) for the modified B positive algorithm, 66.9% for the original B positive, 60% for the APASL guidelines, and just over 50% for the AASLD guidelines. Costs of CHB surveillance ranges from 17.5% for the modified B positive protocol to 30.1% of the program expenditure if using AASLD guidelines. The con-

Table 3 Calculated costs (in Australian dollars) of implementing a program of chronic hepatitis B management in hepatitis B e antigen-negative patients according to the B positive algorithms and published hepatitis B treatment guidelines, *n* (%)

Discounted costs of management strategies	B positive	Modified B positive	APASL	EASL, United States experts	AASLD
Cost/QALY (discounted)	13 465	15 770	11 746	19 622	8867
Total program cost (discounted)	13 979 224	16 372 320	12 194 905	20 371 117	9 205 680
Cost components					
Initial CHB screening cost	767 728 (5.5)	800 792 (4.9)	755 971 (6.2)	845 613 (4.2)	720 357 (7.8)
Drug treatment costs	9 347 662 (66.9)	11 493 535 (70.2)	7 360 940 (60.4)	15 447 510 (75.8)	4 951 419 (53.8)
CHB surveillance costs	2 827 093 (20.2)	2 866 053 (17.5)	2 866 053 (23.5)	2 866 053 (14.1)	2 767 073 (30.1)
HCC surveillance costs	917 783 (6.6)	1 092 983 (6.7)	1 092 983 (9.0)	1 092 983 (5.4)	647 874 (7.0)
Total cost per person in the program	9634	11 283	8404	14 039	6344
% change with equivalent unit costs/QALY	100	117	87	146	66

Tabulates the costs of chronic hepatitis B screening, surveillance and treatment as per Australian, Asia-Pacific, United States and European treatment guidelines scenarios. Costs are discounted by 5% and calculated in Australian dollars (AUD). EASL: European Associations for the Study of Liver Disease; APASL: Asia-Pacific Associations for the Study of Liver Disease; AASLD: American Associations for the Study of Liver Disease; HCC: Hepatocellular cancer; CHB: Chronic hepatitis B; QALY: Quality-adjusted life year.

tribution of the cost of HCC screening and surveillance remains relatively small in all scenarios, ranging from a low of 5.4% for the EASL and United States guidelines to 9% for the APASL guidelines.

Compared to the B positive algorithm, the lowest cost to achieve an equivalent unit cost/quality-adjusted life year is incurred by applying the AASLD guidelines (34% cost saving), followed by the APASL guidelines (13% cost saving); costs would be 17% higher with the modified B positive algorithm (because more patients aged 35-50 years would be in receipt of treatment) and 46% higher for the EASL and United States Expert Group recommendations.

DISCUSSION

This modeling exercise demonstrates that in a population-based CHB management program informed by current treatment guidelines, the majority of patients (ranging from 50 to 70%) have low viral loads (< 2000 IU/mL) and low ALT levels and may be effectively managed at the primary care level. Between a quarter and a third of patients have elevated viral loads and would benefit from more comprehensive follow-up, which we termed “enhanced surveillance”, which could still effectively be delivered at the primary care level and free up specialist resources. These calculations suggest that the proportion of patients requiring tertiary-level assessment for consideration of antiviral therapy is variable, ranging from a low of 8% if the AASLD guidelines are followed to a high of 25% if the more stringent EASL and United States Expert Group guidelines are used. Program costs range from approximately 9 million AUD (if AASLD guidelines are used) to more than double that figure (20 371 117 AUD) if the EASL or United States Expert Group recommendations are applied. Correspondingly, this leads to variations in costs per patient enrolled, ranging from 6344 AUD (for AASLD guidelines) to 14 039 AUD (for EASL and United States Expert Group guidelines). The B positive algorithm steers a mid-course with regards to total (13 979 224-16 372 320 AUD) and per patient costs (9634-11 283 AUD, depending on whether

the viral load cut-off is set at 2000 IU/mL for all patients, or only for those aged > 50 years.

The model assumes that all patients with elevated viral loads and ALT levels receive specialist assessment and liver biopsy to assess the degree of fibrosis prior to treatment initiation, although since November 2011, liver biopsy is no longer mandatory for treatment initiation.

In all modeled programs, the greatest contribution to cost is that of antiviral drug treatment (interferon or entecavir), accounting for 50%-75% of the program budget. By comparison, the costs of CHB surveillance are relatively low for a primary-care-based program (ranging from 14% to 30% of total costs); even lower are the costs associated with HCC screening and surveillance (ranging from 5% to 9%). The original B positive modeling did not include HCC surveillance in the subgroup presumed to have inactive disease; this is at variance with current published guidelines, which recommend HCC surveillance for all Asian men aged > 40 years or women aged > 50 years, irrespective of their disease stage or viral load^[11]. Although the original intention was to balance cost containment with ensuring that HCC cases are not missed, it appears that the contribution of HCC screening to costs would remain modest even if the program embraces the recommended screening guidelines. Sherman in a recent review concedes that it may be possible that, as more information about HCC risk stratification becomes available, patients with long-term inactive disease may not require the same intensive HCC surveillance^[11], something that is supported by the REVEAL data^[20].

Our model has a number of limitations related to our assumptions and the lack of clear data in some areas. For example, we used a 1.5 × ULN cut-off for the ALT levels prompting treatment. The different ALT cut-off levels in various guidelines indicate that there is no agreement about what level of ALT should prompt treatment initiation, and information to clarify this is keenly awaited. Similarly, we assumed that all patients with elevated ALT levels would require antiviral therapy, although in reality, ALT elevation may not always relate to disease reactivation, but to other factors, such as high

body mass index, non-alcoholic fatty liver disease, chronic alcohol consumption or coexisting hepatitis C infection^[21,22]. We also acknowledge that the rate of progression to HCC development is variable in different patient populations, being dependent on the degree of fibrosis, genotype, and other associated risk factors. Although the short-term goals of antiviral therapy have been achieved in recent clinical trials^[22], more answers are needed as to the extent to which this affects liver cancer incidence and the number of cancer deaths. As this is also the major assumption that underlies our analysis, more data confirming the impact of treatment on HCC risk reduction would strengthen the economic model findings.

Our original model factored in the cost of liver biopsy for all patients being considered for antiviral therapy, reflecting the recommendations of guidelines referred to in the paper, suggesting that a liver biopsy is helpful for determining the degree of necroinflammation and fibrosis. However the relatively low cost of a liver biopsy and the relatively small numbers of patients in this subgroup (we estimated that only about 12.5% of patients have ALT levels in that range and even fewer also have low-medium viral loads) means that this procedure will have a minimal impact on overall program costs, although it may influence the number of patients willing to accept drug therapy.

The effectiveness and cost-effectiveness of different interventions need to be corroborated by other types of studies and real-life outcomes data utilized to validate the economic models. We are currently collecting program data for this purpose.

Ideally our findings would need to be compared to those of other studies using decision models and against real-life data from clinical studies, but available data are limited. A recent European review^[23] identified only two studies addressing the cost-effectiveness of screening high-risk groups for CHB: both a United States^[24] and a Dutch study^[25] suggested that screening of migrant groups for HBV was both clinically effective and cost-effective findings that were corroborated by our Australian study^[10].

A clinical study estimating the efficacy and cost of HCC screening in a clinic population in Australia^[26] confirmed our model costs. We need to bear in mind however that direct comparisons between studies are of limited value, due to differences in study design, model assumptions, cut-offs used, and variable cost structures in different countries.

As the stated aim of this short paper was to examine the cost implications of utilizing different guidelines to treat patients with CHB, in order to assist funding decisions, we did not include a broader discussion of cost-effectiveness, effectiveness and efficacy of antiviral treatment, but agree that a more comprehensive review of effectiveness and cost-effectiveness may be warranted.

Although we incorporated a wide range of supporting evidence into our economic model, the relatively limited information available on the composition of cohorts of patients with CHB receiving treatment and the possible differences in treatment response in patients with

HBeAg-negative disease (which has been less extensively studied) may limit the generalizability of our findings. In the absence of relevant data, we assumed that the effectiveness and durability of current interventions can be extrapolated over a lifetime horizon, but acknowledge that the lack of long-term evidence precludes confident estimates of treatment outcomes.

From a policy perspective, the high cost of antiviral therapy makes population-based CHB screening and treatment unaffordable for all but well-resourced countries. For example, Hutton *et al.*^[27] published their analysis of the cost-effectiveness of interventions aiming to combat hepatitis B in the United States and China. They found that in an American setting it is cost-effective to screen adult Asian and Pacific Islanders (APIs) for CHB, with a view to providing them with appropriate treatment, as well as to vaccinate close contacts. This work led to a change in United States public health policy on hepatitis B screening, with a recommendation that all adult APIs and adults born in areas of intermediate HBV prevalence be screened for CHB. The authors found that in a Chinese setting catch-up adolescent vaccination is cost-effective, but that drug treatment costs would need to be halved (to about 1000 United States dollars/year) before the benefits of vaccination would be surpassed by those of instituting treatment^[27]. China's economic successes makes population level CHB screening and treatment a possibility in the future, but effective and inexpensive treatments are needed to reduce the burden of CHB in developing economies^[4].

Our original economic model assumptions attempted to reflect the Australian practice prevalent in 2006 and 2007, when entecavir was becoming first-line treatment for CHB. At that time interferon was still in common use, therefore, we modeled 30% of the cohort as receiving first-line interferon.

However, the therapeutic landscape has changed in recent years, with interferon being used now in only about 10% of patients as first-line treatment for CHB. We therefore repeated our calculations estimating that 10% of patients receive first-line interferon and found the incremental total cost was only about 1.5% higher (data not shown), as a result of interplay between a more intensive and more costly specialist follow-up during interferon treatment, a small proportion of patients who clear the infection, and the overall small number of patients affected.

As both available resources and local clinical preferences guide drug treatment, generic lamivudine may be the only affordable antiviral for low-income countries in Asia and Africa, where CHB is most prevalent, because replacing it with a more potent antiviral agent is associated with a more than 10-fold increase in drug costs^[28].

Consequently, we repeated the analysis, substituting lamivudine for entecavir and assuming that the cost of lamivudine represented only a tenth of the cost of entecavir. This led to significant reductions in drug costs (ranging from 75.3% to 77.6%) and in overall program costs (ranging from 40.5% to 58.9% - data not shown), which further emphasizes the important role played by

drug costing in program feasibility.

Our modeling has provided estimates of the cost of CHB management programs that could be useful for policy makers and health care providers in different settings, to inform program development. It would appear that population-based CHB management programs targeting at-risk groups are affordable in high-resource settings, but remain unattainable for many of the world's population with CHB, living in low- or middle-resourced countries. The high cost of antiviral therapies represents the largest cost component of a CHB management program. It is hoped that reductions in the cost of antiviral drugs will lead to more equitable access to treatment and address the global burden of chronic hepatitis B.

COMMENTS

Background

Chronic hepatitis B (CHB) is a leading cause of cirrhosis and hepatocellular carcinoma (HCC), and in Australia, HCC incidence has been rising faster than that of any other cancer, mostly related to changing migration patterns over recent decades. A population-level disease control is predicated upon early disease detection, regular monitoring and timely institution of antiviral treatment for people with active disease, but antiviral treatment is unaffordable for the great majority of people with CHB who live in resource-limited countries. Therefore the cost and cost-effectiveness of CHB management programs is an important consideration for program funders and needs to be factored in by those tasked with guideline development. The authors previously carried out modeling work that showed that screening and treating migrants born in high prevalence countries is cost-effective, which corroborated the findings of research groups in the United States and the Netherlands. This paper examines the cost of treatment of hepatitis B applied to a hypothetical population of people with CHB diagnosis, treated according to CHB screening and treatment guidelines in common use in Asia (issued by the Asia-Pacific Society for the Study of Liver Disease), Australia (issued by the Gastroenterological Society of Australia), Europe (issued by the European Association for the Study of Liver Disease, EASL) and the United States (issued by the American Association for the Study of Liver Disease, AASLD, as well as by an expert United States advisory group).

Research frontiers

This work builds the body of evidence suggesting the cost-effectiveness of screening high-risk groups for hepatitis B, by examining the implications of implementing a public health program of screening and treatment using different treatment initiation parameters, as recommended by different guideline developers. The proportion of patients in this hypothetical cohort requiring antiviral therapy ranges from 8% (if the AASLD guidelines are used) to 25% (if EASL guidelines are applied). The most substantial component of program cost relates to antiviral treatment (representing up to 75% of program costs), while screening for CHB and cancer surveillance account for a small part of total costs. Treatment with generic lamivudine (instead of entecavir) leads to substantially lower total program costs, although this must be balanced against the greater suppression of viral replication and lack of drug resistance associated with entecavir treatment.

Innovations and breakthroughs

Here the authors propose and cost a scheme for the diagnosis and subsequent monitoring of patients from countries with high prevalence of hepatitis B, resident in Australia. In the B positive algorithm, antiviral treatment is offered for patients with alanine aminotransferase (ALT) elevation above 1.5 × normal, treating the "middle ground" between treating everyone with an elevated ALT level (which may have significant resource implications for countries with high disease prevalence, but limited resources) and treating only people with advanced disease (and running the risk of limiting the effectiveness of the program).

Applications

These findings are relevant for the design of interventions with the potential to make a significant impact on hepatitis B disease burden at a population level, in both well-resourced and low-resource settings. The authors hope that this type of work may be of interest to experts involved in CHB treatment guideline develop-

ment, policy makers and clinicians working in areas with a large hepatitis B load.

Peer review

This is a study on the cost of treatment of hepatitis B, using Markov models. The authors propose a scheme for the diagnosis and subsequent monitoring of patients from countries with high prevalence of hepatitis B, resident in Australia. They also compared the relative cost with the proposed guidelines from other major hepatology associations. They found that the AASLD recommendations were more cost-effective. This type of study is important in view of the increasing cost of drug treatment of HBV infection but also of the increasing cost of diagnostic tests related to HCC surveillance.

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Anemia after gastrectomy for early gastric cancer: Long-term follow-up observational study

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Abstract

AIM: To identify the incidence and etiology of anemia after gastrectomy in patients with long-term follow-up after gastrectomy for early gastric cancer.

METHODS: The medical records of those patients with early gastric adenocarcinoma who underwent curative gastrectomy between January 2006 and October 2007 were reviewed. Patients with anemia in the preoperative workup, cancer recurrence, undergoing systemic chemotherapy, with other medical conditions that can cause anemia, or treated during follow up with red cell transfusions or supplements for anemia were excluded. Anemia was defined by World Health Organization criteria (Hb < 12 g/dL in women and < 13 g/dL in men). Iron deficiency was defined as serum ferritin < 20 µg/dL. Vitamin B₁₂ deficiency was defined

as serum vitamin B₁₂ < 200 pg/mL. Iron deficiency anemia was defined as anemia with concomitant iron deficiency. Anemia from vitamin B₁₂ deficiency was defined as megaloblastic anemia (mean cell volume > 100 fL) with vitamin B₁₂ deficiency. The profile of anemia over 48 mo of follow-up was analyzed.

RESULTS: One hundred sixty-one patients with gastrectomy for early gastric cancer were analyzed. The incidence of anemia was 24.5% at 3 mo after surgery and increased up to 37.1% at 48 mo after surgery. The incidence of iron deficiency anemia increased during the follow up and became the major cause of anemia at 48 mo after surgery. Anemia of chronic disease and megaloblastic anemia were uncommon. The incidence of anemia in female patients was significantly higher than in male patients at 12 (40.0% vs 22.0%, $P = 0.033$), 24 (45.0% vs 25.0%, $P = 0.023$), 36 (55.0% vs 28.0%, $P = 0.004$), and 48 mo (52.0% vs 31.0%, $P = 0.022$) after surgery. Patients with total gastrectomy showed significantly higher incidence of anemia than patients with subtotal gastrectomy at 48 mo after surgery (60.7% vs 31.3%, $P = 0.008$). The incidence of iron deficiency was significantly higher in female patients than in male patients at 6 (35.4% vs 13.3%, $P = 0.002$), 12 (45.8% vs 16.8%, $P < 0.001$), 18 (52.1% vs 22.3%, $P < 0.001$), 24 (60.4% vs 20.9%, $P < 0.001$), 36 (62.5% vs 29.2%, $P < 0.001$), and 48 mo (66.7% vs 34.7%, $P = 0.001$) after surgery.

CONCLUSION: Anemia was frequent after gastrectomy for early gastric cancer, with iron deficiency being the major cause. Evaluation for anemia including iron status should be performed after gastrectomy and appropriate iron replacement should be considered.

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Key words: Gastrectomy; Stomach neoplasms; Anemia; Iron deficiency; Vitamin B₁₂ deficiency

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INTRODUCTION

Gastric cancer is the most common malignancy in Korea and the second most frequent cause of cancer-related death worldwide^[1,2]. Curative resection has proven to be the only successful treatment modality for locally confined gastric cancer^[3]. Anemia is a frequent complication after gastrectomy and deficiencies of iron, vitamin B₁₂, or folate, either alone or in combination, have been reported after gastric surgery^[4-6]. However, these studies included relatively small numbers of patients, follow-up visits were not systematically scheduled, and the definition and biochemical markers of anemia were ambiguous or insufficient. There are limited reports concerning anemia in patients who undergo gastrectomy for early gastric cancer who have systematically scheduled serial follow-up visits, but do not receive supplements for anemia.

The incidence of gastric bypass surgery for morbid obesity is increasing, and anemia after bypass surgery has been reported^[7-11]. Dietary life after gastric surgery in patients with gastric cancer may differ from that in patients with morbid obesity: in contrast to patients with obesity, patients having gastrectomy for gastric cancer do not restrict their dietary intake for weight reduction. The aim of this study was to identify the natural history of anemia after gastrectomy in a cohort of patients undergoing gastrectomy for early gastric cancer who were systematically followed up in the long term.

MATERIALS AND METHODS

Study population

This study was a retrospective chart review of the registry of all patients who had undergone gastrectomy for early gastric cancer at Seoul St Mary's Hospital, Seoul, Korea between January 2006 and October 2007. Patients with anemia in the preoperative workup, cancer recurrence, undergoing systemic chemotherapy, with other medical conditions that can cause anemia, or treated during follow up with red cell transfusions or supplements for anemia were excluded from the study.

Follow-up program

Patients with early gastric cancer were followed up at 3, 6, 9, 12, 18, 24, 48 and 60 mo after surgery. The follow-up

program consisted of interim history taking, physical examination, imaging studies, endoscopic examination, hematology, and blood chemistry panels. Mean cell volume (MCV), serum iron, serum ferritin, total iron-binding capacity, serum vitamin B₁₂, and serum folate level were included in the follow-up program.

Definition of anemia and related conditions

Anemia was defined by World Health Organization criteria (Hb <12 g/dL in women and <13 g/dL in men)^[9]. Iron deficiency was defined as serum ferritin < 20 µg/dL. Vitamin B₁₂ deficiency was defined as serum vitamin B₁₂ < 200 pg/mL. Iron deficiency anemia was defined as anemia with concomitant iron deficiency. Anemia of chronic illness was defined as anemia with serum ferritin > 20 µg/dL. Anemia from vitamin B₁₂ deficiency was defined as megaloblastic anemia (MCV >100 fL) with vitamin B₁₂ deficiency.

Statistical analysis

Continuous data are presented as the mean ± SD and categorical data are presented as proportions. The data were analyzed using the paired *t* test to assess the differences in continuous data during follow up and a χ^2 test or Fisher's exact test to assess the differences according to sex and the type of gastrectomy. Differences were considered significant if the *P* value was less than 0.05. All statistical analysis were performed using SAS software (SAS Institute, Cary, NC, United States).

RESULTS

Four hundred fifty-eight patients with early gastric adenocarcinoma underwent curative gastrectomy in our institution during the study period. Of these, 297 were excluded from the analysis (anemia in preoperative workup in 45 patients, systemic chemotherapy in 79, other medical conditions that can cause anemia in 13, follow-up loss in 86, insufficient biochemical profile for anemia in 73, red cell transfusion during the follow-up in one). Finally, 161 patients undergoing gastrectomy for early gastric cancer were analyzed. No patient in the study population received iron or vitamin B₁₂ replacement therapy. Follow-up after surgery was possible at 3 mo in 159, 6 mo in 161, 12 mo in 161, 18 mo in 160, 24 mo in 158, 36 mo in 154, and 48 mo in 142.

The characteristics of the study population are shown in Table 1. The mean age was 56.2 ± 10.9 years (range, 23-78 years) and 113 patients were men (70.2%). Thirty-nine (24.2%) patients underwent Billroth I subtotal gastrectomy, 90 (55.9%) Billroth II subtotal gastrectomy, and 32 (19.9%) total gastrectomy.

The incidence of anemia was 24.5% at 3 mo after surgery and increased to 37.1% at 48 mo after surgery. The incidence of iron deficiency anemia increased during the follow-up and became the major cause of anemia at 48 mo after surgery (Figure 1). Anemia of chronic disease and megaloblastic anemia were uncommon. Only one

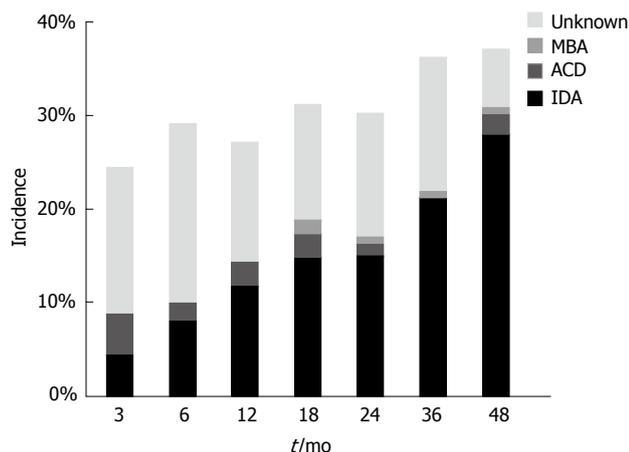


Figure 1 Incidence and distribution of anemia after gastrectomy. IDA: Iron deficiency anemia; ACD: Anemia of chronic disease; MBA: Megaloblastic anemia.

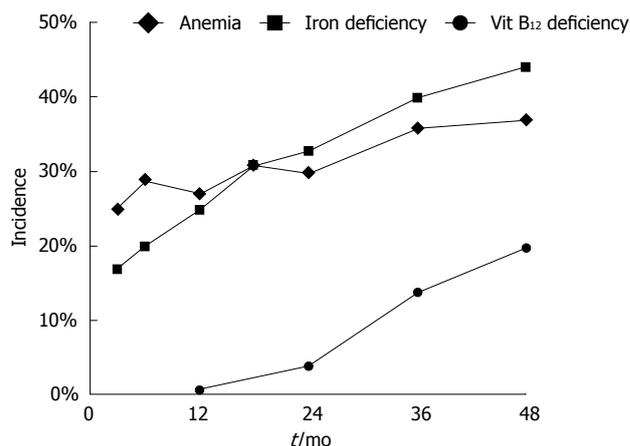


Figure 2 Incidence of anemia, iron deficiency state, and vitamin B₁₂ deficiency state after gastrectomy.

Variable	Value n (%)
Age (yr) ¹	56.2 ± 10.9
Gender	
Male	113 (70.2)
Female	48 (29.8)
Preoperative hemoglobin (g/dL) ¹	14.2 ± 1.1
Male	14.6 ± 0.9
Female	13.2 ± 0.8
Combined disease	
Hypertension	20 (12.4)
Diabetes mellitus	19 (11.8)
Pulmonary disease	2 (1.2)
Coronary artery disease	2 (1.2)
Chronic liver disease	2 (1.2)
Type of operation	
Billroth I subtotal gastrectomy	39 (24.2)
Billroth II subtotal gastrectomy	90 (55.9)
Total gastrectomy	32 (19.9)
Nodal Stage	
N0	143 (88.8)
N1	15 (9.4)
N2	2 (1.2)
N3	1 (0.6)

¹Data are presented as mean ± SD.

patient had a picture consistent with megaloblastic anemia with vitamin B₁₂ deficiency at 48 mo after surgery. The incidence of anemia, iron deficiency, and vitamin B₁₂ deficiency increased during follow-up after surgery (Figure 2). The incidence during follow-up of iron deficiency without anemia was considerable. Most vitamin B₁₂ deficiency occurred in patients who had total gastrectomy. At 48 mo after gastrectomy, the incidence of vitamin B₁₂ deficiency was 3.2% in patients with Billroth I subtotal gastrectomy, 7.5% in Billroth II subtotal gastrectomy, and 76.9% in total gastrectomy. The incidence of vitamin B₁₂ deficiency in patients with total gastrectomy was 0%, 16.1%, 50.0% and 76.9% at 12, 24, 36 and 48 mo after gastrectomy, respectively.

Serial follow-up hematology and blood chemistry data are shown in Table 2. Hemoglobin level, serum

ferritin level, and vitamin B₁₂ level decreased during the follow-up. The average serum albumin level was 3.3 ± 0.4 g/dL before surgery and recovered to 4.2 ± 0.3 g/dL at 3 mo after surgery.

The incidence of anemia in female patients was significantly higher than in male patients at 12 mo, 24 mo, 36 mo, and 48 mo after surgery (Figure 3A). Patients with total gastrectomy showed significantly higher incidence of anemia at 48 mo after surgery than patients with subtotal gastrectomy (60.7% *vs* 31.3%, *P* = 0.008) (Figure 3B). There was no significant difference in the incidence of anemia 48 mo after surgery between patients having Billroth I and Billroth II subtotal gastrectomy (18.8% *vs* 36.1%, *P* = 0.078). The incidence of iron deficiency was also significantly higher in female patients than in male patients at all times in the follow-up period except at 3 mo after surgery (Figure 3C). The incidence of iron deficiency was significantly higher in patients with total gastrectomy than those with subtotal gastrectomy only at 36 mo after surgery (58.1% *vs* 35.0%, *P* = 0.024) (Figure 3D). There was no significant difference in the incidence of anemia and iron deficiency between patients with Billroth I and II subtotal gastrectomy at 48 mo after surgery (28.1% *vs* 45.8%, *P* = 0.095).

DISCUSSION

In this study, we identified the incidence and etiology of anemia after gastrectomy in a cohort of patients having gastrectomy for gastric cancer who were systematically followed up over the long term. We found that the incidence of anemia in the patients with gastrectomy increased during follow-up. Iron deficiency anemia became the major cause of anemia after gastrectomy, and megaloblastic anemia with vitamin B₁₂ deficiency was rare.

In this study, anemia was relatively frequent after gastrectomy for early gastric cancer. Anemia was detected shortly after surgery and increased during the follow-up period. The incidence of anemia was 24.5% at 3 mo after surgery and increased to 37.1% at 48 mo after surgery. Anemia has been reported to occur in up to 60% of

Table 2 Biochemical marker after gastrectomy

	Before surgery	3 mo	6 mo	12 mo	18 mo	24 mo	36 mo	48 mo
Hemoglobin (g/dL)	14.2 ± 1.1	13.4 ± 1.2	13.3 ± 1.2 ^a	13.4 ± 1.3	13.2 ± 1.3 ^a	13.2 ± 1.4 ^a	13.1 ± 1.5 ^a	13.1 ± 1.6 ^a
Serum ferritin (μg/L)		70.8 ± 59.1	63.9 ± 52.4 ^a	55.2 ± 43.4 ^a	49.3 ± 51.3 ^a	43.3 ± 46.0 ^a	34.7 ± 32.2 ^a	33.7 ± 30.7 ^a
Serum vitamin B ₁₂ (pg/mL)				1167.7 ± 597.6		1113.9 ± 629.4	773.7 ± 999.9 ^c	560.3 ± 472.9 ^c
Serum albumin (g/dL)	3.3 ± 0.4	4.2 ± 0.3	4.2 ± 0.4	4.3 ± 0.3	4.3 ± 0.3	4.3 ± 0.3	4.3 ± 0.3	4.3 ± 0.3

^a*P* < 0.05 vs 3 mo; ^c*P* < 0.05 vs 12 mo.

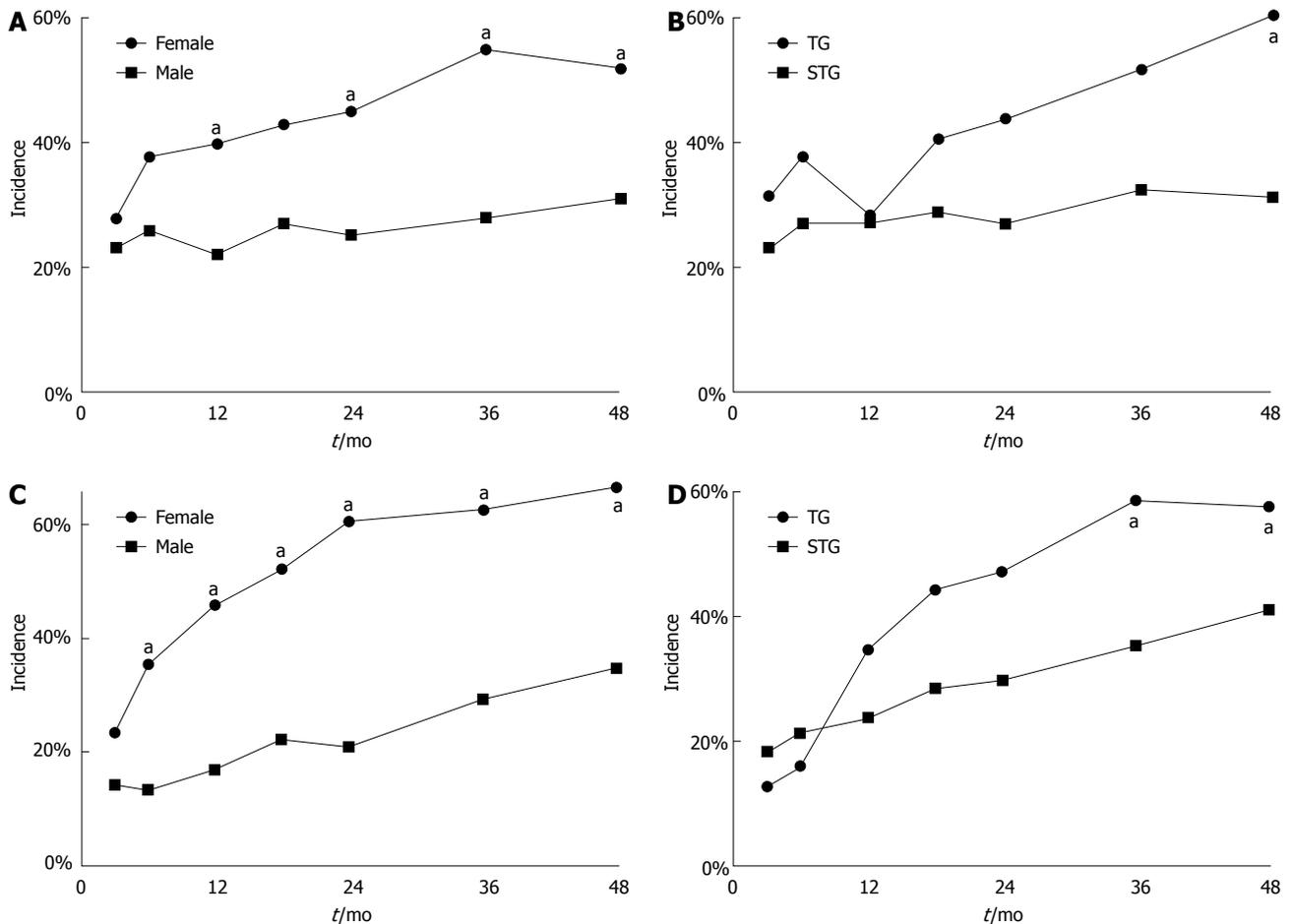


Figure 3 Incidence of anemia and deficiency state after gastrectomy. A: Significantly higher incidence of anemia in female patients than in male patients at 12, 24, 36 and 48 mo after surgery, ^a*P* < 0.05 vs female group; B: Significantly higher incidence of anemia in patients with total gastrectomy (TG) than in patients with subtotal gastrectomy (STG) at 48 mo after surgery, ^a*P* < 0.05 vs STG group; C: Significantly higher incidence of iron deficiency state in female patients than in male patients at all times in the follow-up period except at 3 mo after surgery, ^a*P* < 0.05 vs male group; D: Significantly higher incidence of iron deficiency in patients with TG than in patients with STG at 36 mo after surgery, ^a*P* < 0.05 vs STG group.

patients after partial gastrectomy^[12,13]. The incidence of anemia after bypass surgery has been reported as from 5% to 64%^[7-9,14-17]. A recent study of postsurgical anemia after gastrectomy reported that the incidence of anemia was 37.9%, 33.5% and 34.7% at 3, 6 and 12 mo after surgery, respectively^[18].

Our study showed that iron deficiency anemia increased during follow-up and was the major cause of anemia at 48 mo after surgery. Serial follow-up showed that serum ferritin gradually decreased from 70.8 μg/L to 30.1 μg/L during follow up and the incidence of iron deficiency gradually increased to 44% at 48 mo after gastrectomy. Our results are similar to those of a previous

study of patients after Roux-en-Y gastric bypass^[19]. Iron deficiency anemia was reported to be very common in post-gastrectomy patients at twenty five to thirty years after surgery^[20]. Iron deficiency after gastrectomy or bypass results from malabsorption of iron because of a surgically altered gastrointestinal tract. Inadequate oral intake or occult blood loss may also contribute to this condition. Iron is absorbed in the duodenum and proximal jejunum and its absorption is enhanced by gastric acid secretion. A lack of acidity results in impaired conversion of ingested ferric iron to absorbable ferrous iron^[21]. Duodenal bypass also contributes to iron deficiency in patients with gastrectomy or bypass surgery. Atrophic gastritis

and *Helicobacter pylori* infection were reported to play an important role in iron deficiency after gastrectomy for cancer of the stomach^[22]. In our study, the patients with Billroth I subtotal gastrectomy showed the lowest incidence of iron deficiency compared with all groups of patients with duodenal bypass (total gastrectomy and Billroth II subtotal gastrectomy) at 48 mo after surgery (28.1% *vs* 48.6%, $P = 0.045$). Our study showed a higher incidence of anemia and iron deficiency in patients with total gastrectomy than in those with subtotal gastrectomy. This difference may originate from the more depleted nutritional status of patients with total gastrectomy^[15,23,24]. In contrast to the subjects of other studies of severe obesity, our study population was patients with early gastric cancer and did not need weight reduction. They were recommended an adequate oral intake and serum albumin level was maintained at an adequate level. Therefore, our results show an etiology and natural course of postgastrectomy anemia that is close to reality.

The incidence of vitamin B₁₂ deficiency gradually increased to 20% of patients in our study at 48 mo after gastrectomy. The majority of the patients with vitamin B₁₂ deficiency at 48 mo were those with total gastrectomy. Vitamin B₁₂ deficiency causes megaloblastic anemia and neurological symptoms, and is known to develop within 5 or 6 years after total gastrectomy, a delay that reflects the time needed to exhaust cobalamin stores after cobalamin absorption ceases^[25]. Our study showed that 16.1%, 50.0% and 76.9% of the patients with total gastrectomy presented with vitamin B₁₂ deficiency at 24, 36 and 48 mo after gastrectomy, respectively. Although the incidence of vitamin B₁₂ deficiency at 48 mo after gastrectomy was high in our study, only one patient with vitamin B₁₂ deficiency presented with megaloblastic anemia. Because 70% of patients with vitamin B₁₂ deficiency at 48 mo after gastrectomy showed anemia and the majority of these were iron-deficient, the generation of macrocytosis by concomitant iron deficiency should be considered^[26]. Vitamin B₁₂ deficiency can develop within 2 years after total gastrectomy and vitamin B₁₂ replacement may be considered for post-gastrectomy patients with persistent anemia despite iron replacement or with neurological symptoms combined with vitamin B₁₂ deficiency.

Our study has some limitations. This study was a single-center retrospective study. The symptoms of anemia and other nutritional parameters such as body weight or body mass were not investigated. However, a strength of our study is the tightly controlled long-term follow-up of the patient cohort after gastrectomy for early gastric cancer, with systematically and prospectively scheduled serial follow-up visits and without any supplement for anemia. Our results enabled us to understand the development of post-gastrectomy anemia.

In conclusion, anemia was frequent after gastrectomy for early gastric cancer and iron deficiency was the major cause. Evaluation for anemia including iron status should be performed after gastrectomy and appropriate iron replacement should be considered.

COMMENTS

Background

Curative resection has proven to be the only successful treatment modality for locally confined gastric cancer. Anemia is a frequent complication after gastrectomy. The present study identified the natural history of anemia after gastrectomy in a cohort of patients undergoing gastrectomy for early gastric cancer who were systematically followed up in the long term.

Research frontiers

Anemia was frequent after gastrectomy for early gastric cancer, with iron deficiency being the major cause.

Innovations and breakthroughs

Anemia is a common complication of gastrectomy, particularly in female patients and it worsens as follow-up lengthens in a cohort with long-term follow-up for 48 mo. The incidence of iron deficiency gradually increased during follow up and became the major cause of anemia. Megaloblastic anemia is uncommon although the incidence of vitamin B₁₂ deficiency gradually increased after gastrectomy, particularly particularly after total gastrectomy.

Applications

The results of this study suggest that evaluation for anemia including iron status should be performed after gastrectomy and appropriate iron replacement should be considered.

Peer review

This study examined anemia in a large cohort of patients undergoing gastrectomy for early gastric cancer. The results of this study can serve as a benchmark in the follow up of patients undergoing partial or total gastrectomy for gastric carcinoma.

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Association of chronic viral hepatitis B with insulin resistance

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viral hepatitis B (CVHB) and insulin resistance (IR) in Korean adults.

METHODS: A total of 7880 adults (3851 men, 4029 women) who underwent a comprehensive medical examination were enrolled in this study. Subjects diagnosed with either diabetes mellitus, or any other disorder that could influence their insulin sensitivity, were rejected. Anthropometry, metabolic risk factors, hepatitis B surface antigen, hepatitis B surface antibody, hepatitis B core antibody, fasting plasma glucose and insulin were measured for all subjects. Homeostasis model assessment (HOMA), quantitative insulin check index (QUICKI), and M_{fm} index were used for determining insulin sensitivity. Each participant was categorized into a negative, recovery, or CVHB group. To compare variables between groups, a t-test and/or one-way analysis of variance were used. Partial correlation coefficients were computed to present the association between insulin resistance and other variables. Multiple logistic regression analysis was used to assess the independent association between CVHB and IR.

RESULTS: The mean age of men and women were 48.9 and 48.6 years, respectively. Subjects in the CVHB group had significantly higher waist circumference [(86.0 ± 7.7 cm vs 87.3 ± 7.8 cm, $P = 0.004$ in men), (78.3 ± 8.6 cm vs 80.5 ± 8.5 cm, $P < 0.001$ in women)], cystatin C [(0.96 ± 0.15 mg/dL vs 1.02 ± 0.22 mg/dL, $P < 0.001$ in men), (0.84 ± 0.15 mg/dL vs 0.90 ± 0.16 mg/dL, $P < 0.001$ in women)], fasting insulin [(5.47 ± 3.38 μU/mL vs 6.12 ± 4.62 μU/mL, $P < 0.001$ in men), (4.57 ± 2.82 μU/mL vs 5.06 ± 3.10 μU/mL, $P < 0.001$ in women)] and HOMA index [(1.24 ± 0.86 vs 1.43 ± 1.24, $P < 0.001$ in men), (1.02 ± 0.76 vs 1.13 ± 0.87, $P = 0.033$ in women)] compared to control group. The HOMA index revealed a positive correlation with body mass index (BMI) ($r = 0.378$, $P < 0.001$), waist circumference ($r = 0.356$, $P < 0.001$), percent body fat ($r = 0.296$, $P < 0.001$), systolic blood pressure ($r = 0.202$, $P < 0.001$), total cholesterol ($r = 0.134$, $P < 0.001$), triglycerides ($r = 0.292$, $P < 0.001$),

Abstract

AIM: To investigate the relationship between chronic

cystatin C ($r = 0.069$, $P < 0.001$) and uric acid ($r = 0.142$, $P < 0.001$). The QUICKI index revealed a negative correlation with BMI ($r = -0.254$, $P < 0.001$), waist circumference ($r = 0-0.243$, $P < 0.001$), percent body fat ($r = -0.217$, $P < 0.001$), systolic blood pressure ($r = -0.132$, $P < 0.001$), total cholesterol ($r = -0.106$, $P < 0.001$), triglycerides ($r = -0.205$, $P < 0.001$), cystatin C ($r = -0.044$, $P < 0.001$) and uric acid ($r = -0.096$, $P < 0.001$). For subjects identified with IR, the odds ratio of an accompanying diagnosis of chronic hepatitis B was 1.534 (95% CI: 1.158-2.031, HOMA index criteria) or 1.566 (95% CI: 1.124-2.182, QUICKI criteria) after adjustment for age, gender, BMI, and amount of alcohol consumption.

CONCLUSION: Our study demonstrates that CVHB is associated with IR. CVHB may need to be monitored for occurrence of IR and diabetes mellitus.

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Key words: Hepatitis B; Insulin resistance; Diabetes mellitus, type 2; Metabolic syndrome

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INTRODUCTION

Insulin resistance (IR) is the principal indication for development of metabolic syndrome and type 2 diabetes^[1,2]. IR appears as a consequence of the inability of insulin to induce the appropriate effect on glucose metabolism. Inordinately large amounts of insulin are required to achieve a normal response in a state of IR. A hyperinsulinemic state causes several clinical abnormalities to appear in the blood vessels, kidneys, and liver, and these represent the major features of metabolic syndrome^[3].

Metabolic syndrome generally refers to a combination of metabolic diseases such as abdominal obesity, high blood pressure, dyslipidemia and elevated blood glucose, that appear together in an individual patient^[1]. Because metabolic syndrome is recognized as a serious risk factor for cardiovascular disease, prevention and comprehensive management are important in treating this condition^[4].

Hepatitis B is one of the most common health problems and it is estimated that, of the world's total population, one third (over 2 billion people) have been infected with hepatitis B virus (HBV)^[5]. Approximately two thirds of chronic viral hepatitis B (CVHB) patients live in Asia

and the Pacific Islands. HBV infection may cause acute and/or chronic hepatitis and premature death from liver cirrhosis, liver failure or hepatocellular carcinoma^[6]. Moreover, CVHB infection is related to other diseases such as polyarteritis nodosa (PAN)^[7], glomerulonephritis (GN)^[8], serum sickness-like syndrome (prodrome)^[9], arthritis^[10], and acrodermatitis^[11].

Recently, an experimental study suggested that hepatitis B X protein (HBx) impairs the hepatic insulin signaling pathway, and that HBV infection is associated with IR^[12]. A previous clinical studies also suggest that hyperinsulinemia occurs in CVHB and hepatitis C^[13], and this association has been elucidated in hepatitis C virus (HCV) infection^[14,15]. HCV may disturb the insulin signaling pathway by activation of the tumor necrosis factor (TNF) system^[16]. IR has been proposed as an important risk factor in patients with chronic hepatitis C, mainly due to its relationship to steatosis development^[17] and fibrosis progression^[18], and non-response to peginterferon plus ribavirin^[19]. However, the effect of HBV infection on human insulin sensitivity remains unclear. In this study, we tested the hypothesis that HBV infection may associate with IR and metabolic syndrome, by comparing incidence of IR and prevalence of metabolic syndrome between HBV-infected study participants and a healthy control group.

MATERIALS AND METHODS

Study subjects

This consecutive study conducted at the Center for Health Promotion, Pusan National University Hospital in Busan, South Korea. Data for this study were obtained from 7880 Koreans (3851 men, 4029 women) who underwent a comprehensive medical examination between January 2007 and September 2008. The study participants were eligible if they met all of the following criteria: age ≥ 18 years, no history of diabetes and hypertension requiring medication, negative for anti-hepatitis C antibody, serum aspartate aminotransferase or alanine aminotransferase (ALT) < 80 IU/L, serum gamma-glutamyl transferase (GGT) < 80 mg/dL, serum creatinine < 1.5 mg/dL, prostate specific antigen < 5.0 ng/mL, α -fetoprotein < 10.0 IU/mL, carcinoembryonic antigen < 5.0 ng/mL (for smokers) or < 2.5 ng/mL (for non-smokers), WBC count $< 10\ 000/\mu\text{L}$, and high sensitivity C-reactive protein (hs-CRP) < 1 mg/dL.

Measurements

The health checkup which provided our study data included a physical examination, anthropometric measurements and blood tests. Height and weight were measured to the nearest 0.1 cm and 0.1 kg using standard protocol with subjects wearing a light gown and without shoes. Body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). Waist circumference was measured at the narrowest point between the lower border of the rib cage and the iliac crest, at the end of a normal expiration of breath and to the nearest 0.1 cm.

Percentage of body fat and total fat mass were measured by bioelectric impedance analysis (Inbody 3.0, Biospace Co, Ltd, Korea). Blood pressure was measured using the right arm of subjects assuming a sitting position, and after they had rested for at least 10 min. By use of an automated blood pressure measurement device (BP-203RV II, Colin Corp, Aichi, Japan). Medication history, alcohol intake and smoking habits were obtained by patient interview. The questions relating to alcohol intake included descriptions of the type of alcohol beverage consumed, the weekly frequency of alcohol consumption, and the amount consumed daily. Smoking status was classified as either non-smoker or smoker (former or current). Blood samples were obtained from an antecubital vein after 12 h fasting, typically between 8 and 9 AM. The blood samples were subsequently analyzed at a certified laboratory at Pusan National University Hospital. Lipid profiles, uric acid, and GGT concentrations were measured using an autoanalyzer with the enzymatic colorimetric method (Hitachi7600, Hitachi Ltd, Japan). Cystatin C was measured by turbidimetric immunoassay (HBI Co, Ltd, Korea) using the Modular Analytics E170 (Roche Diagnostics, Switzerland). Hs-CRP was measured using a Behring Nephelometer (DadeBehring, Germany). Fasting plasma glucose was measured by the glucose oxidase method using a Synchron LX 20 (Beckman Coulter, Fullerton, CA, United States). Fasting insulin was measured using a radioimmunoassay (Diagnostic Product Corporation, Los Angeles, CA, United States) with antibody-coated tubes. The mean intra- and interassay coefficient of variation (CV) values were 4.2% and 6.3%, respectively.

Hepatitis B surface antigen (HBsAg), hepatitis B surface antibody, and hepatitis B core antibody were measured by enzyme-linked immunosorbent assay (Bio Focus Co, Ltd, Korea). Hepatitis B viral status was classified into three groups (negative/recovery/CVHB), according to serologic patterns. Insulin sensitivity was estimated using homeostasis model assessment (HOMA)-IR [fasting insulin ($\mu\text{IU/mL}$) \times fasting glucose (mg/dL)/405]^[20], quantitative insulin check index (QUICKI) $\{1/[\log \text{glucose (mg/dL)} + \log \text{insulin } (\mu\text{IU/mL})]\}$ ^[21,22] and Mf_{im} index $(\exp^{2.63 - [0.28 \times \ln(\text{insulin})] - [0.31 \times \ln(\text{triglycerides})]})$ ^[23].

Definition of metabolic syndrome

The prevalence of metabolic syndrome reported in this study was estimated using definitions proposed in 2005 by the American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI)^[24] and the International Diabetes Federation criteria^[25] for the diagnosis of metabolic syndrome. We defined central obesity as a waist circumference ≥ 90 cm in males and ≥ 85 cm in females, according to geography-specific cut points for waist circumference^[26].

Ethical approval

All participants gave informed consent, and this study was approved by the Institutional Review Board at Pusan National University Hospital and is in accordance with the Declaration of Helsinki (E2010-055).

Table 1 Baseline characteristics of study subjects

Variables	Men (n = 3851)	Women (n = 4029)
Age (yr)	48.9 \pm 10.7	48.6 \pm 10.4
BMI (kg/m ²)	24.5 \pm 2.8	23.5 \pm 2.9
Abdominal circumference (cm)	86.5 \pm 7.4	79.2 \pm 8.4
Percentage body fat (%)	22.7 \pm 5.1	30.1 \pm 5.7
Systolic BP (mmHg)	126.3 \pm 15.3	122.3 \pm 15.4
AST (IU/L)	23.8 \pm 8.6	20.9 \pm 10.5
ALT (IU/L)	26.8 \pm 16.6	19.0 \pm 11.5
GGT (IU/L)	38.0 \pm 20.1	20.0 \pm 12.6
Fasting plasma glucose (mg/dL)	91.6 \pm 14.7	88.3 \pm 14.1
Fasting insulin ($\mu\text{U/mL}$)	5.48 \pm 3.49	4.64 \pm 2.78
Total cholesterol (mg/dL)	196.3 \pm 33.3	195.8 \pm 35.2
Triglycerides (mg/dL)	138.0 \pm 80.9	103.8 \pm 63.6
HDL-cholesterol (mg/dL)	50.8 \pm 12.5	59.8 \pm 14.1
LDL-cholesterol (mg/dL)	124.8 \pm 29.5	121.0 \pm 31.8
high-sensitivity CRP (mg/dL)	0.15 \pm 0.47	0.11 \pm 0.37
TSH ($\mu\text{U/mL}$)	1.79 \pm 1.79	2.29 \pm 2.15
Uric acid (mg/dL)	5.88 \pm 1.25	4.20 \pm 0.91

Differences between men and women were statistically significant except for age and total cholesterol ($P < 0.05$ by *t* test). BMI: Body mass index; BP: Blood pressure; AST: Aspartate transferase; ALT: Alanine transferase; GGT: Gamma-glutamyl transferase; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; CRP: C-reactive protein; TSH: Thyroid stimulating hormone.

Statistical analysis

To compare variables between groups, a *t* test and/or one-way analysis of variance followed by a Scheffé post hoc test or Kruskal-Wallis test were used as appropriate. Pearson partial correlation coefficients were computed to present the association between fasting plasma glucose concentration and other variables after adjustments for age, gender and alcohol consumption. Using multiple logistic regression analysis and adjusting for age, gender and alcohol intake, we estimated the existence of any independent association between IR and HBV status. Statistical analysis was performed using SPSS 12.0 for Windows. A *P* value of less than 0.05 was considered statistically significant. All statistical tests were two-sided.

RESULTS

Baseline characteristics of study subjects

The subjects were classified as men ($n = 3851$) and women ($n = 4029$), and their baseline clinical characteristics were compared (Table 1). The mean age of men and women were 48.9 years and 48.6 years, respectively. Age and total cholesterol level were not statistically different between men and women ($P > 0.05$). Men had significantly higher results for BMI, abdominal circumference, systolic blood pressure, aspartate aminotransferase, ALT, GGT, fasting plasma glucose, insulin, triglycerides, low-density lipoprotein-cholesterol, hs-CRP, and uric acid ($P < 0.001$).

Metabolic characteristics according to hepatitis groups

The metabolic data of study participants are shown in Table 2. In both men and women, subjects in the CVHB group were significantly older with larger waist circum-

Table 2 Means and frequencies of metabolic risk factors associated with hepatitis B virus status in men and women (mean \pm SD)

Variables	Negative (<i>n</i> = 1292)	Recovery from hepatitis B (<i>n</i> = 1956)	Chronic hepatitis B (<i>n</i> = 603)	<i>P</i> value ¹
Hepatitis B virus status in men				
Age (yr)	44.4 \pm 11.7	51.4 \pm 9.4	50.8 \pm 10.7	0.000
Body mass index (kg/m ²)	24.5 \pm 2.9	24.5 \pm 2.7	24.7 \pm 2.9	0.493
Waist circumference (cm)	86.0 \pm 7.7	86.5 \pm 7.0	87.3 \pm 7.8	0.004
Percentage body fat (%)	22.5 \pm 5.7	22.8 \pm 4.6	23.0 \pm 5.0	0.094
Systolic blood pressure (mmHg)	126.4 \pm 15.5	126.3 \pm 15.0	126.1 \pm 15.6	0.922
Total cholesterol (mg/dL)	196.0 \pm 33.2	196.8 \pm 33.1	195.5 \pm 34.5	0.680
Triglyceride (mg/dL)	142.8 \pm 84.5	135.6 \pm 79.2	135.2 \pm 78.2	0.029
HDL-cholesterol (mg/dL)	51.0 \pm 12.7	50.9 \pm 12.8	50.4 \pm 11.4	0.602
Cystatin C (mg/L)	0.96 \pm 0.15	0.98 \pm 0.16	1.02 \pm 0.22	0.000
Fasting glucose (mg/dL)	90.7 \pm 15.3	92.1 \pm 14.4	91.9 \pm 14.4	0.039
Fasting insulin (μ U/mL)	5.47 \pm 3.38	5.29 \pm 3.10	6.12 \pm 4.62	0.000
HOMA index	1.24 \pm 0.86	1.22 \pm 0.80	1.43 \pm 1.24	0.000
QUICKI index	0.386 \pm 0.065	0.386 \pm 0.044	0.385 \pm 0.134	0.922
Mf _{fm} index	8.41 \pm 2.43	8.58 \pm 2.31	8.39 \pm 2.63	0.072
Hepatitis B virus status in women				
Age (yr)	45.9 \pm 10.7	50.9 \pm 9.3	51.6 \pm 10.3	0.000
Body mass index (kg/m ²)	23.2 \pm 3.0	23.7 \pm 2.7	24.0 \pm 2.9	0.000
Waist circumference (cm)	78.3 \pm 8.6	79.8 \pm 8.0	80.5 \pm 8.5	0.000
Percentage body fat (%)	29.6 \pm 6.8	30.4 \pm 4.6	31.0 \pm 4.9	0.000
Systolic blood pressure (mmHg)	121.3 \pm 15.4	123.0 \pm 15.5	124.5 \pm 14.3	0.000
Total cholesterol (mg/dL)	193.3 \pm 35.4	197.9 \pm 35.0	198.1 \pm 34.3	0.000
Triglyceride (mg/dL)	102.0 \pm 63.5	106.4 \pm 66.2	100.3 \pm 47.5	0.061
LDL-cholesterol (mg/dL)	118.3 \pm 31.6	123.2 \pm 31.8	123.7 \pm 31.7	0.000
Cystatin C (mg/L)	0.84 \pm 0.15	0.87 \pm 0.15	0.90 \pm 0.16	0.000
Fasting glucose (mg/dL)	88.0 \pm 14.6	88.7 \pm 13.7	88.3 \pm 12.9	0.357
Fasting insulin (μ U/mL)	4.57 \pm 2.82	4.62 \pm 2.67	5.06 \pm 3.10	0.010
HOMA index	1.02 \pm 0.76	1.03 \pm 0.67	1.13 \pm 0.87	0.033
QUICKI index	0.401 \pm 0.056	0.398 \pm 0.051	0.392 \pm 0.045	0.013
Mffm index	9.77 \pm 2.62	9.58 \pm 2.53	9.36 \pm 2.25	0.006

¹Analysis of variance except triglyceride (Kruskal-Wallis test). HDL: High-density lipoprotein; LDL: Low-density lipoprotein; HOMA: Homeostasis model assessment; QUICKI: Quantitative insulin check index.

Table 3 Partial correlation coefficients of insulin sensitivity index to metabolic parameters after adjusting for age, gender, and alcohol consumption

	BMI	WC	BFP	SBP	TC	TG	HDL-C	Cys-C	Uric acid
FPG	0.150	0.151	0.114	0.138	0.087	0.141	-0.102	-0.108	-0.010
Insulin	0.395	0.368	0.313	0.196	0.132	0.293	-0.206	0.105	0.168
HOMA index	0.378	0.356	0.296	0.202	0.134	0.292	-0.202	0.069	0.142
QUICKI index	-0.254	-0.243	-0.217	-0.132	-0.106	-0.205	0.164	-0.044	-0.096
Mf _{fm} index	-0.374	-0.366	-0.318	-0.199	-0.258	-0.628	0.386	-0.116	-0.203

BMI: Body mass index; WC: Waist circumference; BFP: Percent of body fat; SBP: Systolic blood pressure; TC: Total cholesterol; TG: Triglyceride; HDL: High-density lipoprotein; Cys-C: Cystatin-C; FPG: Fasting plasma glucose; HOMA: Homeostasis model assessment; QUICKI: Quantitative insulin check index. All correlation coefficients are statistically significant ($P < 0.001$).

ferences, and had higher percentages of body fat, cystatin C, fasting insulin, and HOMA index compared to other groups. There were significant differences between men in the CVHB group and negative group in terms of fasting plasma glucose, but no significant differences were observed for women. QUICKI ($P < 0.05$) and Mf_{fm} index ($P < 0.01$) results were significantly lower for women in the CVHB group.

Correlation of insulin sensitivity index with metabolic factors

The HOMA index revealed a positive correlation with BMI ($r = 0.378$, $P < 0.001$), waist circumference ($r = 0.356$,

$P < 0.001$), percent body fat ($r = 0.296$, $P < 0.001$), systolic blood pressure ($r = 0.202$, $P < 0.001$), total cholesterol ($r = 0.134$, $P < 0.001$), triglycerides ($r = 0.292$, $P < 0.001$), cystatin C ($r = 0.069$, $P < 0.001$) and uric acid ($r = 0.142$, $P < 0.001$) (Table 3). QUICKI and Mf_{fm} index produced a negative correlation with BMI, waist circumference, percent body fat, systolic blood pressure, total cholesterol, triglycerides, cystatin C, and uric acid ($P < 0.001$).

Association of insulin resistance and chronic hepatitis B

For the presence of IR, adjusted odds ratios for the CVHB group was 1.534 (95%CI: 1.158-2.031, HOMA index criteria) or 1.566 (95% CI: 1.124-2.182 in QUICKI

Table 4 Logistic regression analysis with insulin resistance as a dependent variable

Variables	Insulin resistance					
	HOMA index criteria			QUICKI criteria		
	β	SE	Odds ratio (95% CI)	β	SE	Odds ratio (95% CI)
Age (yr)	0.009	0.005	1.009 (0.999-1.019)	0.016	0.006	1.016 (1.005-1.028)
Gender						
Men	0.487	0.109	1.627 (1.314-2.015)	0.452	0.131	1.571 (1.215-2.031)
Women			1.000			1.000
Body mass index (kg/m ²)	0.316	0.017	1.372 (1.328-1.417)	0.347	0.020	1.746 (1.663-1.833)
Alcohol consumption (kcal)	0.000	0.000	1.000 (0.999-1.000)	0.000	0.000	1.000 (0.999-1.000)
Hepatitis B virus status						
Negative			1.000			1.000
Recovery from hepatitis B	-0.027	0.112	0.974 (0.782-1.212)	-0.075	0.135	0.928 (0.712-1.210)
Chronic hepatitis B	0.428	0.143	1.534 (1.158-2.031)	0.449	0.169	1.566 (1.124-2.182)

HOMA: Homeostasis model assessment; QUICKI: Quantitative insulin check index.

criteria) (Table 4). Adjusting factors included age, gender, BMI and amount of alcoholic consumption.

DISCUSSION

In this study, CVHB was observed to be associated with IR in subjects free of prior diabetes mellitus. CVHB independently predicted a clinically significant increase in the odds ratio for the development of IR. These results indicate that patients with CVHB may need to be carefully monitored for occurrence of IR and diabetes mellitus. As the present study reports basic data on the association between CVHB and IR in a large community population, these findings support previous proposals that CVHB infection is related to IR.

HBV is the prototype member of a steadily growing family of viruses called hepadnaviruses^[27]. It is a partially double stranded virus that uses reverse transcriptase in its replication cycle. CVHB infection is a common health issue in Asia and the Pacific Islands. In Korea, approximately 3.7% of total population are affected in chronic hepatitis B^[28]. Most were infected directly from their mother during birth or through contact between children. CVHB infection may increase the occurrence of hepatic fibrosis, liver cirrhosis, and hepatocellular carcinoma^[29]. In addition, CVHB infection is related to diseases such as PAN, GN, and arthritis^[7-9]. Moreover, there is experimental evidence that CVHB infection increases the appearance of both IR and associated diabetes mellitus. A recent animal study suggested that HBx impairs the insulin signaling pathway^[12]. These findings provide the basis of a hypothesis for mechanism and are consistent with our study results.

IR is assumed to be caused by an inadequate glucose metabolism capacity which leads to more insulin to be secreted to achieve the same biologic response^[30]. Hyperinsulinemia may induce a large variety of abnormalities in blood vessels, kidneys, and muscles, and is the major pathogenesis associated with metabolic syndrome. Diabetes mellitus and metabolic syndrome are also independent risk factors for atherosclerotic disease^[31]. Thus,

early screening of high risk groups is very important to successful health promotion. The gold standard parameter for determining insulin sensitivity is the hyperinsulinemic euglycemic clamp technique. The HOMA model^[20], QUICKI^[21], and M_{fim} index^[23] used in this study show good correlation with the clamp technique and are easily utilized in primary practice.

Previous studies have proposed association of HBV infection and IR. One previous, retrospective study proposed that maternal HBsAg carrier status was a risk factor for development of gestational diabetes^[32]. Sangiorgio *et al.*^[33] also reported increased frequency of HCV and HBV infection in type 2 diabetic patients. One study reported concordant results using the HOMA model that concluded that hyperinsulinemia occurs in chronic viral hepatitis B and hepatitis C^[13]. However, another previous study reported that HBV carriers were not associated with IR^[34]. But that study had limitations due to small number of study subjects and high prevalence of fatty liver disease in the subjects.

The mechanism IR plays in CVHB infection remains unknown. There are four proteins that originate from the HBV genome including polymerase, a surface protein, a core protein, and the HBx protein. Among these proteins, HBx may be most closely associated with hepatic steatosis, inflammation, and HBV-related disease^[34]. Previous reports proposed that hepatic steatosis and systemic inflammation are associated with IR^[35]. HBx protein can induce hepatic steatosis and inflammation, thus CVHB infection is possibly associated with an impaired insulin signaling pathway^[36]. A recent report concluded that chronic inflammation had effect on IR^[37]. HBV may induce activation of proinflammatory cytokines TNF- α , interleukin (IL)-6, and IL-1 β associated with fat accumulation^[29]. Hepatic steatosis has already been demonstrated to be related to IR, and this association has been clearly identified in HCV infection. HCV proteins present due to infection may also disturb the insulin signaling pathway. IR with chronic hepatitis C has also been related to steatosis development and fibrosis progression^[13-15].

The strength of this study was inclusion of healthy volunteers, which provided greater validity compared to hospital-based or institutional based populations. Other strengths included the large sample size, characterization of multiple confounders that influence IR, and the availability of 3 IR index or insulin sensitivity markers which are validated and widely used, and were also used for determining the degree of IR in previous studies.

This study had several limitations. Because of the cross-sectional nature of this study, it was difficult to prove a causal relationship between HBV infection and IR. Also, our results may not be able to be generalized to other ethnic groups because the present study was conducted exclusively using ethnic Koreans. A weakness in terms of clinical data is that daily variability of insulin within individuals is high, and a single, daily sample may not accurately characterize the actual level. Moreover, there are numerous other factors that influence IR such as condition of skeletal muscles, engagement in physical activity, and severity of liver injuries. Future studies that overcome these limitations are needed to confirm our findings.

In conclusion, CVHB may be associated with IR as identified by HOMA-index, QUICKI, and Mf_{im} index results. These findings should be explored further.

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COMMENTS

Background

Insulin resistance (IR) is the principal indication for development of metabolic syndrome and type 2 diabetes. Chronic viral hepatitis B (CVHB) is one of the most common health problems and previous clinical studies also suggest that hyperinsulinemia occurs in CVHB. However, the effect of hepatitis B virus (HBV) infection on human insulin sensitivity remains controversial. The authors therefore investigated the hypothesis that HBV infection may associate with IR and metabolic syndrome, by comparing incidence of IR between HBV-infected subjects and healthy group.

Research frontiers

There are four proteins that originate from the HBV genome including polymerase, a surface protein, a core protein, and the hepatitis B X protein (HBx) protein. Among these proteins, HBx may be most closely associated with hepatic steatosis, inflammation, and HBV related disease. Moreover, recent experimental study suggested that HBx impairs the hepatic insulin signaling pathway.

Innovations and breakthroughs

CVHB was observed to be associated with IR in subjects free of prior diabetes mellitus. CVHB independently predicted a clinically significant increase in the odds ratio for the development of IR. These results indicate that patients with CVHB may need to be carefully monitored for occurrence of IR and diabetes mellitus.

Applications

CVHB infection is a common health issue in Asia and the Pacific Islands. CVHB may need to be monitored for occurrence of insulin resistance and diabetes mellitus.

Terminology

Homeostasis model assessment index is calculated by equation of [fasting insulin (μ U/mL) \times fasting glucose (mg/dL)/405], quantitative insulin check index is calculated by equation of $\{1/[\log \text{glucose (mg/dL)} + \log \text{insulin } (\mu\text{U/mL})]\}$, Mf_{im} index is obtained by equation of $(\exp^{2.63 - [0.28 \times \ln(\text{insulin})] - [0.31 \times \ln(\text{triglycerides})]})$.

Peer review

This study investigated the association of insulin resistance and chronic viral hepatitis B. The authors evaluated a numerous population of HBV infected patients and compared their metabolic features with control group. This manuscript reinforce further evaluations on a real clinical impact of this association.

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Treatment of functional dyspepsia with sertraline: A double-blind randomized placebo-controlled pilot study

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Abstract

AIM: To evaluate sertraline, a selective serotonin reuptake inhibitor in the treatment of patients with functional dyspepsia.

METHODS: Consecutive tertiary hospital patients with a clinical diagnosis of functional dyspepsia (FD) according to the Rome II criteria with a Hong Kong dyspepsia index (HKDI) of greater than 16 were recruited. Patients commenced enrolment prior to the inception of the Rome III criteria for functional dyspepsia. All patients were ethnic Chinese, had a normal upper endoscopy and were *Helicobacter pylori* negative prior to enrolment. Study patients were randomized to receive sertraline 50 mg or placebo daily for 8 wk. HKDI symptom scores, quality of life, hospital anxiety and depression (HAD) scale and global symptom relief were evaluated before, during and after treatment. Adverse effects were monitored during and after treatment.

RESULTS: A total of 193 patients were randomized in the intention to treat (ITT), and 150 patients were

included in the per protocol (PP) analysis. In both the ITT and PP, there was no difference in the primary outcome of global dyspepsia symptoms between the sertraline and placebo groups at week 8. In the ITT analysis, 98 and 95 patients were randomized to the sertraline and placebo groups respectively. A total of 43 patients withdrew from the study (22.3%) by week 8, with 23 of the 24 drop-outs in the sertraline group occurring prior to week 4 (95.8%). In contrast, in the placebo arm, 11 of 19 patients dropped out by week 4 (57.9%). Utilizing the last response carried forward to account for the drop-outs, there were no differences between the sertraline and placebo groups at baseline in terms of the HKDI, HKDI 26.08 ± 6.19 vs 26.70 ± 5.89 , $P = 0.433$; and at week 8, HKDI 22.41 ± 6.36 vs 23.25 ± 7.30 , $P = 0.352$ respectively. In the PP analysis, 74 and 76 patients were randomized to the sertraline and placebo groups respectively. At baseline, there were no statistically significant differences between the sertraline and placebo groups, HKDI 25.83 ± 6.313 vs 27.19 ± 5.929 respectively, $P = 0.233$; however by week 8, patients in the sertraline group demonstrated a statistically significant difference in their Hong Kong Dyspepsia Index compared to placebo, HKDI 20.53 ± 6.917 vs 23.34 ± 7.199 , $P = 0.02$, respectively). There was also no statistically significant difference in overall quality of life measures or the HAD scale related to treatment in either the ITT or PP analysis at week 8.

CONCLUSION: This pilot study, the first to examine sertraline, a selective serotonin reuptake inhibitor, for the management of FD, did not find that it was superior to placebo.

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Key words: Dyspepsia; Chinese; Gastrointestinal diseases; Drug therapy; Sertraline; Selective serotonin reuptake inhibitors

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INTRODUCTION

Functional dyspepsia (FD) is defined as persistent or recurrent pain and/or discomfort centered in the upper abdomen for at least 12 wk in the preceding 12 mo according to the Rome II criteria in the absence of structural disease^[1]. The Rome III criteria, published in 2006, further refines FD into epigastric pain syndrome and/or postprandial distress syndrome with the criteria fulfilled in the last 3 mo with symptoms onset at least 6 mo prior to diagnosis, again in the absence of structural disease^[2,3]. The prevalence of dyspepsia in the Asia Pacific region varies from 10% to 20%, with a FD prevalence of 7.9%-12% which is lower than that seen in the west^[4-6]. FD or non-ulcer dyspepsia is a significant cause of morbidity and work-related productivity lost^[7]. The pathogenesis of FD is not known. A number of studies have shown an important role of psychological factors in the pathogenesis of this condition^[8-10]. We have demonstrated previously that anxiety and depression are important co-factors in its pathogenesis^[11]. There is no definitive treatment for this condition. Acid suppression therapy has been shown to be ineffective for the treatment of this condition in Chinese patients^[12] despite benefit of proton pump inhibitor therapy in patients with ulcer-like and reflux like dyspepsia^[13]. *Helicobacter pylori* (*H. pylori*) eradication confers only small benefit relative to placebo^[14] and studies of itopride, a dopamine D2 antagonist with acetyl cholinesterase effects although initially promising, conferred no benefit in a subsequent and larger study^[15,16]. Visceral hypersensitivity appears to be important in the pathogenesis of FD, as evidenced by a small study utilizing capsaicin to generate a desensitization of gastric nociceptive C fibers^[17]. Similarly, antidepressants were investigated in FD for their ability to modulate visceral hypersensitivity^[18]. Earlier antidepressant therapy studies demonstrated some effectiveness in the treatment of functional gastrointestinal symptoms, however a recent study utilizing venlafaxine (a selective serotonin and noradrenaline reuptake inhibitor) did not show any benefit^[19-23]. In terms of antidepressants studied in FD, tricyclic compounds are the class of antidepressants best studied for this application, however selective serotonin

reuptake inhibitors (SSRIs) are more commonly used in clinical practice because of their safer side-effect profile. The precise mechanism of action of SSRIs in the treatment of depression is not fully understood. However, long-term treatment with SSRIs has been reported to down regulate the serotonin transmitter responsible for serotonin reuptake as well as serotonergic receptors^[24], which may down regulate visceral hypersensitivity.

An open label study found that the SSRI, fluoxetine, was superior to no treatment in depressed patients with FD, however had methodological flaws including the open label nature of the study^[25]. To date, there are no published randomized controlled studies on the effect of sertraline for the treatment of FD. We performed a double-blind, randomized, placebo-controlled trial consisting of 8 wk of therapy in Chinese patients with FD. We aimed to assess the efficacy of SSRI in the treatment of FD and to identify potential responders to SSRI therapy in subgroups of patients with dyspepsia as their chief symptom.

MATERIALS AND METHODS

Patient enrollment

Consecutive patients referred to the Department of Medicine, Queen Mary Hospital, Hong Kong between June 2002 and June 2008 were screened for enrolment. FD was defined as persistent or recurrent dyspepsia (pain or discomfort centered in the upper abdomen) with no evidence of organic disease, chronic severe constipation, or irritable bowel syndrome to explain the symptoms, for at least 12 wk, which need not be consecutive, within the preceding 12 mo, in accordance with the Rome II criteria^[26]. The Rome III criteria for FD had not yet been conceived when the study was commenced^[3]. Patients aged 18-80 years with symptoms of dyspepsia within two weeks prior to the endoscopy visit were eligible for the study. Informed written consent was obtained from all patients. Patients were also required to have a dyspepsia score of greater than 16 by our validated questionnaire^[27] and have had no prior investigations performed for this episode of dyspepsia within the 6 mo prior to the study. All enrolled patients were ethnic Chinese. Exclusion criteria included patients who were pregnant or breast feeding, had a history of alcohol or drug abuse; recent malignancy or significant medical illnesses or concurrent medication, which may interact with or contra-indicate the use of sertraline. Patients with a history of or current anti-depressant use were excluded. Patients with classical heartburn or acid regurgitation as their only symptom without epigastric discomfort or pain were also excluded to avoid recruitment of patients with non-erosive gastro-oesophageal reflux disease. All patients had normal upper endoscopy. The study was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (EC 1774-02).

Study protocol

Patients were randomized to receive either sertraline 50

mg or placebo once daily for eight weeks. Randomization was performed by drawing a sealed envelope that contained a pre-assigned randomized treatment generated by computer on entry to the study. Both the investigators and patients were blinded to the assigned treatment throughout the study. The sertraline and placebo capsules were identical in appearance. Patients were given a diary in which they recorded side effects and symptoms during therapy. After enrolment by gastroenterologists, patients returned for follow up at four and eight weeks where one of two gastroenterologists assessed their symptoms and quality of life.

Dyspepsia symptoms were assessed by a locally validated dyspepsia questionnaire, the Hong Kong dyspepsia index (HKDI) which consisted of 12 questions (epigastric pain, upper abdominal bloating, upper abdominal dull ache, epigastric pain before meals, epigastric pain when anxious, vomiting, nausea, belching, acid regurgitation, heartburn, feeling of acidity in the stomach, loss of appetite) graded on a five point Likert scale as follows: 1 (none), no symptoms; 2 (mild), symptoms can be easily ignored; 3 (moderate), awareness of symptoms but easily tolerated; 4 (severe), symptoms sufficient to cause interference with normal activities; and 5 (incapacitating), incapacitating symptoms with an inability to perform daily activities and/or require days off work. Test-retest reproducibility and internal consistency were good, with an intra-class correlation coefficient of 0.89 and Cronbach's alpha coefficient of 0.90. A cut off score of ≥ 16 was discriminative between controls and dyspeptic patients. Moreover, the HKDI score was significantly correlated to patients who reported a subjective improvement in symptoms and those who reported no change or worsening after therapeutic intervention (Kendall's $\tau = 0.21$, $P = 0.02$)^[27]. Patients were then sub-classified into four dyspepsia subgroups according to their predominant symptoms: (1) ulcer-like dyspepsia-predominant epigastric pain; (2) dysmotility-like dyspepsia-predominant discomfort that may be characterised by upper abdominal fullness, early satiety, bloating, or nausea; (3) reflux-like dyspepsia-predominant reflux symptoms (heartburn or acid regurgitation); and (4) unspecified-symptoms do not fulfill the criteria for ulcer-like, dysmotility-like, or reflux-like dyspepsia. Although reflux-like dyspepsia was discarded in the Rome II criteria, we felt that a certain proportion of patients with FD still belong to that particular subgroup and there is considerable overlap between FD and non-erosive or negative endoscopy reflux disease^[28,29]. Furthermore, inclusion of reflux-like dyspepsia allows comparison with previous randomized controlled trials^[30].

Quality of life was assessed by a locally validated questionnaire [Chinese translated form of 36-item short-form (SF-36)]^[31]. The SF-36 consisted of 36 items to measure eight aspects of psychological general well being (physical functioning, role physical, bodily pain, general health, vitality, social functioning, role emotional, and mental health). A generic quality of life instrument was utilized to assess general well being as at the commence-

ment of this study, there were no dyspepsia specific quality of life questionnaires validated in the Chinese language. The symptoms pertaining to anxiety and depression were assessed by the hospital anxiety and depression (HAD) scale questionnaire which consists of 14 questions. Finally, subjective global symptom relief was graded by patients, from a scale of 1 to 5, representing the spectrum from complete resolution of symptoms to worsening of symptoms, respectively.

Study intervention: Sertraline

The SSRI utilized in this study was sertraline (Zoloft, Pfizer Corporation) at a dose of 50 mg orally daily. Study participants were provided with pre-sealed boxes containing either sertraline or placebo and were asked to take a capsule per day for 8 wk in total. Patients were provided with a 4 wk supply of capsules and were contacted weekly to ensure compliance with treatment. Patient compliance was checked by counting returned study medications. Subjects who took less than 75% of the study medication were excluded from the final per protocol (PP) analysis.

Statistical analysis

Mean dyspepsia score, the eight aspects of the SF-36 scores and the two HAD scores before and after treatment were collected on Excel (Microsoft) databases in the two treatment groups. The change in mean HKDI, SF-36 and HAD scores from baseline, at the four and eight week visit were calculated and compared between the sertraline and the placebo groups. Patient diaries, detailing the presence and severity of symptoms, were also compared between groups at weeks 4 and 8. The primary end point was defined as an improvement in clinical symptoms at week 8. Secondary endpoints included an improvement or resolution of the clinical symptoms, or an improvement in any of the scales including HKDI, SF-36 or HAD at week 8. Continuous variables were expressed as mean \pm SD, and categorical data expressed as percentages. Continuous variables were compared using Student's t tests. Categorical variables were compared using Fisher exact or χ^2 tests as appropriate. The Mann-Whitney test was used for data with a skewed distribution. The intention to treat (ITT) analysis included all patients who had taken at least one tablet. In the PP analysis, patients with poor drug compliance ($< 75\%$ intake of any study drugs) and drop outs (due to adverse effects) were excluded. Multiple logistic regression analysis was performed to determine independent factors (age, sex, *H. pylori* status, smoking, alcohol intake, dyspepsia duration, predominant symptoms, and type of treatment given) associated with treatment response.

All calculated P values were two-sided and P values < 0.05 were considered statistically significant. Statistical analysis was performed using SPSS Ver. 16.0 for Windows (SPSS Inc., Chicago, IL, United States).

Power of the study

This was a pilot study, so assuming a placebo response

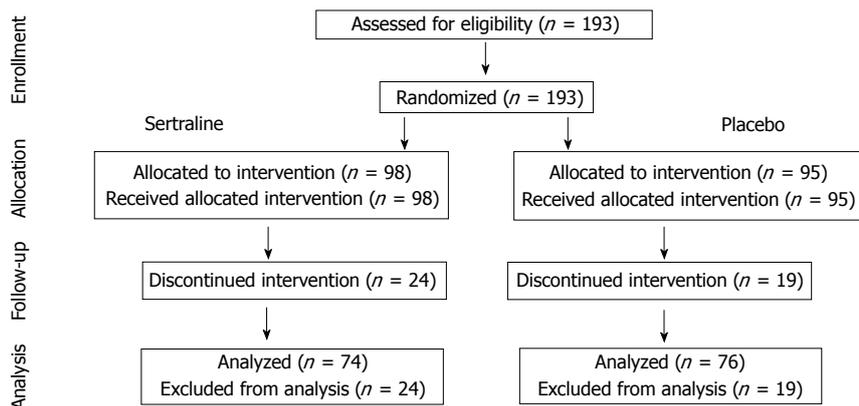


Figure 1 Study patient flow chart.

Table 1 Demographics of study patients

	Sertraline	Placebo	P value
Number of patients	98	95	
Age (yr)	43.0	41.6	0.515
Sex (male)	27	27	1.000
Current smokers (%)	3.2	7.3	0.122
Alcohol (%)	6.2	8.3	0.295
<i>H. pylori</i> positive (%)	8.4	7.3	0.843
NSAID use (%)	3.1	2.6	1.000
Predominant symptom <i>n</i> (%)			
Ulcer like	17 (44.7)	21 (55.3)	
Dysmotility like	60 (49.2)	62 (51.8)	
Reflux like	8 (57.1)	6 (42.9)	
Non-specific	13 (68.4)	6 (31.6)	

H. pylori: *Helicobacter pylori*; NSAIDs: Non-steroidal anti-inflammatory drugs.

rate of 25%-30%, a drug response rate of 30 % above placebo and a drop out rate of 20%^[11], 166 patients will be needed to demonstrate 95% confidence interval with power of 0.8 and alpha of 0.05 (i.e., 166 patients with dyspepsia with 83 patients in each arm). It was aimed to recruit 190 patients.

RESULTS

Baseline demographics

We recruited 193 eligible patients (patients for the ITT analysis). A total of 98 patients were randomized to receive sertraline 50 mg and 95 patients were randomized to receive placebo (Figure 1).

All recruited patients were ethnic Chinese. Baseline characteristics of the patients in the two treatment groups are given in Table 1. 75.5% and 80% of patients took more than 75% of the medications in the sertraline and placebo groups, respectively. Poor compliance patients, those who refused follow up, and those who discontinued treatment because of adverse events were excluded from the PP analysis (*n* = 150).

Baseline characteristics of the patients and their dyspepsia subtypes are listed in Table 1. Mean age of these patients was 42.4 years (range: 18-71 years) with a median dyspepsia score of 26.5 (range: 17-46). There were 54 males (mean age 45.2 years) and 139 females (mean age

41.4 years). Mean age, sex distribution, smoking history, alcohol consumption and *H pylori* positivity at baseline were similar between the treatment groups (Table 1). Baseline mean HKDI score, SF-36 and HAD scales in the PP analysis (Table 2) assessments were similar between the treatment groups.

Dyspepsia scores

In the PP analysis at week 8, 28.4% *vs* 27.6%, of patients experienced complete resolution of their dyspepsia symptoms, whilst 64.9% *vs* 59.2% of patients experienced no difference in their dyspepsia symptoms in the sertraline and placebo groups (*P* = 0.511 for difference between the two cohorts) respectively. Sub group analysis for complete response at weeks 4 and 8 was also unrevealing. Complete response was similar between the treatment groups at weeks 4 and 8.

In the PP analysis the baseline mean HKDI score was 25.83 (SD = 6.313) and 27.19 (SD = 5.929) for sertraline and placebo arms respectively (*P* = 0.233). Mean HKDI score improved in all groups at week 4 compared to baseline. HKDI score in the sertraline group improved the most but was not statistically significant. By week 8, the sertraline group had a mean HKDI score of 20.53 (SD = 6.917), whilst the placebo group's mean dyspepsia score was 23.34 (SD = 7.199), (*P* = 0.02). The change in HKDI between week 0 to 8 was 5.3 and 3.85 in the treatment and placebo groups respectively (*P* < 0.001 for both sertraline and placebo groups). There were no consistent significant differences in the parameters of the quality of life assessment and the HAD scale at week 8 (Table 2).

For the ITT analysis, where the method utilized was the last response carried forward, the mean HKDI at week 0 was 26.08 and 26.70 (SD = 6.19 and 5.89, *P* = 0.433) for sertraline and placebo cohorts respectively. Although improvement of the HKDI was seen at weeks 4 and 8, the results were not statistically significant at (HKDI week 8 = 22.41 and 23.25, SD = 6.36 and 7.30 for sertraline and placebo respectively, *P* = 0.352). Again, there was no consistent significant differences in the parameters of quality of life assessment, HAD scale or complete responses at week (data not shown).

Table 2 Dyspepsia index, 36-item short-form score and hospital anxiety scale results

	Week 0	<i>P</i> value	Week 4	<i>P</i> value	Week 8	<i>P</i> value
Mean dyspepsia score						
Sertraline	25.83	0.124	22.59	0.740	20.53	0.02
Placebo	27.19		22.94		23.34	
SF36-physical functioning						
Sertraline	81.79	0.585	79.30	0.39	75.61	0.15
Placebo	83.11		81.63		80.39	
SF36-role physical						
Sertraline	57.91	0.30	52.62	0.40	52.70	0.22
Placebo	52.11		57.87		62.17	
SF36-bodily pain						
Sertraline	49.10	0.002	54.22	0.89	53.97	0.50
Placebo	41.06		53.78		51.63	
SF36-general health						
Sertraline	33.76		39.06		35.39	
Placebo	32.54	0.65	41.85	0.34	34.72	0.84
SF36-vitality						
Sertraline	47.69	0.57	47.91	0.73	49.05	0.68
Placebo	46.76		48.88		50.33	
SF36-social function						
Sertraline	67.75		72.38		68.41	
Placebo	67.68	0.98	72.19	0.96	69.74	0.73
SF36-role emotional						
Sertraline	51.82		53.49		52.70	
Placebo	50.75	0.85	54.19	0.90	51.32	0.85
SF36-mental health						
Sertraline	58.12		54.89		65.24	
Placebo	58.59	0.83	61.57	0.03	64.37	0.69
HAD scale						
Anxiety score						
Sertraline	14.27	0.52	13.58		14.29	
Placebo	13.88		13.66	0.90	13.68	0.41
HAD scale						
Depression score						
Sertraline	15.51	0.14	15.50	0.88	14.27	0.45
Placebo	14.84		15.56		13.70	

HAD: Hospital Anxiety and Depression Scale; SF36: 36-item short-form.

Adverse events

At week 8, a total of 43 patients (24 on sertraline and 19 on placebo) discontinued treatment. The main reasons for discontinuation of the study medication were drug side effect (41.2%), no reason given (41.9%) or other reason which included the development of conditions for which sertraline could interfere with prescribed treatment (7%) (Table 3). Of particular interest is the pattern of withdrawal from the study, 23 of 24 patients withdrawing from the sertraline group did so before week 4, representing 95.8% of all drop-outs from the sertraline group. By contrast, in the placebo group, approximately half of the patients withdrew prior to week 4 (57.9%), whilst the other half withdrew prior to week 8. Patients experiencing drug adverse effect were noted to have multiple symptoms including insomnia, constipation and agitation, however there was no significant difference in the rate of adverse effects experienced by the two cohorts. Nine percent of all study patients withdrew from the study without explanation (were lost to follow up).

Table 3 Default patient profile *n* (%)

	Default week 4	Default week 8	Reason for default week 8		
			No reason given ¹	Adverse effect of drug ¹	Other ¹
Sertraline	23	24	7 (16.3)	14 (32.6)	3 (7)
Placebo	11	19	11 (25.6)	8 (18.6)	0 (0)

¹Represents percentage of all default patients; *P* = 0.259 for drug adverse effect between sertraline and placebo groups.

Factors associated with response

Age, sex, *H. pylori* status, smoking, alcohol consumption, and dyspepsia duration were not associated with response to sertraline. Multiple logistic regression analysis did not identify any independent predictors of favorable outcome.

A *post hoc* analysis comparing reflux like dyspepsia *vs* all other types of dyspepsia showed similar results to the PP analysis, in the non reflux like group the HKDI was 25.70 and 27.00 at week 0, whilst at week 8, HKDI was 20.85 and 23.42 respectively (*P* = 0.004). Similarly, the SF 36, HAD scales and complete response rates did not show any statistically significant differences (data not shown). In the reflux like group where *n* = 14, HKDI was 28.88 and 28.00 at week 0, and at week 8, HKDI was 21.00 and 23.80 (*P* = 0.426).

DISCUSSION

We have reported a double-blind, randomized, placebo-controlled pilot study of sertraline 50 mg *vs* placebo for the treatment of FD. We found that there was a statistically significant improvement in the mean HKID score at week 8 in the sertraline group compared to the placebo group in the PP but not the ITT analysis. There were also no differences in measures of quality of life, depression and anxiety and subjective global symptom resolution.

This study is a pilot study examining the effect on sertraline in patients with FD. The sertraline dose that was administered is a clinically relevant dose (the initial treatment dose for depression and obsessive-compulsive disorder and in some studies, depression)^[32]. The trial duration of 8 wk seems adequate given that a steady-state plasma sertraline level is expected after 1 wk with once daily dosing and the beneficial effects of antidepressants in functional gastrointestinal disorders are often observed after shorter treatment duration than in depression^[33]. However, longer term follow-up may yield more significant results given that individual responses to sertraline can vary and up to 12 wk may be required to see the full onset of action. Furthermore, although the dosage of sertraline utilized was appropriate for the reasons cited above, several studies have indicated that due to ethnic differences, Chinese patients may tolerate lower doses better^[33,34].

One of the limitations of the study is the drop out rate, 17.6% at week 4 and 22.3% at week 8. Our drop

out rates are similar to those observed when antidepressants are utilized in functional gastrointestinal disorders^[35]. There are many factors which could account for the drop out rate in our study. Sertraline's known side effects include sleep disturbance, headache, tremors, agitation and gastrointestinal upset^[24]. In our study, adverse drug effect was the cause of study withdrawal in 41.2% of cases. Interestingly, of the patients who dropped out, 41.9% withdrew from the study without giving a reason. Multiple studies have indicated a cultural bias in the Chinese population against a diagnosis of psychiatric or functional disorders^[36-38] and the authors hypothesize that the cultural stigma attached to treatment for a functional disorder with a known anti-depressant would contribute significantly to the drop out rate. Furthermore, the majority of the drop outs in the sertraline group occurred at week four, which could possibly be explained by the short term side effects of sertraline usually seen during the run in period of SSRIs^[39] which could be mitigated by more intensive support and education during the first few weeks of treatment.

Another limitation of the study is the failure to discern a difference in the generic quality of life measures utilized between the sertraline and placebo cohorts. This may ostensibly be a reflection of the fact that generic quality of life instruments are not sensitive enough to detect changes in overall well being in patients with FD particularly with treatment^[40]. This has been seen with other gastrointestinal disorders and has resulted in the development of disease specific quality of life instruments^[41,42].

Finally, the most important limitation of the study is the failure to find a difference in global symptom scores in the ITT analysis, and only a small difference in the HKDI, but not global symptoms score in PP analysis. The authors believe this small finding suggests a possible benefit of sertraline in patients with FD, but perhaps this study was under-powered to detect this difference due to the unexpectedly high dropout rate, particularly in the first 4 wk when SSRI adverse effects are at their maximal, and for this reason warrants further larger studies utilizing sertraline to clarify the issue. We assert that our findings are important given that clinicians not uncommonly are utilizing SSRIs to treat FD despite the fact that to date, our study included, there is no strong justification for its use^[43].

In conclusion, our data suggest that an SSRI, sertraline was not superior to placebo for the management of FD in Chinese patients. Further studies are warranted to confirm these results as this study was likely under powered to determine an effect in the context of a higher than expected drop out rate and there is a suggestion that with more support, a longer follow up period, and perhaps a reduction in the dose of sertraline in Chinese patients an effect may be seen.

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COMMENTS

Background

Early studies with tricyclic antidepressants demonstrated efficacy in the treatment of functional dyspepsia yet there have been no double-blind, randomized, placebo-controlled trials examining the role of selective serotonin reuptake inhibitors (SSRIs) in this condition.

Research frontiers

SSRIs, the most commonly utilized antidepressant in clinical practice, may improve the symptoms of functional dyspepsia through modulation of visceral hypersensitivity. In this study, the authors examine the effects of sertraline, a SSRI, on global symptoms, a locally validated dyspepsia index, the 36-item short-form (SF-36) and the hospital anxiety and depression scale.

Innovations and breakthroughs

Tricyclic antidepressant medication has been shown to be efficacious in functional dyspepsia, however tricyclic antidepressant medications have significant side effects, prompting the study of the utility of newer antidepressants in this condition. Venlafaxine, a selective serotonin and noradrenaline reuptake inhibitor did not show any benefit in functional dyspepsia however an open label study of fluoxetine, a SSRI, found benefit. This is the first double-blind, randomized, placebo-controlled study examining an SSRI in the treatment of functional dyspepsia.

Applications

This study found that treatment with the SSRI, sertraline, improved the Hong Kong dyspepsia index score at week 8 compared to baseline but did not find overall improvement in global dyspepsia symptoms, SF-36 or the hospital anxiety and depression scale, possibly due to the higher than expected drop out rate in the sertraline group by week 4. Larger studies are warranted to further examine the effects of sertraline in functional dyspepsia.

Peer review

This is a nicely designed study showing that SSRI sertraline is of no benefit in functional dyspepsia. The authors acknowledge the main limitation of a negative study represented by the scarce numerosity and high drop out rate, based on the optimistic calculations adopted to evaluate sample size. A more realistic hypothesis will substantially raise the number of patients needed to be included.

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Transcatheter arterial chemoembolization for gastrointestinal stromal tumors with liver metastases

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Abstract

AIM: To evaluate the efficacy and safety of transcatheter arterial chemoembolization (TACE) for gastrointestinal stromal tumor (GIST) with liver metastases after the failure of tyrosine kinase inhibitors (TKIs).

METHODS: Patients with histologically confirmed CD117-positive GIST with liver metastases who were resistant and/or intolerant to prior imatinib and/or sunitinib and who received TACE for at least one treatment cycle or only best supportive care and TKI reintroduction were eligible for the study. The patients were divided into two groups: those in TACE group received TACE treatment containing 5-20 mL iodized oil and 40-80 mg doxorubicin hydrochloride and TKI reintroduction or best supportive care, those in control group only received TKI reintroduction or best supportive care. The primary end-point was overall survival

and the secondary end-points were, progression-free survival (PFS), response rates, and safety.

RESULTS: Sixty patients admitted between June 2008 and October 2011 were eligible for this study, including 22 in TACE group and 38 in control group. In the TACE group, 12 (54.5%) achieved liver partial response, 5 (22.7%) had stable disease, and 5 (22.7%) had liver progressive disease. Disease control rate of liver metastases was 77.3% in the TACE group and 39.5% in the control group. The median liver PFS in TACE group was 47.1 wk (95% CI: 23.9-70.3). The median PFS in TACE group was longer than in control group (30.0 wk, 95% CI: 20.1-39.9 vs 12.9 wk, 95% CI: 11.9-13.9) ($P = 0.0001$). The median overall survival in TACE group was also longer than in control group (68.5 wk, 95% CI: 57.4-79.6 vs 25.7 wk, 95% CI: 23.2-28.2) ($P = 0.0001$). TACE treatment significantly reduced the risk of death (hazard ratio: 0.109). Patients without extrahepatic metastases treated with TACE had significantly better prognosis. Most of the adverse events were of grade 1 or 2 and tolerable.

CONCLUSION: TACE is effective and well tolerated in GIST patients with liver metastases after TKI failure, and it may be an optional treatment for this disease.

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Key words: Gastrointestinal stromal tumor; Liver metastases; Transcatheter arterial chemoembolization; Tyrosine kinase inhibitor failure; Overall survival

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal (GI) tract and account for about 2% of gastrointestinal tract tumors^[1,2]. Tyrosine kinase inhibitors (TKIs) imatinib and sunitinib have demonstrated efficacy against GISTs, and are referred to as the first- and second-line therapeutic drugs^[3-5]. However, resistance to such kind of TKIs remains a substantial problem. Around 4%-5% patients showed evidence of primary resistance and nearly half of the patients will experience secondary resistance within two years^[4,6,7]. At present, there is still no standard treatment for metastatic GIST after imatinib and sunitinib failure. The National Comprehensive Cancer Network (NCCN) guideline (2010)^[8] recommended considering reintroduction of a TKI for palliation of symptoms in patients with GIST progression despite prior imatinib and sunitinib.

Liver is the most common site of metastasis from GISTs, with a reported incidence of 55%-72% in patients with recurrence, and metastatic liver disease is a major determinant of patient survival^[9,10]. Some studies^[11-14] have shown favorable results of transcatheter arterial chemoembolization (TACE) for GIST with liver metastases. However, there are few studies about the role of TACE in the treatment of GIST patients after TKIs failure, moreover, there is no control study comparing TACE with best supportive care (BSC) and/or TKI reintroduction. Herein we retrospectively analyzed the survival benefit of TACE, BSC and/or TKI reintroduction in the patients with liver metastatic GISTs treated in the Peking University Cancer Hospital when resistance and/or intolerance occurred to imatinib and/or sunitinib.

MATERIALS AND METHODS

Study design

It is an open, retrospective, controlled study to evaluate the efficacy and safety of TACE in Chinese GIST patients with liver metastases after TKI treatment failure. Patients with histologically confirmed CD117-positive GIST with liver metastases who were resistant and/or intolerant to prior imatinib and/or sunitinib and who received TACE for at least one treatment cycle or only BSC and TKI reintroduction were eligible for the study. Following a retrospective review of the medical records of the patients seen at our hospital between June 2008 and October 2011, a total of 60 patients were found to meet the study criteria. There were 22 in TACE treatment group and 38 in BSC/TKI reintroduction group, which served as control group.

Patient characteristics: The following demographic

and clinicopathological information was retrospectively obtained from the patient records: gender, age, extent of liver disease, and extrahepatic metastases.

Treatment: Data of TKI reintroduction and TACE treatment, including dose of TKI, interval between TKI and TACE, TACE procedure, and cycles of TACE, were collected.

Follow-up: Overall survival (OS) and progression-free survival (PFS) were acquired.

Study end-points

The primary end-point was OS and the secondary end-points were PFS, disease control rate (DCR) of liver metastases defined as a combination of complete response + partial response (PR) + stable disease (SD), and safety. Response rate was evaluated every 6 wk. OS was defined as the time from the first TACE or BSC/TKI reintroduction to the occurrence of death from any cause. The PFS was defined as the time from the first time of TACE or BSC/TKI reintroduction to the occurrence of disease progression or death from any cause. Disease control rate was assessed by Response Evaluation Criteria in Solid Tumors (RECIST 1.1). Adverse events were graded according to the National Cancer Institute Common Toxicity Criteria version 3.0.

Statistical analysis

All the statistical analyses were performed using the SPSS 15.0 (SPSS Inc., Chicago, IL, United States). PFS and OS curves were constructed by the Kaplan-Meier method and compared with log-rank test. In order to adjust for confounding variables, we used Cox proportional hazards models to estimate the simultaneous effects of prognostic factors on survival. Frequency and percentage descriptions were used for categorical variables and the χ^2 test was used to compare the incidence of different events. If the theoretical frequency was lower than 1, *F* test was conducted. Continuous variables were expressed as mean \pm SD and mean differences between two groups were compared using Student's *t* test.

RESULTS

Patient characteristics

There were 45 males and 15 females with a median age of 55.0 years (95% CI: 51.8-58.2). All the patients at registration were assessed to have the Eastern Cooperative Oncology Group (ECOG) performance status grade 0-2 and had received imatinib treatment. Among them, 35 took sunitinib after imatinib failure prior to TACE or BSC/TKI introduction treatment. Thirty-four (56.7%) had liver metastases and the others had extrahepatic metastases. Clinical features of the patients in the two groups are shown in Table 1.

In TACE group, 15 (68.2%) had an extent of liver involvement within 50%, 6 (27.3%) within 50%-70%,

Table 1 Clinicopathologic features of the patients

Clinicopathologic features	TACE n = 22 (%)	Control n = 38 (%)	Statistical test	P value
Sex			$\chi^2 = 0.310$	0.578
Male	16 (72.7)	29 (76.3)		
Female	6 (27.3)	9 (23.7)		
Age (yr)	53.0 (49.3-59.6)	55.0 (48.0-62.0)	$U = 5.000$	0.279
ECOG PS			$\chi^2 = 2.344$	0.126
0-1	16 (72.7)	20 (52.6)		
2	6 (27.3)	18 (47.4)		
Primary location			$\chi^2 = 0.012$	0.994
Stomach	9 (40.9)	15 (39.5)		
Small intestinal	9 (40.9)	16 (42.1)		
Other	4 (18.2)	7 (18.4)		
Number of liver lesions			$\chi^2 = 1.805$	0.406
1	8 (36.4)	8 (21.1)		
2-5	9 (40.9)	20 (52.6)		
> 5	5 (22.7)	10 (26.3)		
Extrahepatic metastases			$\chi^2 = 0.083$	0.773
Yes	9 (40.9)	17 (44.7)		
No	13 (59.1)	21 (55.3)		
Sunitinib second-line therapy before TACE			$\chi^2 = 0.992$	0.319
Yes	11 (50.0)	24 (63.2)		
No	11 (50.0)	14 (36.8)		
TKI reintroduction			$\chi^2 = 1.778$	0.182
Yes	10 (45.5)	24 (63.2)		
No	12 (54.5)	14 (36.8)		

TACE: Transcatheter arterial chemoembolization; ECOG PS: The Eastern Cooperative Oncology Group performance status; TKI: Tyrosine kinase inhibitors.

and 1 more than 70%. Eight patients (36.4%) had only 1 liver metastasis, 9 (40.9%) had 2-5 liver metastases, and the others had more than 5. The mean TACE treatment cycles received by all the patients in TACE group was 2.64, with 6 (27.3%) receiving only one TACE, and 16 (72.7%) received more than one TACE. Fifteen patients (68.2%) showed a good blood supply of liver metastases.

Treatment in TACE group

TACE protocol: Eligibility criteria for TACE included well-preserved hepatic and renal function, the Child-Pugh classification within A and B, adequate hematologic function, and ECOG performance status of 0-2. Patients with high-risk factors, such as portal vein occlusion, no hepatopetal flow, massive ascites, encephalopathy, or active cardiac failure, were excluded.

Local anesthesia was obtained with 1% lidocaine. After the introduction of a selective catheter through the femoral artery using the Seldinger technique, the localization of the hepatic arteries was checked with celiac and mesenteric arteriography. This was performed to define vascular anatomy. Next, indirect portography was performed to outline the portal circulation in the venous phase. A 5 French catheter was placed in the celiac trunk to identify the hepatic artery. Depending on the size, loca-

tion, and arterial supply to the tumor, a micro-catheter was advanced further into the segmental feeding arteries to perform embolization. An emulsion containing 5-20 mL iodized oil and 40-80 mg doxorubicin hydrochloride was used according to the tumor size. Additional embolization was performed using 1-2 mm diameter gelled sponge particles according to the status of blood supply. The ideal embolization end-point is the stasis of flow in tumor-feeding branches. Follow-up abdominal imaging (computed tomography) was generally performed two months after the first embolization. The follow-up images were assessed by two radiologists (Cao K and Cui Y) and compared with the baseline images to assess response.

TKI reintroduction: Ten patients received TKI reintroduction during the intermittent period of TACE. Among them, 6 patients took imatinib 400 mg/d and 4 patients took sunitinib 37.5 mg/d. The interval between TKI therapy and TACE was 2 wk.

Treatment in control group

All the patients in control group had GIST resistant to imatinib and 35 had tumor resistant to sunitinib. Among them, 9 patients received imatinib 400 mg/d and 15 received sunitinib 37.5 mg/d reintroduction, and the others only received best supportive care. Efficacy was evaluated every 6-8 wk according to the RECIST criteria.

Response rate: All the patients had measurable metastatic disease according to the RECIST criteria and tumor assessment was performed at least once. In TACE group, 12 (54.5%) achieved liver PR, 5 (22.7%) had SD, and 5 (22.7%) showed liver disease progression (PD) after TACE treatment. The DCR of liver metastases was 77.3%. In addition, 8 patients had PD when all the lesions were evaluated, and the DCR of all the lesions was 63.6%. In the control group, 12 patients receiving TKI reintroduction and 3 patients receiving BSC had SD, and the others had PD. The DCR in the control group was 39.5%.

PFS: As of May 2012, 19 (86.0%) patients in TACE group had liver metastasis progression. The median liver PFS of all 22 patients was 47.1 wk (95% CI: 23.9-70.3). In control group, all the patients had tumor progression. The median PFS in TACE group was longer than in control group (30.0 wk, 95% CI: 20.1-39.9 *vs* 12.9 wk, 95% CI: 11.9-13.9) ($P = 0.0001$, Figure 1A).

OS: As of May 2012, 4 patients in TACE group and 2 patients in control group were alive, and deaths occurred because of tumor progression. The median OS in TACE group was longer than in control group (68.5 wk 95% CI: 57.4-79.6 *vs* 25.7 wk 95% CI: 23.2-28.2) ($P = 0.0001$, Figure 1B). TACE significantly reduced the risk of death in GIST patients with liver metastases according to the Cox proportional hazards regression model [hazard ratio (HR): 0.109; 95% CI: 0.044-0.271].

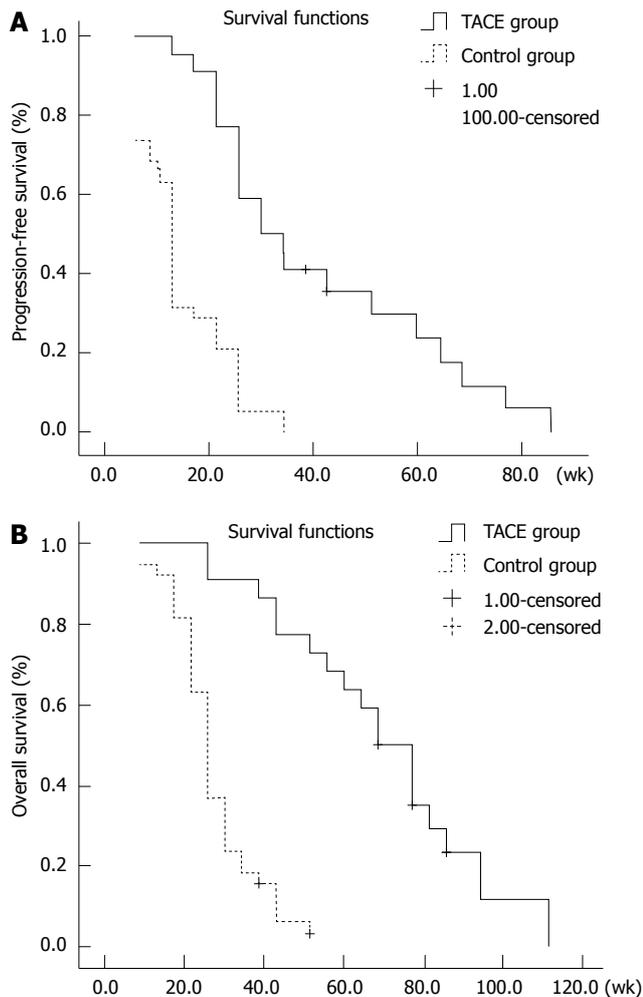


Figure 1 The median progression-free survival (A) and overall survival (B) were longer in transcatheter arterial chemoembolization group than in control group ($P = 0.0001$). TACE: Transcatheter arterial chemoembolization.

Univariate and multivariate analysis

Results of the univariate and multivariate analysis are summarized in Table 2. The results showed the P value of number of liver metastases is 0.086. The patients without extrahepatic metastases and the patients treated with TACE were the two factors significantly associated with good survival. The two factors led to a reduction of death risk by 53.7% (HR: 0.463, $P = 0.007$) and 58.5% (HR: 0.415, $P = 0.005$), respectively.

In TACE group, univariate and multivariate analysis showed that absence of extrahepatic metastases, more than one session of TACE, and DCR of more than 3 mo after TACE were significantly associated with a good survival ($P = 0.006$, $P = 0.02$, $P = 0.012$).

Adverse events

Most patients in TACE group developed post-embolization complications, which included abnormal liver function, abdominal pain, fever and nausea. The incidence of fever, alanine aminotransferase increase and nausea in TACE group was higher than in control group ($P < 0.05$). However, the majority of adverse events were of grade

Table 2 Univariate analysis by each variable

Variable	<i>n</i>	OS (wk)	<i>P</i> value
Gender			0.133
Male	45	34.3	
Female	15	25.7	
Primary tumor location			0.825
Stomach	24	25.7	
Intestine	25	34.3	
Others	11	30.0	
ECOG PS			0.102
0-1	36	35.8	
2	24	24.5	
Number of liver metastases			0.079
1	16	42.9	
2-5	29	25.7	
> 5	15	38.6	
Extrahepatic metastases			0.005
Yes	26	25.7	
No	34	42.9	
TKI reintroduction			0.657
Yes	34	30.0	
No	26	30.0	
TACE treatment			0.0001
Yes	22	68.5	
No	38	25.7	

OS: Overall survival; ECOG PS: The Eastern Cooperative Oncology Group performance status; TKI: Tyrosine kinase inhibitors; TACE: Transcatheter arterial chemoembolization.

1-2, and in most cases, these symptoms were effectively resolved with supportive measures. No patient died within 1 mo after TACE. Other adverse events included anemia, neutropenia, thrombocytopenia, ascites, pleural effusion and hemorrhage (Table 3). No one discontinued treatment because of adverse events.

DISCUSSION

There is still no standard treatment for the GIST patients after imatinib and sunitinib failure. TKI reintroduction, BSC or drugs in clinical trial are recommended for these patients. Some studies^[15-19] reported that the novel TKIs had potential activity against metastatic GIST, but the efficacy remains to be validated in prospective randomized controlled trials. Liver is the most common metastatic site of GIST and some patients even have only liver metastases other than other diseases till death. Resection of liver metastases has improved the overall survival^[20-22], again the efficacy of resection should be further confirmed by prospective clinical trials. Some retrospective studies^[11-14] showed that TACE may be potentially effective for GIST resistant to TKI. In this study, the patients with liver metastatic GIST receiving TACE after imatinib and/or sunitinib failure gained better PFS and OS than the patients receiving TKI reintroduction or BSC. In the sunitinib phase III trial^[23], the median time to progression of the patients receiving placebo was only 6.4 wk. The results demonstrated that TACE may benefit the patients with liver metastases.

In TACE group, 68.2% patients had good blood

Table 3 Adverse events in the two groups

Adverse events	All grades (%)			Grade 3-4 (%)		
	TACE group (n = 22)	Control group (n = 38)	P value	TACE group (n = 22)	Control group (n = 38)	P value
Fever	20 (90.9)	5 (13.2)	0.0001	2 (9.1)	0 (0)	0.061
Fatigue	16 (72.7)	28 (73.7)	0.936	5 (22.7)	8 (21.1)	NA
Abnormal ALT	16 (72.7)	6 (15.8)	0.0001	5 (22.7)	0 (0)	0.005
Nausea	14 (63.6)	14 (36.8)	0.045	1 (4.5)	0 (0)	0.367
Ascites	5 (22.7)	10 (26.3)	0.757	0 (0)	0 (0)	NA
Diarhoea	4 (18.2)	5 (13.2)	0.712	0 (0)	0 (0)	NA
Hemorrhage	3 (8.3)	4 (10.5)	0.700	1 (4.5)	2 (5.3)	1.000
Neutropenia	12 (54.5)	16 (42.1)	0.352	3 (12.6)	3 (7.9)	0.659
Anemia	7 (31.8)	24 (63.2)	0.019	3 (12.6)	6 (15.8)	1.000
Thrombocytopenia	7 (31.8)	10 (26.3)	0.649	2 (10.5)	1 (2.6)	0.548

TACE: Transcatheter arterial chemoembolization; ALT: Alanine aminotransferase; NA: Not applicable.

supply of liver metastases. Some suspensions such as iodized oil (lipiodol) can occlude small tumor vessels and cause obstruction in the vascular bed of liver metastases. Unresectable or metastatic GIST resists the conventional cyto-toxic chemotherapy^[9,24,25], so cyto-toxic drugs are not recommended in TACE. Further to a earlier report^[24] which showed that doxorubicin had slight efficacy in metastatic GIST, it has been reported recently that the chemo-embolization with doxorubicin elusion with the iodized oil demonstrated a potential efficacy^[11-14]. Lipiodol and microspheres concentrate and prolong the retention of the chemotherapeutic agent (doxorubicin) in the tumor^[26].

The results of this study showed that TACE significantly reduced death risk by 89.1%. In the subgroup analysis, DCR of more than 3 mo after TACE was correlated to good survival, indicating the benefit of TACE with regard to the overall survival. In the univariate and multivariate analysis, absence of extrahepatic metastases and TACE treatment were the independent prognostic factors. The similar results were seen in subgroup analysis in TACE group. These results showed that the patients without extrahepatic metastases can enjoy a longer survival after TACE. At the same time, a single session of TACE may not be adequate enough to control liver metastases. The results were consistent to the earlier report^[11]. However, bias of the patient selection may exist in this retrospective study. More prospective trials are expected to confirm the efficacy of TACE in this group of patients. In this study, all cases enrolled had advanced GIST with relatively larger liver lesions after the TKI failure. TACE still yielded a good control rate in this group of patients. Whether TACE procedure should be recommended earlier even before TKI failure warrants future studies.

NCCN guideline recommended considering reintroduction of a TKI for palliation of symptoms in patients with GIST progression despite prior imatinib and sunitinib. Does TKI reintroduction combining with TACE improve PFS and OS of GIST with liver metastases, especially for the patients with extrahepatic metastases? In this study it seemed that the patients receiving combined TACE and TKI reintroduction had longer overall sur-

vival than those receiving TACE alone, but there was no statistical significance ($P = 0.638$). This may be attributed to the small case number in this study. However, TKI reintroduction did not increase the incidence of complication during TACE treatment. The interval time of 2 wk between TKI and TACE is appropriate. For the patients without extrahepatic metastases, the combined TACE and TKI reintroduction may be an optional method of treatment.

Many patients in TACE group suffered from post-embolization complications, such as abdominal pain, fever and nausea. Most of them were of grade 1 or 2, and 22.7% patients were of grade 3. But all the adverse events were ameliorated within 1-2 wk with supportive measures. No adverse events out of expectation happened and no one discontinued treatment because of severe adverse events. The combined TACE and TKI reintroduction was well tolerated in the majority of the patients.

In summary, TACE may be an optional treatment for GIST with liver metastases after TKI failure. TACE can better benefit the patients without extrahepatic metastases.

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COMMENTS

Background

There is still no standard treatment for metastatic gastrointestinal stromal tumor (GIST) after imatinib and sunitinib failure. Liver is the most common site of metastasis from GISTs and liver metastasis is one of the major causes of death in these patients. The authors evaluated the efficacy and safety of transcatheter arterial chemoembolization (TACE) in GIST with liver metastases after the failure of tyrosine kinase inhibitors.

Research frontiers

There are few studies about the role of TACE in the treatment of GIST patients after tyrosine kinase inhibitors (TKIs) failure, moreover, there is no control study comparing TACE with best supportive care (BSC) and/or TKI reintroduction.

Innovations and breakthroughs

This study is the first controlled report to evaluate the therapeutic effect of TACE combining with BSC and/or TKI reintroduction in GISTs with liver metastases after TKI failure. The results of the paper showed that TACE improved progression-free survival and overall survival of GIST patients with liver metastases after TKI failure as compared with those receiving only BSC and or TKI reintroduction.

Applications

This study provided some evidences that TACE may be an optional treatment for GIST with liver metastases after TKI failure. TACE can better benefit the patients without extrahepatic metastases.

Terminology

GIST is the most common mesenchymal tumor of the gastrointestinal tract and TKI is the standard treatment for metastases GIST. TACE is the abbreviation of transcatheter arterial chemoembolization. TACE is the use of vascular embolizing material combined with cytotoxic drugs to induce tumor ischemic necrosis and prolonged drug transit time, and often used in treatment of hepatocellular carcinoma.

Peer review

This is a nice review of a unique series of patients with metastatic GIST. The authors should specify what the chemotherapy in the TACE procedure actually is. It appears only to be lipid.

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Liver-protecting effects of omega-3 fish oil lipid emulsion in liver transplantation

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Abstract

AIM: To investigate the liver-protecting effect of parenteral nutrition (PN) support with omega-3 fatty acids in a randomized controlled clinical trial.

METHODS: Sixty-six patients with the diagnosis of end-stage liver disease or hepatic cellular carcinoma were admitted to the Affiliated Drum Tower Hospital, Nanjing University, China for orthotopic liver transplantation. The patients were randomly divided into two groups: PN group ($n = 33$) and polyunsaturated fatty acid (PUFA) group ($n = 33$). All patients received isocaloric and isonitrogenous PN for seven days after surgery, and in PUFA group omega-3 fish oil lipid emulsion replaced part of the standard lipid emulsion. Liver function was tested on days 2 and 9 after surgery. Pathological examination was performed after reperfu-

sion of the donor liver and on day 9. Clinical outcome was assessed based on the post-transplant investigations, including: (1) post-transplant mechanical ventilation; (2) total hospital stay; (3) infectious morbidities; (4) acute and chronic rejection; and (5) mortality (intensive care unit mortality, hospital mortality, 28-d mortality, and survival at a one-year post-transplant surveillance period).

RESULTS: On days 2 and 9 after operation, a significant decrease of alanine aminotransferase ($299.16 \text{ U/L} \pm 189.17 \text{ U/L}$ vs $246.16 \text{ U/L} \pm 175.21 \text{ U/L}$, $P = 0.024$) and prothrombin time ($5.64 \text{ s} \pm 2.06 \text{ s}$ vs $2.54 \text{ s} \pm 1.15 \text{ s}$, $P = 0.035$) was seen in PUFA group compared with PN group. The pathological results showed that omega-3 fatty acid supplement improved the injury of hepatic cells. Compared with PN group, there was a significant decrease of post-transplant hospital stay in PUFA group ($18.7 \text{ d} \pm 4.0 \text{ d}$ vs $20.6 \text{ d} \pm 4.6 \text{ d}$, $P = 0.041$). Complications of infection occurred in 6 cases of PN group (2 cases of pneumonia, 3 cases of intra-abdominal abscess and 1 case of urinary tract infection), and in 3 cases of PUFA group (2 cases of pneumonia and 1 case of intra-abdominal abscess). No acute or chronic rejection and hospital mortality were found in both groups. The one-year mortality in PN group was 9.1% (3/33), one died of pulmonary infection, one died of severe intra-hepatic cholangitis and hepatic dysfunction and the other died of hepatic cell carcinoma recurrence. Only one patient in PUFA group (1/33, 3.1%) died of biliary complication and hepatic dysfunction during follow-up.

CONCLUSION: Post-transplant parenteral nutritional support combined with omega-3 fatty acids can significantly improve the liver injury, reduce the infectious morbidities, and shorten the post-transplant hospital stay.

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Key words: Fish oil lipid; Liver; Transplantation; Paren-

teral nutrition; Metabolism

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INTRODUCTION

Liver transplantation has dramatically improved the prognosis of end-stage liver disease. The progress made in the immunosuppressive regimens and surgical techniques has yielded a better outcome of the patients, and the 5-year survival after liver transplantation is 70%-80%^[1]. The liver recipients with liver insufficiency are in fact known to experience a higher incidence of severe protein/calorie malnutrition, which is associated with a greater risk of postoperative complications and mortality in patients undergoing liver transplantation^[2,3]. And ischemia/reperfusion (I/R) injury associated with liver transplantation often leads to hepatic dysfunction despite the improvement in surgical techniques and perioperative medication. I/R injury of the liver is an event involving multiple factors, such as hypoxia during inflow occlusion of the liver and inflammatory reactions after reperfusion^[4,5], and the mechanisms of the reperfusion injury, including the release of inflammatory cytokines, the generation of oxygen free radicals, Kupffer cell activation and leukocyte-endothelial cell interaction^[6,7]. Based on the pathophysiology of hepatic I/R injury, the current study particularly focused on various perioperative approaches to protect the liver from these inflammatory reactions and microcirculatory disturbances.

Omega-3 (N-3) fatty acids, which are derived from fish oil, are essential polyunsaturated fatty acids (PUFAs) for humans. Omega-3 fatty acids exert anti-inflammatory and immunomodulatory properties through their ability to modulate the synthesis of different eicosanoids^[8,9]. Perioperative administration of omega-3 fatty acids reduces plasma and tissue levels of the eicosanoids, specific leukotrienes, thromboxanes, and prostaglandins, all of which have pro-inflammatory effects^[10,11]. Recent studies described that supplementation with omega-3 fatty acids decreases the rate of inflammatory complications, the length of hospital stay, and the mortality after major abdominal surgeries^[12-14]. Their protective effects on hepatic I/R injury and inflammatory responses have been increasingly investigated.

In this study, we investigated the liver-protecting effects of parenteral nutrition (PN) supplemented with omega-3 fish oil lipid emulsion in patients undergoing liver transplantation.

MATERIALS AND METHODS

Ethics

This study was carried out in the Department of Hepatobiliary Surgery of the Affiliated Drum Tower Hospital, Medical School of Nanjing University, China according to the principles and guidelines of the Helsinki Declaration of 1975 revised in 2000. The protocol was approved by the Ethics Committee of the Affiliated Drum Tower Hospital. All patients fully understood the objective and adverse reactions of the study, and signed the written informed consent voluntarily prior to study enrollment.

Patients and randomization

From January 2006 to July 2010, we prospectively investigated 66 patients (45 men and 21 women; mean age, 51.6 years; range, 34-64 years) who underwent orthotopic liver transplantation in the Department of Hepatobiliary Surgery at the Affiliated Drum Tower Hospital of Medical School of Nanjing University. Recipients with manifest metabolic diseases (e.g., diabetes mellitus and hyperthyroidism) or severe renal abnormality were excluded. No acute rejection, primary transplanted liver dysfunction or second operation, which may affect the evaluation of liver function, were seen during the first 9 d after transplantation. The selection criteria for donors in this study included: (1) age < 50 years, matched ABO blood group and no history of chronic liver disease; (2) no evidence of malignant tumor, viral hepatitis or other viral infections; (3) no cirrhosis, mass or severe fatty degeneration of the donor liver seen during organ harvesting; and (4) liver biopsies of each donor liver taken before transplantation were reviewed by two pathologists. Donor livers with normal pathology or mild fatty change (10%-30%) were included in this study (Figure 1).

After transplantation, these patients were randomized into two groups based on the randomization chart generated by the Statistical Analysis System (SAS): (1) PN group (33 patients), without supplementation of omega-3 fatty acids in addition to routine treatment; and (2) PUFA group (33 patients), with PN supplemented with omega-3 fatty acids in addition to routine treatment.

This is a randomized controlled clinical study carried out in the Department of Hepatobiliary Surgery of our hospital. Nutrition Risk Screening 2002 (NRS 2002) scoring system was used, and the post-operative NRS 2002 score was ≥ 3 in all the patients, which meant that all the patients need nutritional support.

Treatment

The PN was given around the clock for seven days from the second day after operation. The two nutritional support groups were isonitrogenous and isocaloric. Nitrogen intake was 0.16 g/kg body weight per day, caloric intake was 104.5 kJ/kg per day, and lipid intake was 1.0 g/kg per day. The nonprotein calories were provided with dextrose (4.0 g/kg per day) and fat emulsion in a ratio of 2:1. The only source of lipids in PN group was

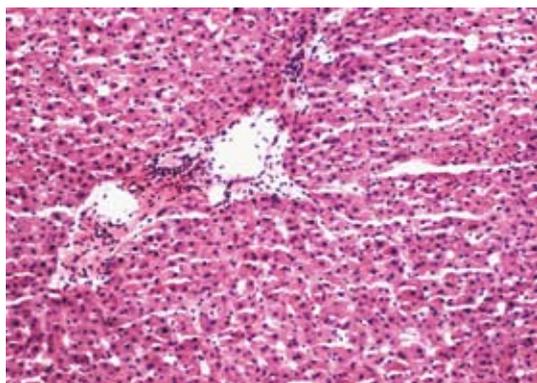


Figure 1 Pathology of liver biopsy of donor liver taken before transplantation. Mild steatosis was observed in donor liver. (Hematoxylin and eosin staining; paraffin-embedded 5 μ m thick sections; $\times 200$).

the standard lipid emulsion (20% emulsion, with a ratio of long-chain triglycerides to medium-chain triglycerides of 1:1, Huarui Pharmaceuticals, Jiangsu, China), and in PUFA group omega-3 fish oil lipid emulsion (Omegaven, 10%, 2 mL/kg per day, Fresenius Kabi Co., Austria) replaced part of the standard lipid emulsion. Both groups received 1.0 g amino acid/kg per day, and they were administered a commercially available branched-chain amino acid solution (Branched-chain amino acid solution 20%, Huarui Pharmaceuticals, Jiangsu, China). The ratio of nonprotein calories to nitrogen in both nutritional support groups was 653 kJ:1 g. The omega-3 fish oil lipid emulsion-containing solutions were prepared by the clinical pharmacist under aseptic condition and adjusted according to the weight of each individual patient. The amino acids, fat emulsion and dextrose mixture with electrolytes, vitamins, and trace elements were administered through a central venous catheter. As soon as the bowel function returned on days 3 or 4 after transplantation, all patients in the two groups were given liquid carbohydrate and cow's milk protein.

The surgical treatment was standardized, and modified piggyback orthotopic liver transplantation was performed by three groups of surgeons using the same approach. After operation, all the patients in the two groups were treated with the same antibiotics and antivirals, and 20 g of albumin was administered intravenously daily for five days to prevent any complications caused by hypoalbuminemia.

Assessment

Venous heparin blood samples were obtained on days 1 (the day before transplantation), 2 and 9 after surgery and liver function assessment was made. Serum total bilirubin (TB), direct bilirubin (DB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and prothrombin time (PT) were measured by an automatic biochemical analyzer (HITA-CHI 7600, Japan).

Liver biopsy with fine needle was conducted after reperfusion of donor liver and on day 9 after surgery, re-

spectively. Hepatic specimens for light microscopy were fixed with formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin for histological examination. Portal inflammation in the liver biopsy specimens was semiquantified by calculating inflammatory cells in portal tracts based on the Knodell histology activity index (HAI)^[15]. Portal inflammation was scored as 0, no portal inflammation; 1, mild (sprinkling of inflammatory cells in $< 1/3$ of portal tracts); 3, moderate (increased inflammatory cells in $1/3$ - $2/3$ of portal tracts); and 4, marked (dense packing of inflammatory cells in $> 2/3$ of portal tracts).

The assessment of clinical outcome was based on post-transplant investigations as shown by: (1) post-transplant mechanical ventilation; (2) total hospital stay; (3) infectious morbidities (pneumonia, intra-abdominal abscess, central line sepsis, wound infection, and urinary tract infection); (4) acute and chronic rejection; (5) mortality (intensive care unit mortality, hospital mortality, 28-d mortality, and survival at one year post-transplant surveillance period).

These post-transplant parameters were investigated and documented daily during the patients' post-transplant hospital stay and the period of one-year postoperative follow-up.

Statistical analysis

The results were expressed as mean \pm SD. Data were analyzed using the SAS. Differences between means were evaluated using Student *t* test when normal distribution was confirmed by Shapiro-Wilks test. When the hypothesis of normal distribution was rejected, differences between groups were tested by nonparametric statistics using Mann-Whitney test for unpaired samples and Wilcoxon criteria for paired samples. Fisher's exact test was used for analysis of categorical values when appropriate. A *P* value of < 0.05 was considered significant.

RESULTS

A total of 66 patients were enrolled in this study, including 33 patients in PN group and 33 patients in PUFA group. The mean age of the subjects was 51.6 years (range, 34-64 years). The clinical diagnosis of these patients included: hepatic cell carcinoma (27 cases), post-hepatitis B liver cirrhosis (35 cases), alcoholic liver cirrhosis (1 case), primary biliary liver cirrhosis (2 cases) and congenital polycystic liver (1 case). Demographic and clinical data (including age, sex, clinical diagnosis, Child-Pugh classification of hepatic function, warm ischemic time, cold ischemic time, operative time, anhepatic phase and post-operative immunosuppression) are summarized in Table 1. With respect to warm ischemic time, cold ischemic time, operative time, anhepatic phase, ratio of Child-Pugh classification, immunosuppression and clinical diagnosis, there were no significant differences between the two groups in any of these above param-

Table 1 Clinical data of the enrolled patients

	PN group	PUFA group
Sex (M/F)	23/10	22/11
Age, yr	48.62 ± 14.61	51.52 ± 12.41
Clinical diagnosis		
Hepatic cell carcinoma	13	14
Post-hepatitis B liver cirrhosis	17	18
Alcoholic liver cirrhosis	1	0
Primary biliary liver cirrhosis	1	1
Congenital polycystic liver	1	0
Child-Pugh classification (A/B/C)	14/10/9	13/10/10
Warm ischemic time (min)	3.91 ± 1.16	4.15 ± 1.32
Cold ischemic time (min)	524.28 ± 132.83	506.56 ± 151.26
Operation period (min)	651.27 ± 181.42	626.39 ± 192.86
Anhepatic phase (min)	119.81 ± 82.35	142.15 ± 58.75
Immunosuppressive therapy (FK506 + P/CSA + P/CSA + P + MMF)	22/11/0	21/11/1

PN: Parenteral nutrition; PUFA: Polyunsaturated fatty acid; P: Prednisone; CSA: Ciclosporin A; MMF: Mycophenolate mofetil; FK506: Tacrolimus.

eters ($P > 0.05$).

Liver function assessment

No significant difference of pre-operative liver function was observed between the two groups. On days 2 and 9 after operation, a significant decrease of ALT ($299.16 \text{ U/L} \pm 189.17 \text{ U/L}$ *vs* $246.16 \text{ U/L} \pm 175.21 \text{ U/L}$, $P = 0.024$) and PT ($5.64 \text{ s} \pm 2.06 \text{ s}$ *vs* $2.54 \text{ s} \pm 1.15 \text{ s}$, $P = 0.035$) was seen in PUFA group compared with PN group. And there was no significant decrease of the following parameters tested on days 2 and 9: AST ($116.31 \text{ U/L} \pm 42.19 \text{ U/L}$ *vs* $121.09 \text{ U/L} \pm 53.14 \text{ U/L}$, $P = 0.682$), TB ($93.93 \text{ } \mu\text{mol/L} \pm 45.49 \text{ } \mu\text{mol/L}$ *vs* $87.20 \text{ } \mu\text{mol/L} \pm 61.12 \text{ } \mu\text{mol/L}$, $P = 1.439$), DB ($42.74 \text{ } \mu\text{mol/L} \pm 17.36 \text{ } \mu\text{mol/L}$ *vs* $36.22 \text{ } \mu\text{mol/L} \pm 21.63 \text{ } \mu\text{mol/L}$, $P = 0.815$) and LDH ($156.12 \text{ U/L} \pm 89.20 \text{ U/L}$ *vs* $119.10 \text{ U/L} \pm 69.72 \text{ U/L}$, $P = 1.112$) in PUFA group compared with PN group (Table 2).

Light microscopy

The histological examination after reperfusion revealed some swelling hepatocytes and inflammatory cell infiltration in the portal areas, and no significant difference of numerical score of portal inflammation was observed between the two groups.

Histological examination on day 9 in PN group revealed more inflammatory cells aggregating in hepatic sinusoid lumen, extensive swelling and some balloon-like degeneration of hepatocytes, extensive congestion, and bilirubin deposit in the hepatic plasma (Figure 2A). These were ameliorated markedly by parenteral nutritional support with omega-3 fatty acids (Figure 2B), and the numerical score of portal inflammation was significantly lowered in PUFA group (Table 3). There was no sign of acute rejection in both groups.

Clinical outcome

There was no significant difference of post-transplant

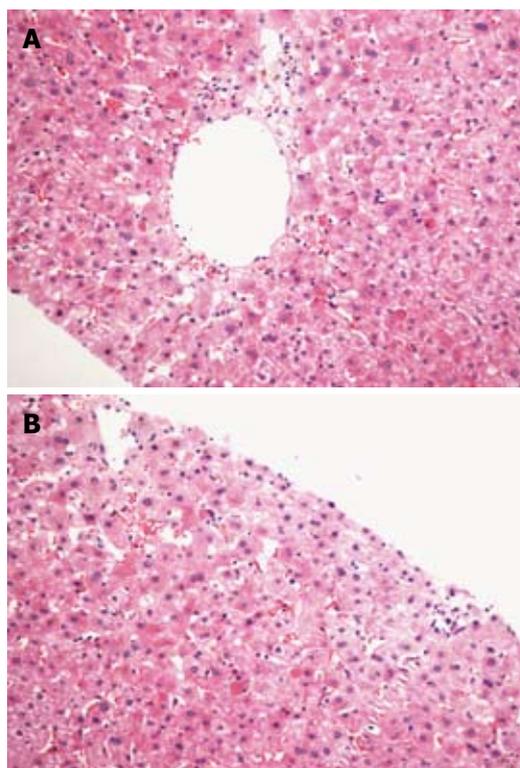


Figure 2 Histological appearance of the transplanted liver on day 9 after surgery. A: Extensive swelling, balloon-like degeneration and necrosis of hepatocytes, extensive congestion and bilirubin deposit were observed in the hepatic plasma in parenteral nutrition group; B: The results of histological examination were ameliorated markedly in the polyunsaturated fatty acid group. (Hematoxylin and eosin staining; paraffin-embedded 5 μm thick sections; $\times 200$).

mechanical ventilation between the two groups ($P > 0.05$). Compared with PN group, the post-transplant hospital stay was significantly shortened in PUFA group ($P < 0.05$). Infectious complications occurred in 6 cases of PN group (2 cases of pneumonia, 3 cases of intra-abdominal abscess and 1 case of urinary tract infection), and in 3 cases of PUFA group (2 cases of pneumonia, 1 case of intra-abdominal abscess). No acute or chronic rejection and hospital mortality were found in the two groups. All patients were followed up, and the one-year mortality in PN group was 9.1% (3/33), one died of pulmonary infection, one died of severe intra-hepatic cholangitis and hepatic dysfunction and the other of hepatic cell carcinoma recurrence. Only one patient in PUFA group (1/33, 3.1%) died of biliary complication and hepatic dysfunction during follow-up (Table 4).

DISCUSSION

An impairment of nutritional status is a frequent finding in patients with end-stage liver disease. Malnutrition adversely affects the prognosis of these patients and is associated with the morbidity and mortality after liver transplantation^[16]. Malnutrition has been shown to be the only independent risk factor for the length of stay in the intensive care and the total number of days spent in the hospital, and the liver recipient's nutritional status

Table 2 Effect of parenteral nutritional support with Omega-3 fatty acids on liver function

	Normal value	Group	Day 1	Day 2	Day 9	Decrease (Day 2-Day 9)
ALT (U/L)	5-40	PN group	198.16 ± 117.13	401.32 ± 215.35	155.16 ± 108.41 ^b	246.16 ± 175.21
		PUFA group	227.16 ± 121.17	410.98 ± 201.64	101.82 ± 71.24 ^b	299.16 ± 189.17 ^c
AST (U/L)	8-40	PN group	95.12 ± 61.79	203.25 ± 73.49	82.16 ± 46.16 ^b	121.09 ± 53.14
		PUFA group	115.62 ± 81.27	185.12 ± 42.16	68.81 ± 24.32 ^b	116.31 ± 42.19
TB (μmol/L)	5-20.5	PN group	92.16 ± 42.15	158.32 ± 65.41	71.12 ± 55.12 ^a	87.20 ± 61.12
		PUFA group	116.82 ± 61.65	160.34 ± 68.24	66.41 ± 61.52 ^b	93.93 ± 45.49
DB (μmol/L)	1.7-6.8	PN group	52.15 ± 32.95	76.46 ± 31.28	40.24 ± 26.69 ^b	36.22 ± 21.63
		PUFA group	47.39 ± 27.19	81.25 ± 26.32	38.51 ± 19.87 ^b	42.74 ± 17.36
LDH (U/L)	109-245	PN group	266.25 ± 132.42	476.25 ± 98.15	357.15 ± 192.52 ^a	119.10 ± 69.72
		PUFA group	226.45 ± 172.24	416.38 ± 151.14	260.26 ± 111.32 ^b	156.12 ± 89.20
PT (s)	10-15	PN group	18.76 ± 3.21	17.16 ± 4.05	14.62 ± 3.87 ^a	2.54 ± 1.15
		PUFA group	19.12 ± 4.16	17.81 ± 3.82	12.17 ± 3.69 ^b	5.64 ± 2.06 ^c

PN: Parenteral nutrition; PUFA: Polyunsaturated fatty acid; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TB: Total bilirubin; DB: Direct bilirubin; LDH: Lactate dehydrogenase; PT: Prothrombin time. ^a*P* < 0.05, ^b*P* < 0.01 *vs* day 2; ^c*P* < 0.05 *vs* PN group.

Table 3 Effect of parenteral nutritional support with Omega-3 fatty acids on numerical score of portal inflammation

Group	Day 0	Day 9
PN group	3.3 ± 0.5	2.7 ± 0.9
PUFA group	3.6 ± 0.4	1.8 ± 0.6 ^a

PN: Parenteral nutrition; PUFA: Polyunsaturated fatty acid. ^a*P* < 0.05 *vs* PN group.

also influences the incidence of post-transplant complications and may therefore increase the costs of liver transplant^[17,18]. After liver transplantation, surgical stress, postoperative fasting, and the possible occurrence of complications suggest the need for nutritional support. The primary goal of the nutrition support in the immediate post-transplant period is to provide adequate nutrition to promote recovery and replenishment of the depleted nutrient stores. Although most transplant centers use the similar post-transplant nutritional support as for other major abdominal operations, few studies have elucidated the role of postoperative nutritional support in the liver recipients.

Enteral nutrition is safer and less expensive than PN, and enteral nutrition has the potential advantage of maintaining intestinal trophism more effectively^[19]. This effect may help prevent bacterial translocation and enteric-origin infections in patients treated with transplantation^[20,21]. All patients in this study resumed their daily oral diet postoperatively as soon as bowel function returned to maintain intestinal trophism, but the recipients could not endure a large amount of liquid diet even with nasogastric tube at the early phase after transplantation because of obvious abdominal pain, distention or diarrhea in our previous experience. The bowel function in all the patients in this study returned on day 3 or 4 after transplantation, and PN support discontinued on day 8 after surgery when the patients were able to maintain an adequate oral intake.

Omega-3 fatty acids mainly act as eicosapentaenoic acids (EPA) and docosahexaenoic acids (DHA), both

Table 4 Effect of parenteral nutritional support with Omega-3 fatty acids on clinical outcome

	PN group	PUFA group
Post-transplant mechanical ventilation (h)	12.1 ± 5.1	10.8 ± 5.4
Post-transplant hospital stay (d)	20.6 ± 4.6	18.7 ± 4.0 ^a
Infectious morbidities	6/33	3/33 ^a
Acute/chronic rejection	0/33	0/33
ICU mortality	0/33	0/33
Hospital mortality	0/33	0/33
28-d mortality	0/33	0/33
One-year mortality	3/33	1/33

PN: Parenteral nutrition; PUFA: Polyunsaturated fatty acid; ICU: Intensive care unit. ^a*P* < 0.05 *vs* PN group.

had anti-inflammatory effects. EPA and DHA reduce the release of arachidonic acid-derived pro-inflammatory eicosanoids, and generate a group of lipid mediators called resolvins (E- and D-series) and protectins with potent anti-inflammatory and inflammation resolution properties^[22,23]. Studies with experimental models of liver reperfusion injury have reported the beneficial actions of n-3 PUFA-derived resolvins and protectins in preventing liver DNA damage and oxidative stress, thus significantly ameliorating the necroinflammatory liver injury and hepatic steatosis^[24-26]. The liver-protecting effects of postoperative PN support supplemented with omega-3 fatty acids were evaluated in this study. Liver enzyme of ALT released after I/R was significantly suppressed by the supplements of omega-3 fatty acids. PT, as an important parameter in evaluating the synthesis function of liver, was significantly decreased in PUFA group. And the results of histological examination on day 9 revealed that the hepatocyte injury and inflammatory cell aggregation were ameliorated markedly in PUFA group. PUFA therapy could also decrease the infectious morbidities, and shorten the post-transplant hospital stay significantly. The possible mechanisms of omega-3 fatty acids include down-regulation of the inflammatory responses to surgery and immune modulation rather than a sole nutritional effect.

Some of the patients exhibited an obvious nitrogen accumulation disorder reflected by either encephalopathy or an excessive rise in blood urea nitrogen in the immediate postoperative period. Branched-chain amino acids were chosen for this study because it can promote protein synthesis in patients with chronic liver diseases and avoid the additional metabolic load of transplanted liver^[27]. Medium-chain triglycerides were included in the regimen to avert glucose intolerance and deposits in the transplanted liver. Based on the results of this study, we think that post-transplant nutritional support in the form of a solution enriched with branched-chain amino acids, dextrose, medium-chain triglycerides and omega-3 fatty acids might offer a benefit in terms of preserved liver function and better clinical outcome, including the decreased infectious morbidities and post-transplant hospital stay.

In conclusion, we have shown that omega-3 fatty acids-supplemented PN significantly improves the injury of transplanted liver, decreases the infectious morbidities, and shortens the post-transplant hospital stay.

ACKNOWLEDGMENTS

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COMMENTS

Background

The liver recipients with liver insufficiency are known to experience a higher incidence of severe protein/calorie malnutrition, and malnutrition is associated with a greater risk of postoperative complications and mortality in patients undergoing liver transplantation. And ischemia/reperfusion injury associated with liver transplantation often leads to hepatic dysfunction despite the improvements in surgical techniques and perioperative medication.

Research frontiers

Omega-3 fatty acids exert anti-inflammatory and immunomodulatory properties through their ability to modulate the synthesis of different eicosanoids. Recent studies have described that supplementation with omega-3 fatty acids decreases the rate of inflammatory complication, the length of hospital stay, and the mortality after major abdominal surgeries.

Innovations and breakthroughs

Although most transplant centers use the similar post-transplant nutritional support as for other major abdominal operations, few studies have elucidated the role of postoperative nutritional support in the organ recipient. Based on the results of this study, the authors have shown that post-transplant nutritional support in the form of a solution enriched with branched-chain amino acids, dextrose, medium-chain triglycerides and omega-3 fatty acids might offer a benefit in terms of preserved liver function and better clinical outcome, including the decreased infectious morbidities and post-transplant hospital stay.

Applications

This study has shown that omega-3 fatty acids-supplemented parenteral nutrition (PN) significantly improves the injury of transplanted liver, decreases the infectious morbidities, and shortens the post-transplant hospital stay. The nutritional support strategy is recommended in patients undergoing liver transplantation.

Peer review

The manuscript evaluates the potential for supplementation with polyunsaturated fatty acid to ameliorate hepatic injury associated with reperfusion and PN. It provides evidences about an efficient nutritional support strategy for liver transplanted patients.

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Colorectal cancer lymph node staining by activated carbon nanoparticles suspension *in vivo* or methylene blue *in vitro*

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Abstract

AIM: To investigate whether activated carbon nanoparticles suspension (ACNS) or methylene blue (MB) can increase the detected number of lymph nodes in colorectal cancer.

METHODS: Sixty-seven of 72 colorectal cancer patients treated at our hospital fulfilled the inclusion criteria of the study which was conducted from December 2010 to February 2012. Seven patients refused to participate. Eventually, 60 patients were included, and randomly assigned to three groups (20 in each group): ACNS group (group A), MB group (group B) and non-stained conventional surgical group (group C). In group A, patients received subserosal injection of 1 mL ACNS in a 4-quadrant region around the mass. In group B, the main artery of specimen was identified and isolated after the specimen was removed, and 2 mL MB was slowly injected into the isolated, stretched and fixed vessel. In group C, no ACNS and MB were injected. All the mesentery lymph nodes were isolated and removed systematically by visually inspecting and

palpating the adipose tissue.

RESULTS: No difference was observed among the three groups in age, gender, tumor location, tumor diameter, T-stage, degree of differentiation, postoperative complications and peritoneal drainage retention time. The total number of detected lymph nodes was 535, 476 and 223 in the three groups, respectively. The mean number of detected lymph nodes per patient was significantly higher in group A than in group C (26.8 ± 8.4 vs 12.2 ± 3.2 , $P < 0.001$). Similarly, there were significantly more lymph nodes detected in group B than in group C (23.8 ± 6.9 vs 12.2 ± 3.2 , $P < 0.001$). However, there was no significant difference between group A and group B. There were 50, 46 and 32 metastatic lymph nodes dissected in 13 patients of group A, 10 patients of group B and 11 patients of group C, without significant differences among the three groups. Eleven of the 60 patients had insufficient number of detected lymph nodes (< 12). Only one patient with T_{4a} rectal cancer had 10 lymph nodes detected in group B, the other 10 patients were all from group C. Based on the different diameter categories, the number of detected lymph nodes in groups A and B was significantly higher than in group C. However, there was no statistically significant difference between group A and group B. The metastatic lymph nodes were not significant different among the three groups. Similarly, tumor location, T stage and tumor differentiation did not affect the staining results. Body mass index was a minor influencing factor in the two different staining methods. The stained lymph nodes can easily be identified from the mesenteric adipose tissues, and the staining time for lymph nodes was not significantly different compared with unstained group. None of the patients in groups A and B had drug-related complications.

CONCLUSION: Both activated carbon nanoparticles suspension *in vivo* and methylene blue *in vitro* can be used as tracers to increase the detected number of lymph nodes in colorectal cancer.

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Key words: Nanotechnology; Activated carbon nanoparticles suspension; Methylene blue; Lymph nodes; Colorectal cancer

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INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of cancer-related death among men and women in the United States with an estimated 143 460 new cases and 51 690 deaths in 2012 according to the statistics of American Cancer Society^[1]. Accurate lymph node metastasis staging is of prognostic and therapeutic importance in patients with CRC. Previous researches have found that the number of lymph nodes evaluated after surgical resection was positively associated with the survival of CRC patients^[2,3]. However, population-based data suggest that lymph node evaluation is not adequate in the majority of patients with CRC^[4,5]. In addition, computed tomography (CT)^[6], even positron emission tomography^[7], are not efficacious enough in identifying nodal status. Therefore, a variety of techniques, including lymph node staining or radionuclide scan^[8], have been applied to make lymph nodes retrieval more efficient.

The biological application of nanoparticles is a rapidly developing area of nanotechnology^[9,10]. Particles have been observed passing through the lymphatic vessels but not the blood capillaries mainly due to the difference in permeability. Activated carbon nanoparticles suspension (ACNS), using smooth carbon particles at a diameter of 21 nm added with suspending agents, is a stable suspension of carbon pellets of 150 nm in diameter. ACNS is obviously inclined to the lymphatic system. After macrophage phagocytosis, ACNS quickly gathers in the lymph nodes and dyes them black. This unique selective bio-distribution is being extensively studied in recent years, such as sentinel lymph node staining, drug carriers and thermotherapy^[11-13].

In our previous study^[14], 62 patients with CRC were divided into two groups. The experimental group, using a simple lymphatic staining method, was injected with methylene blue (MB) into the regional main blood vessels immediately after specimens were resected *in vitro*. More and smaller lymph nodes could be detected, which significantly improved the lymph node harvest of resected colorectal specimens. However, the detection

sensitivity for lymph node metastasis was low and the staining could not be done *in vivo* before the destruction of the lymphoid structures. Therefore, we conducted a randomized controlled trial to test whether MB and ACNS as tracers can increase the detected number of lymph nodes in the systematic nodal dissected tissues from CRC resection and compare the staining effect of the two methods in order to choose the best one for further clinical application.

MATERIALS AND METHODS

Patient selection

This trial was performed in the Department of Surgical Oncology, Second Affiliated Hospital Zhejiang University College of Medicine, China from December 2010 to February 2012. The study was approved by the Ethics Committee of Zhejiang University. Informed consent was obtained from all the patients. Inclusion criteria were as follows: 18-80 years of age; endoscopic biopsy confirmed; performance status of 0-1 on the Eastern Cooperative Oncology Group scale; good compliance; able to tolerate radical resection; adequate hematologic function [white blood cell (WBC) count > 4000/mL, absolute neutrophil count > 1500/mL, platelet count > 100 000/mL, and hemoglobin > 10 g/dL]; normal hepatic function [bilirubin < 1.5 the upper-normal limits (UNL) and alanine aminotransferase or aspartate aminotransferase < 2.5 UNL]; and normal renal function (creatinine < 1.5 mg/dL). Exclusion criteria included: clinical stage IV CRC according to the American Joint Committee on Cancer (AJCC); patients received chemotherapy, radiotherapy or biological therapy prior to surgery; previous abdominal surgery; significant neurological or mental disorder. Of the 72 CRC patients, 67 fulfilled the inclusion criteria. Seven patients refused to participate. The enrollment was completed when 60 patients were included. The patients were randomly allocated to three groups (20 patients in each group): ACNS group (group A), MB group (group B) and non-staining conventional surgical group (group C).

Surgical technique

All surgical procedures were completed by the same team of surgeons. Each patient was administrated with 2 g cephalosporin for antibiotic prophylaxis within 30 min before surgery, and the same dose was repeated if the operation lasted more than 2 h.

In group A, patients received subserosal injection of 1 mL ACNS (Chongqing LUMMY Pharmaceutical Co., Chongqing, China) in a 4-quadrant region around the mass (Figure 1A). To avoid surgical destruction of the lymphatic system along the bronchi and vessels, we waited for 10 min after injection. In group B, MB was injected by the same methods (Jiangsu Jumpcan Medicines Group, Taixing, Jiangsu Province, China), however, staining effect was very poor partly because MB was quickly absorbed *in vivo* and then excreted with urine.

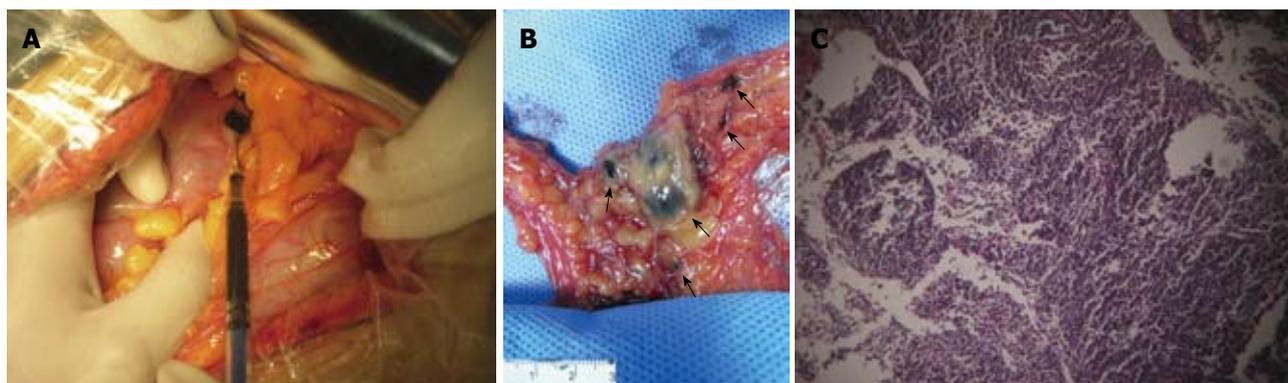


Figure 1 Activated carbon nanoparticles suspension *in vivo* is effective as a tracer in colorectal cancer lymph node detection. A: Subserosal injection of activated carbon nanoparticles suspension (ACNS) into a 4-quadrant region around the mass; B: Lymph nodes can easily be identified from the mesenteric adipose tissues, arrow points to the black dyed lymph nodes; C: ACNS migrating to the lymph node (hematoxylin and eosin, $\times 100$).

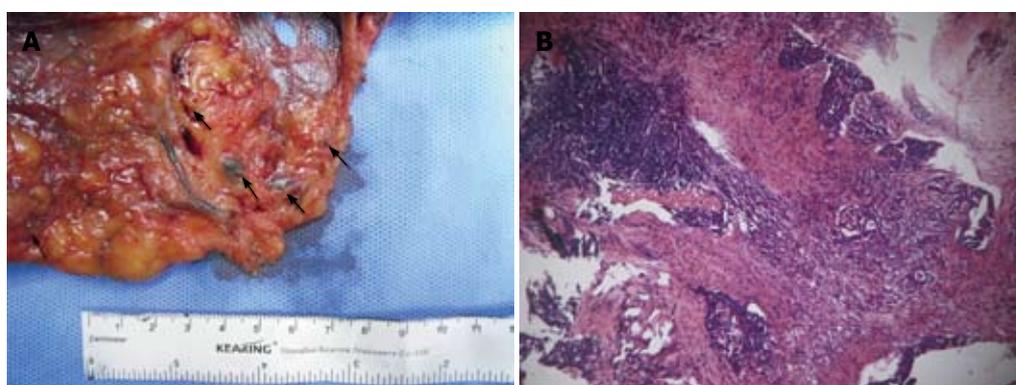


Figure 2 Methylene blue *in vitro* used as a tracer in colorectal cancer lymph node detection. A: The main artery of the specimen was isolated and methylene blue (MB) was injected into the vessel. Lymph nodes can easily be identified, arrow points to the blue dyed lymph nodes; B: MB migrating to the lymph node (hematoxylin and eosin, $\times 100$).

Therefore, we established a method for lymph nodes staining *in vitro*. After the specimen was dissected, we immediately identified the main artery of the specimen and isolated it at the root for 1 cm, and injected 2 mL MB slowly into the isolated, stretched and fixed vessel (Figure 2A). We also waited for 10 min after injection. In group C, neither ACNS nor MB was used for the staining.

All the mesentery lymph nodes were isolated and removed systematically by visually inspecting and palpating the adipose tissues. After identification and excision, all the black or blue nodes were collected for subsequent pathological examinations. Postoperative pain was relieved by intravenous opioid administration.

Statistical analysis

Statistical analysis was performed using the SPSS 17.0 for Windows (SPSS, Inc, Chicago, IL, United States). Factors considered to be possible determinants of the number of lymph nodes examined were first checked with the analysis of variance analysis and the influence of possible determinants was then tested in regression analysis. Kruskal-Wallis test was used when there was heterogeneity of variance. *P* value (two-tailed) of less than 0.05 was considered statistically significant.

RESULTS

Patient population

The 60 patients were randomly assigned to three groups. No difference was observed among the three groups in age, gender, tumor location, tumor diameter, T-stage and degree of differentiation. There was also no statistically significant difference in postoperative complications and peritoneal drainage retention time (Table 1).

Lymph nodes detected

The total number of detected lymph nodes was 535, 476 and 223 in the three groups, respectively. The mean number of detected lymph nodes per patient in group A was significantly higher than in group C (26.8 ± 8.4 vs 12.2 ± 3.2 , $P < 0.001$). Similarly, there were significantly more lymph nodes detected in group B than in group C (23.8 ± 6.9 vs 12.2 ± 3.2 , $P < 0.001$). However, there was no significant difference between group A and group B (26.8 ± 8.4 vs 23.8 ± 6.9 , $P > 0.1$) (Figure 3A). There were 50, 46 and 32 metastatic lymph nodes dissected in 13 patients of group A, 10 patients of group B, and 11 patients of group C, without significant difference among the three groups ($P > 0.1$). According to the

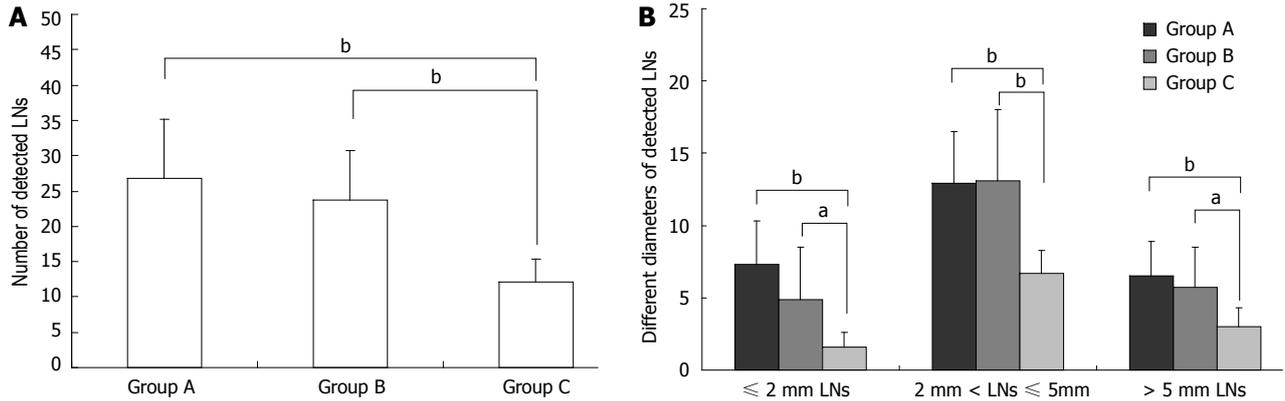


Figure 3 Lymph nodes detected in the three groups. A: The mean number of lymph nodes detected; B: Distribution of lymph node diameters. ^a*P* < 0.05, ^b*P* < 0.01 vs group A. LN: Lymph node.

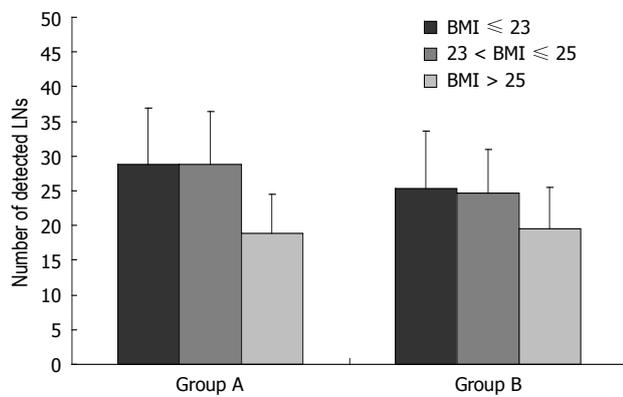


Figure 4 Body mass index as an influencing factor in the two different staining methods. LN: Lymph node; BMI: Body mass index.

AJCC guideline, more than 12 detected lymph nodes are required for the accurate clinical staging. In this study, 11 of the 60 patients had insufficient number of detected lymph nodes. Among them, only one patient in group B with T_{4a} rectal cancer had 10 lymph nodes detected, the other 10 patients were all from group C (*P* < 0.001). According to the different diameter range, lymph nodes (LNs) detected were divided into three categories: LNs ≤ 2 mm, 2 mm < LNs ≤ 5 mm, and LNs > 5 mm. We analyzed the different diameter categories of detected lymph nodes and metastatic lymph nodes in each group, and found that the number of detected lymph nodes was significantly higher in groups A and B than in group C. However, there was no statistically significant difference between group A and group B. Data is shown in Figure 3B. There was no significant difference in metastatic lymph nodes between each group (*P* > 0.05). Similarly, tumor location, T stage and tumor differentiation exerted no influence on the staining results (*P* > 0.05). Mesenteric hypertrophy may affect the lymph node detection by different staining methods^[15]. To explore whether the mesenteric hypertrophy affects the staining results in our trial, patients in the two staining groups were re-classified according to the Asian body mass index (BMI) criteria

Table 1 Clinicopathological details of the 60 patients in the three groups

	Group A	Group B	Group C
No. of patients	20	20	20
Age (yr)	57.5 ± 11.5	58.9 ± 17.8	64.9 ± 7.4
Gender			
Male	14	14	13
Female	6	6	7
Tumor location			
Right colon	2	2	4
Transverse colon	1	1	1
Left colon	5	1	1
Sigmoid	5	4	4
Rectum	7	12	11
Tumor diameter (mm)	5.0 ± 1.7	4.7 ± 2.3	3.7 ± 1.1
T-stage			
T1	0	1	0
T2	2	4	4
T3	6	7	7
T4	12	8	9
Degree of differentiation			
Well	7	7	8
Moderate	10	11	11
Poor	3	2	1
Postoperative complications			
Bleeding	1	0	0
Infection	0	1	0
Fistula	1	1	2
Abdominal tube drainage (d)	6.7 ± 2.8	6.1 ± 3.3	7.8 ± 3.6

as BMI ≤ 23 kg/m², 23 kg/m² < BMI ≤ 25 kg/m² and BMI > 25 kg/m². Statistical analysis showed that BMI was a minor influencing factor in the two different staining methods while mesenteric hypertrophy did not influence the staining results (Figure 4). Using either ACNS (Figure 1B) or MB (Figure 2A), stained lymph nodes can easily be visualized from the mesenteric adipose tissues, and the staining time for lymph nodes was not significant different compared with unstained group (*P* > 0.05). Hematoxylin and eosin stained micrograph confirmed that ACNS migrates to the lymph nodes (Figure 1C), so does the MB (Figure 2B). None of the patients injected with either ACNS or MB had drug-related complications.

DISCUSSION

Lymph nodes status of colorectal cancer played a vital role in tumor staging, classification, postoperative sequential treatment and prognosis. The number of detected lymph nodes is a significant prognostic factor in colon cancer patients^[16]. AJCC and College of American Pathologists recommend at least 12 lymph nodes detected for more accurate diagnosis of stage II CRC^[17]. However, recent studies indicated that lymph node detection rate was still low in CRC^[4], which can not accurately reflect the patient's disease status. There are many factors affecting the lymph node detection, including patient's age, gender, tumor grade, extent of surgical resection and the pathologist's expertise^[18]. Palpation is still the most important method for lymph node detection^[19]. Numerous studies have been conducted to improve the methods for lymph node detection. Cawthorn *et al.*^[20] used xylene alcohol clearance technique to facilitate the identification of lymph nodes (23.1 ± 1.18 vs 10.5 ± 0.6). Quadros *et al.*^[21] performed lymphoscintigraphy using technetium-99 m-phytate and patent blue to detect lymph nodes of rectal adenocarcinoma patients, which significantly increase the lymph nodes detection rate, particularly lateral pelvic lymph node metastasis. However, these techniques were not widely used in clinical practice because they are time-consuming, labor-intensive and toxic to doctors. This clinical trial used the novel nanomaterials ACNS and MB *in vivo* or *in vitro*. The results suggested that both staining techniques can significantly improve the lymph node detection compared with the conventional palpation method (26.8 ± 8.4 vs 23.8 ± 6.9 vs 12.2 ± 3.2). In our research, none of the patients had insufficient number of detected lymph nodes in ACNS stained group, only one patient in MB stained group, but 10 patients did so from unstained group C. Statistical results showed that in different diameter categories, the number of the detected lymph nodes was significantly higher in both the two stained groups than in the unstained group (Figure 4), especially in the ACNS group. This demonstrated the obvious advantages of the two staining methods in detecting the smaller diameter lymph nodes. Micrometastasis is defined as cohesive deposits of tumor cells of 2 mm or less, but larger than 0.2 mm. This definition has been extended in the AJCC 7th edition to include non-cohesive infiltrate of > 200 cells as micrometastasis^[22]. No definitive conclusions have been drawn about the role of sentinel lymph node biopsy and micrometastasis on the prognosis of CRC^[23,24]. Taking into account the advantage of the detection for lymph node micrometastasis in this study, our team will further study the effects of sentinel lymph node biopsy and micrometastasis in the prognosis of CRC patients. In this trial, we failed to find significant differences between the two staining methods with regard to the total number of detected lymph nodes, lymph node diameters or lymph node metastasis, which may be attributed to the limitations of sample size.

The biological application of nanoparticles is a rap-

idly developing area of nanotechnology that raises new possibilities in the diagnosis^[25] and treatment of human cancers^[26,27]. Nanoparticles are being developed for contrast at T1-weighted magnetic resonance imaging^[28], radionuclides for single photon emission computed tomography and positron emission tomography^[29], iodine for CT^[30] and gas-containing bubbles for ultrasonography^[31]. Several nanotechnologies have been used to improve the delivery of chemotherapeutic agents to cancer cells^[32,33], which promoted the microdosing clinical studies^[34]. A review by Schroeder *et al.*^[35] showed that nanoparticle therapies will improve the outcome for patients with metastatic cancer. Using ACNS combined with preoperative lymphoscintigraphy and intraoperative gamma probe detection, the detecting sensitivity was 100% for internal mammary sentinel node biopsy of breast cancer patients^[36]. A clinical trial from him^[37] showed that ACNS and MB both were effective as tracers in lymph nodes detection for non-small cell lung cancer. However, ACNS staining in colorectal cancer lymph nodes detection remains largely unexplored. MB is being widely used in clinical practice, however, when it was used in lymph nodes staining in CRC *in vivo*, it played a minor role in lymph nodes detection because it was absorbed and excreted too quickly. We thus used a modified method as described in the Surgical Technique, injected MB into the main blood vessels of the tumor drainage region *in vitro*, and achieved much better staining results in lymph node detection. An ideal tracer should possess the following properties: not influenced by external conditions, easy to perform, effective and free from side-effects. We found that T stage, degree of tumor differentiation, tumor location, and BMI had minor influence on the two staining methods. In this study, black-stained or blue-stained lymph nodes can be easily identified from the mesenteric adipose tissues, and no side-effect was found among patients, surgeons and pathologists. However, the results obtained in this study may be limited by the sample size. Further studies with a larger sample will be conducted.

In conclusion, both ACNS *in vivo* and MB *in vitro* are effective as a tracer in increasing the detected number of lymph nodes in CRC. They may play a key role in the studies of sentinel lymph nodes biopsy and micrometastasis in CRCs.

COMMENTS

Background

Accurate lymph node metastasis staging has important prognostic and therapeutic implications in patients with colorectal cancer (CRC). Activated carbon nanoparticles suspension (ACNS) is obviously inclined to lymphatic system. The unique selective biodistribution is being extensively studied in recent years, such as sentinel lymph node staining, drug carriers, and thermotherapy. Additionally, methylene blue (MB), as an efficacious and cost-effective tracer, was widely used in clinical practice.

Research frontiers

Numerous studies have been conducted to improve the methods of lymph node detection. The biological application of nanoparticles is a rapidly developing area of nanotechnology. However, ACNS as a tracer for lymph nodes detection

in CRC patients has not been reported. This study found that ACNS *in vivo* is effective in increasing the detected number of lymph nodes in CRC. Similarly, the authors used a modified method, injecting MB into the main blood vessels of the tumor drainage region *in vitro*, and achieved analogous clinical effects. Moreover, the two staining methods were mildly influenced by T stage, degree of tumor differentiation, tumor location, and body mass index.

Applications

This is the first research that compares the staining effects of ACNS and MB in colorectal cancer lymph nodes detection. Both ACNS *in vivo* and MB *in vitro* are effective as tracers in increasing the detected number of lymph nodes in CRC.

Terminology

ACNS is a stable suspension of carbon pellets with a diameter of 150 nm, which is obviously inclined to lymphatic system. After macrophage phagocytosis, ACNS quickly gathers in the lymph nodes and dyes them black.

Peer review

The topic is very interesting and is of great clinical importance as the number of removed and histologically analyzed lymph nodes does determine the prognosis of CRC patients. An easy, time- and cost-effective method to help the surgeons' work is needed.

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Inverted Meckel's diverticulum as a cause of occult lower gastrointestinal hemorrhage

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Abstract

Meckel's diverticulum is a common asymptomatic congenital gastrointestinal anomaly, but rarely it can present with hemorrhage. Over the last few years inverted Meckel's diverticulum has been reported in the literature with increasing frequency as an occult source of lower gastrointestinal hemorrhage. Here, we report a case of a 54-year-old male, who was referred for surgical evaluation with persistent anemia and occult blood per rectum after a work up which failed to localize the source over 12 mo, including upper and capsule endoscopy, colonoscopy, enteroclysis, Meckel scan, and tagged nuclear red blood cell scan. An abdominal computed tomography scan showed a possible mid-ileal intussusception and intraluminal mass. During the abdominal exploration, inverted Meckel's diverticulum was diagnosed and resected. We review the literature, discuss the forms in which the disease presents, the diagnostic

modalities utilized, pathological findings, and treatment. Although less than 40 cases have been reported in the English literature from 1978 to 2005, 19 cases have been reported in the last 6 years alone (2006-2012) due to improved diagnostic modalities. Successful diagnosis and treatment of this disease requires a high index of clinical suspicion, which is becoming increasingly relevant to general gastroenterologists.

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Key words: Inverted Meckel's diverticulum; Gastrointestinal hemorrhage; Lower gastrointestinal bleeding; Intussusceptions

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INTRODUCTION

Meckel's diverticulum is the most common congenital abnormality in the gastrointestinal tract, but is usually asymptomatic^[1]. When symptomatic it may present with hemorrhage in association with ectopic gastric and/or pancreatic mucosa, intestinal obstruction, intussusceptions, or inflammation. This abnormality can also present in the setting of an inverted diverticulum causing a lower gastrointestinal bleed. There is no role for non-operative

management in inverted Meckel's diverticulum, which mandates early surgical removal. Here, we present a case which first underwent an extensive diagnostic evaluation of persistent gastrointestinal hemorrhage over a 12-mo period before this entity was diagnosed and then appropriately treated. The aim of this report is to alert surgeons and gastroenterologists of this important source of persistent gastrointestinal hemorrhage which requires a high clinical suspicion to diagnose because of how difficult it is to detect. We conducted an extensive review of the previously reported cases in the literature, and discuss the presentations of this readily curable disease, the utility of various diagnostic modalities, pathological findings, and the appropriate management.

CASE REPORT

A 54-year-old male with no significant past medical history was seen by a gastroenterologist for anemia and stools positive for occult blood. An extensive diagnostic evaluation was undertaken over a 12-mo period before the patient was referred for surgical evaluation. He had a negative upper gastrointestinal and capsule endoscopy, and a negative colonoscopy, despite adequate bowel preparation. Enteroclysis revealed a polypoid lesion in the mid jejunum. The differential diagnosis at that point included tumor, lipoma, carcinoid, or sarcoma. He underwent a Meckel scan, which was negative. Given the continued anemia and occasional bright red blood per rectum, he underwent a tagged nuclear red blood cell scan, which failed to demonstrate an acute hemorrhagic source. An abdominal computed tomography (CT) scan was obtained, which demonstrated a possible mid-ileal intussusception and an intraluminal mass. The patient was referred for a surgical evaluation.

The patient underwent an exploratory laparoscopy which demonstrated a hard intraluminal mass in the mid-ileum. The remainder of the small bowel was normal to the level of the ligament of Treitz. A 5 cm mini midline-laparotomy was performed overlying the small bowel lesion. The lesion containing portion of the small bowel was delivered out of the abdomen and resected. Small bowel continuity was established *via* a stapled side-to-side anastomosis.

On gross examination, the specimen consisted of a segmental resection of the small bowel with a small dimple shaped defect penetrating through the bowel wall. This defect was proven to be patent by a probe. Opening of the specimen revealed a 5.0 cm × 1.8 cm × 1.5 cm polypoid lesion that terminated in a club-shaped head (Figure 1). The small bowel mucosa was contiguous with that of the stalk and extended to within 1.5 cm of the tip of the lesion. The dumbbell shaped tip was covered by attenuated mucosa that was granular in appearance. A small area of erythema was seen at the tip and appeared grossly consistent with an area of hemorrhage.

Microscopic sections revealed well-defined intestinal mucosa that surrounded the core of the lesion (Figure 2). The most internal component of the core consisted of

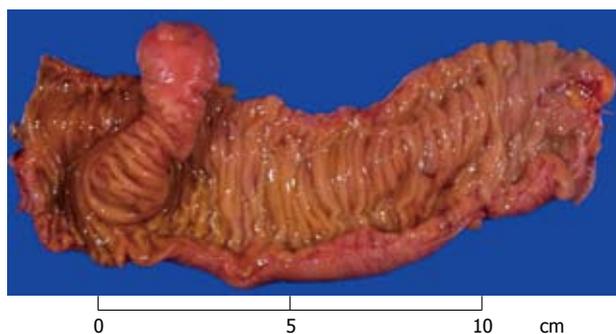


Figure 1 Gross specimen of the inverted Meckel's diverticulum arising in the segmental resection of the small bowel.

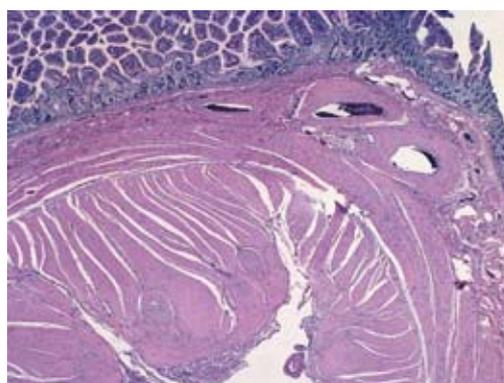


Figure 2 Histopathology reveals a central core consisting of serosa and muscle. The cross section reveals small intestinal mucosa lining the fragment.

the serosa and the muscle of the bowel wall. These layers were circumscribed by the intestinal submucosa and mucosa that became attenuated toward the tip of the specimen. The mucosa seen at the tip was that of intestinal-type with scattered paneth cells (exocrine serous cells) and goblet cells. No heterotopic gastric or pancreatic tissue was identified. The patient had an uneventful post-operative recovery. He was discharged on post-operative day three, and never had further episode of lower gastrointestinal hemorrhage nor any complications or sequelae during the subsequent two years of follow up.

DISCUSSION

Meckel's diverticulum is the most common congenital anomaly in the gastrointestinal tract^[1]. In an autopsy series, the incidence was reported as 1%-3%^[2]. Johann F Meckel, a famous anatomist, was the first to describe this entity. In 1808, he stated that the diverticulum comprised a remnant of the vitelline duct, the duct between the intestinal tract and the yolk sac^[3]. In normal human embryology, the vitelline duct closes by the 10th week of gestation. If it persists, presentations include incidental meckel's diverticulum, fibrous cord connecting the bowel to the anterior abdominal wall, persistent omphalenteric fistula, enterocystoma, torsion, and intussusception^[4]. Although the location of the diverticulum varies, they have been classically described on the antimesenteric surface of

Table 1 Clinical characteristics, diagnostic modalities, and pathological finding of patients with inverted Meckel's diverticulum

Clinical characteristics	n (%)
Gender ^[4,7,9-12,14-43]	
Male	41/59 (69)
Female	18/59 (30)
Signs and symptoms ^[4,7,9-12,14-34,36-43]	
Bleeding	48/59 (81)
Abdominal pain	41/59 (69)
Intussusception	23/59 (39)
Anemia	47/59 (80)
Diagnostic modalities ^[4,7-9,12,14-34,36-43]	
Positive upper endoscopy	2/58 (3)
Positive lower endoscopy	4/59 (7)
Positive abdominal ultrasonography	12/13 (92)
Positive tagged RBC scan	0/3 (0)
Positive Meckel's scan	0/3 (0)
Positive barium enema	0/0
Positive enteroclysis	7/7 (100)
Positive upper GI series with small bowel follow through	18/21 (86)
Positive abdominal CT scan	24/24 (100)
Pathologic findings ^[4,7-9,12,14-34,36-43]	
Ulceration	40/59 (68)
Ectopic gastric tissue only	18/59 (31)
Ectopic pancreatic tissue only	13/59 (22)
Ectopic gastric and pancreatic tissue	4/59 (7)
No ectopic tissue	24/59 (41)

GI: Gastrointestinal; CT: Computed tomography; RBC: Red blood cells.

the ileum within 100 cm of the ileocecal valve^[5]. Possible complications include hemorrhage, obstruction, diverticulitis, hernia, tumor, and inflammation, one of which an estimated 2% of those with Meckel's diverticulum will develop^[1,2]. The presentation of these complications often produces a complex constellation of recurrent symptoms consistent with obstruction, chronic abdominal pain and lower gastrointestinal hemorrhage which commonly delays diagnosis and definitive surgical treatment^[5].

An inverted Meckel's diverticulum is a condition where the Meckel's diverticulum literally inverts on itself; however, the pathophysiology underlying this rare phenomenon is not clearly understood. One theory is that there is abnormal peristalsis of the bowel segment in the proximity of the Meckel's diverticulum, possibly due to the tissue present at the base of the diverticulum itself, which causes the diverticulum to invert. Because of the inversion of the Meckel's diverticulum it may be difficult for diagnostic studies which rely on access to the lumen of diverticula, such as capsule endoscopy and colonoscopy, to identify this rare lesion. This inversion of the Meckel's diverticulum can then also lead to a complete intussusception of the bowel or to a compromise in blood flow to that bowel, ulceration and then gastrointestinal hemorrhage^[6].

Because of the clinical challenge of diagnosing this rare entity, inverted Meckel's diverticulum has been reported in less than 70 cases reported in the English literature. However, as diagnostic modalities have improved the reports of this disease as an occult source of hemorrhage

has increased from 40 cases from 1978 to 2005, to 19 cases reported in the last 6 years alone (Table 1). The largest series thus far reported included 18 cases, between 1971-1995 from the Armed Forces Institute of Pathology (AFIP)^[7], and the most recent systematic review was in 2005 before the surge in reports^[5]. Therefore, our report and review comprehensively reviews all these cases in the literature to further guide clinicians in the approach to the diagnosis and treatment of this readily curable disease (Table 1).

The median age of presentation of inverted Meckel's diverticulum is 27.7, slightly younger than reported by the AFIP, which was 33, with a male to female ratio of approximately 2.33:1. The most common presenting complaint was bleeding in 48 of 59 cases (80%), anemia 47 of 59 cases (78%), and abdominal pain (68%) (Table 1). With the most common presentation being lower gastrointestinal bleeding, it is not surprising that most reported cases included a thorough work up for the source of hemorrhage^[5]. In most cases involving bleeding and anemia, patients underwent an upper and lower endoscopy with negative results.

The first reported radiographic description of an inverted Meckel's diverticulum was by Fetterman *et al*^[8] in 1968 and there has been a great proliferation of diagnostic modalities available to clinicians^[5]. A Meckel's scan, a radionucleotide scan that detects gastric mucosa, can be a useful diagnostic tool, especially in the pediatric population. It has a high sensitivity but low specificity. Only 50% of cases are believed to be associated with ectopic gastric or pancreatic mucosa, but it is seen in 75% of those presenting with symptoms^[9]. Meckel scans were reported in only three cases^[4,10,11] which were negative. One of these actually had both ectopic gastric and pancreatic mucosa^[4]. This strongly suggests that a negative Meckel scan does not rule out the diagnosis, which was exemplified in our case.

Other radiologic diagnostic modalities include ultrasonography, which demonstrated positive or clinically influential findings in 12 of 13 cases (Table 1). These findings were often non-specific and only prompted surgical exploration in one case of a post-operative bowel obstruction caused by an intussusception from the inverted Meckel's diverticulum which was detected by ultrasound^[12]. Some of the nonspecific findings include "eggplant shaped mass within the bowel"^[13], fluid filled target^[14], and distended loops of bowel with free fluid^[15]. The use of a tagged nuclear red blood cell scan was reported in three cases with negative results, and barium enema has been of little use (Table 1). When a patient presents with massive hemorrhage, angiography may be useful, especially in a hemodynamically unstable patient. In a single reported case of angiography used for the diagnosis of Meckel's diverticulum, angiography revealed a vitelline artery centrally located in the ileal lumen^[10].

The three most useful tools employed for the diagnosis of inverted Meckel's diverticulum include small bowel follow-through, enteroclysis, and abdominal CT scans^[4,13,16]. When the scan reveals a mass, it often is as-

sociated with a central area of fat density^[5]. Small bowel follow-through was helpful in 18 of 21 cases. Findings include mass-like lesions, polypoid filling defects, and ulcerations. CT scans have been extremely helpful, especially recently with improving technology. CT scan was used in 24 of the reported cases, and all revealed useful information that ultimately led to an operation. They were especially useful when intussusceptions were found in association with the characteristic "target sign". In adults, intussusceptions with clinical symptoms are a clear indication for operation. In the pediatric population, however, even intussusceptions caused by an inverted Meckel's diverticulum can be treated non-surgically with barium enema reduction^[17]. When the small bowel is highly suspected as the source of hemorrhage, enteroclysis has been suggested as the single best study in the diagnosis of inverted Meckel's diverticulum. In 7 reported cases, all seven were useful, as they revealed filling defects and polypoid lesions (Table 1).

In the literature review of the specimens, the average length of the inverted segment was approximately 3.99 cm. Ulceration has been reported in the adjacent ileal mucosa, in the Meckel's segment, and in the tip. There appears to be no direct correlation with the presence or absence of ectopic tissue. The ulceration seen in cases without gastric mucosa may be explained by either ischemia and/or trauma^[5]. Fifty-eight percent of the reported cases were associated with ectopic mucosa of either gastric or pancreatic origin (Table 1).

The preferred treatment of any symptomatic Meckel's diverticulum is surgical. Whenever an inverted Meckel's diverticulum is diagnosed either pre-operatively or intra-operatively, the surgical procedure should be segmental resection with reestablishment of bowel continuity. Intussusception was noted in our case preoperatively. In our literature review, twenty three cases documented active intussusception at the time of operation (Table 1). There was one report of an endoscopic mucosal resection which resulted in iatrogenic perforation requiring emergent laparotomy^[18]. It has been the general consensus that intussusceptions in the adult should be treated with resection and primary anastomosis^[11]. Although most reports have described laparotomy, some minimally invasive techniques have been described in the literature. El-Dhuwaib *et al.*^[14] and Karahasanoglu *et al.*^[15] reported exploration and resection laparoscopically for an inverted Meckel's diverticulum. However, others have reported that manual palpation or laparoscopic inspection of the small bowel itself is not enough and may lead surgeons to miss the diagnosis^[17]. In our case, we planned on an initial abdominal exploration laparoscopically, and if unsuccessful, had planned on conversion to an open laparotomy with possible intra-operative endoscopy. Fortunately, we were able to find the lesion laparoscopically and performed the resection *via* a mini-laparotomy, which has the potential to provide less morbidity than a larger laparotomy incision. Our approach provided adequate exposure to achieve appropriate margins if the

lesion had been found to have been malignant. In cases where even open laparotomy fails to localize the lesion, the successful use of intra-operative endoscopy to localize the lesion and guide treatment of inverted Meckel's diverticulum has been reported^[19].

Bleeding seen in inverted Meckel's diverticulum cannot be attributed entirely to ulceration secondary to gastric mucosa, and it may be due to trauma or inversion induced mucosal ischemia^[5]. In fact, most cases did not have gastric cells present (Table 1). Trauma, due to its location within the lumen, is likely a primary source of bleeding in most cases reported in association with normal intestinal mucosa^[5,13,20-22].

There is some debate as to the treatment of incidentally discovered Meckel's diverticulum in the asymptomatic patient. Resection is generally recommended for patients younger than 40, diverticulum longer than 2 cm, divertula with narrow necks, fibrous bands, ectopic gastric tissue, and/or when the diverticulum appears thickened and inflamed. When a Meckel's diverticulum is discovered as the lead point to an intussusception, it is thought to be a primary pathologic process, and not a secondary process^[5,17]. The exact cause of the inversion is not yet understood. Intussusceptions are primarily seen in children under the age of 2, and only 5% of all intussusceptions are seen in adults^[1]. In children however, there is no lead point in 95% of the cases^[23].

Multiple diagnostic modalities have been described in the diagnosis of inverted Meckel's diverticulum. In instances of lower gastrointestinal hemorrhage, it is appropriate to first exclude an upper gastrointestinal source and a colonic source. Based upon a review of the literature, the studies recommended when inverted Meckel's diverticulum is suspected are CT scans and enteroclysis. However, to make this difficult diagnosis requires a high index of suspicion with an awareness of this important pathologic process and its unique presentation.

Although Meckel's diverticulum is usually an asymptomatic common congenital abnormality of the gastrointestinal tract, it can present with lower gastrointestinal hemorrhage^[35]. In the case of inverted Meckel's diverticulum, the bleeding may be due to the presence of ectopic gastric mucosa, but may also be commonly due to trauma or inversion induced ischemia. In a patient presenting with lower gastrointestinal bleeding, upper and lower endoscopy can be used to rule out a source. If these modalities are negative and Meckel's diverticulum is suspected, CT scan or enteroclysis may be more helpful in the diagnosis than other modalities, and its wider use may account for the increase in reports of this rare disease in the literature. Treatment usually provides a complete cure when it entails operative resection, either *via* an open or laparoscopic approach with possible intra-operative endoscopy. Because of the non-specific presentation of inverted Meckel's diverticulum as an occult source of lower gastrointestinal hemorrhage, it is important for gastroenterologists and surgeons to understand the pathophysiology, appropriate diagnostic approach and therapeutic management of this readily curable disease.

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Enterolithiasis-associated ileus in Crohn's disease

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Abstract

Stasis of the flow of the intestinal contents, ingested material and unfavorable composition of the chylus can lead to the formation of enteroliths inside the bowel. Enterolithiasis represents a rare disorder of the gastrointestinal tract that can be associated with intermittent abdominal pain or more serious complications such as bleeding or obstruction. Enterolithiasis in Crohn's disease represents an extremely rare condition and usually occurs only in patients with a long symptomatic history of Crohn's disease. We report an unusual case of enterolithiasis-related intestinal obstruction in a young male patient with Crohn's disease (A2L3B1 Montreal Classification for Crohn's disease 2005) undergoing emergency laparotomy and ileocecal resection. In addition, we present an overview of the relevant characteristics of enterolithiasis on the basis of the corresponding literature.

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Key words: Crohn's disease; Enterolithiasis; Ileus; Inflammatory bowel disease; Obstruction

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INTRODUCTION

As enterolithiasis of the small bowel is a rare condition, only a few cases of enterolithiasis-related small bowel ileus in patients with Crohn's disease have been published. A PubMed database analysis showed 9 results published between 1972 and 2010 when searching for "enterolithiasis + crohn"^[1,2]. To date, about 20 cases of Crohn's disease-associated enterolithiasis have been reported in the literature. Here we present the case of a Crohn's disease patient with enterolithiasis-related small bowel obstruction and discuss the clinical and radiological features of this rare entity on the basis of the current literature.

CASE REPORT

A 46-year-old male patient (nonsmoker) presented with acute diffuse abdominal pain, constipation and vomiting at the emergency unit of the department of surgery. His past medical history included laparoscopic cholecystectomy for symptomatic cholelithiasis several years ago and a 12-year history of Crohn's disease. At the time of admission the patient was treated with budesonide, enteroclysm and 5-aminosalicylic acid (A2L3B1 Montreal Classification for Crohn's disease 2005). The clinical examination showed no fever, a soft, but distended abdomen without abdominal guarding. Blood investiga-



Figure 1 X-ray of the abdomen with two small radiopaque enteroliths in the lower abdomen. A: Overall picture; B: Enlarged detail. L: Left.



Figure 2 Computed tomography images with two radiopaque enteroliths.

tion revealed a normal leucocyte count and a marginally increased level of C-reactive protein. On the abdominal X-ray, the typical imaging of an incipient small bowel ileus with dilated small bowel loops was apparent (Figure 1A), however, two conspicuous small shadows in the pelvis were also observed (Figure 1B). Consequently, a computed tomography (CT) scan of the abdomen was performed to confirm or exclude a mechanical obstruction. The CT scan showed a significant small bowel stricture and two adjacent intraluminal radiopaque, dense calcified enteroliths with a maximal diameter of 20 mm in the terminal ileum with significant wall thickening, acute inflammatory signs and prestenotic dilatation of the small bowel indicating a mechanical ileus (Figure 2). The patient underwent immediate emergency laparotomy: the intraoperative findings revealed a long (30 cm), dense narrowing of the terminal ileum with irremovable stony palpable masses, poststenotic emptiness and prestenotic dilatation of the bowel (Figure 3). An ileocecal resection of about 40 cm of the terminal ileum with a side to side ileoascendostomy reconstruction was conducted (Figure 4). Histopathological examination of the resected specimen confirmed the diagnosis of an acute inflammatory episode of Crohn's disease without any sign of malignant transformation or perforation. After an uneventful postoperative stay of 11 d, the patient was discharged home with Crohn's disease specific medication (metronidazole, azathioprine) recommended by the gastroenterologists to prevent postoperative bacterial infection and to control inflammatory activity.

DISCUSSION

The first description of enterolithiasis in the scientific literature can be traced back to 1917, when Pfahler *et al.*^[3] published a report on the radiological features of enterolithiasis. It was not until 1959 that Atwell *et al.*^[4] introduced the definition of enteroliths as “endogenous foreign bodies in the gastrointestinal tract”. According to a classification by Grettve *et al.*^[5], enteroliths can be divided into primary and secondary enteroliths. Primary enteroliths always develop inside the bowel, and can be caused by concretions of normal chylus components such as choleic acid, calcium phosphate and calcium carbonate (so called “true” primary enteroliths) or by ingested indigestible material such as hair (trichobezoar), vegetables (phytobezoar) or other exogenous substances such as barium sulfate (so called “false” primary enteroliths). In contrast, secondary enteroliths typically form outside the bowel and then pass into the bowel like gallstones which fistulate into the bowel causing peritonitis and/or mechanical obstruction. The most common localization of enteroliths is the colon including the appendix vermiformis (appendicoliths), however, enteroliths can occur throughout the entire gastrointestinal tract from the stomach to the rectum.

Stasis or decelerated motility of the gastrointestinal tract seems to be the crucial pathogenetic factor in the development of enteroliths, since an unimpaired continuous flow of the gastrointestinal content usually does not allow the formation of enteroliths^[5]. However, when stasis occurs, small particles have enough time to crystal-



Figure 3 Intraoperative illustration of the small bowel ileus with evident stenosis and prestenotic dilation.



Figure 4 Resected specimen of the cecum and small bowel with two fixed enteroliths causing small bowel obstruction.

lize and form accumulating stones. Stasis is always associated with anatomic alterations of the gastrointestinal tract such as harmless anatomic variations (diverticula, duplication cysts)^[6,7] or pathologic conditions (strictures) due to bowel diseases such as Crohn's disease or tuberculosis^[8-10]. In addition, stasis may also develop after bowel resection with a broad side to side enteroanastomosis with a big blind pouch allowing for continuous deposition of intestinal contents^[11]. Enteroliths can be asymptomatic and dissolve spontaneously passing slowly through the gastrointestinal tract until excretion. However, in most cases enterolithiasis is associated with recurrent signs of intestinal obstruction and intermittent abdominal pain. In addition, refractory chronic anemia and perforation have also been reported^[4,12]. Interestingly, the patient in our case report did not really suffer from any of these symptoms, although both enterolithiasis and Crohn's disease represent chronic disorders. Obviously, there was a moderate postinflammatory, quite asymptomatic stricture of the terminal ileum which permitted very slow but continuous development of enteroliths without significant alteration of intestinal passage. The actual inflammatory episode then led to complete intestinal obstruction due to thickening of the bowel wall. We suggest that the enteroliths developed primarily on site, since they were almost completely fixed in the diseased

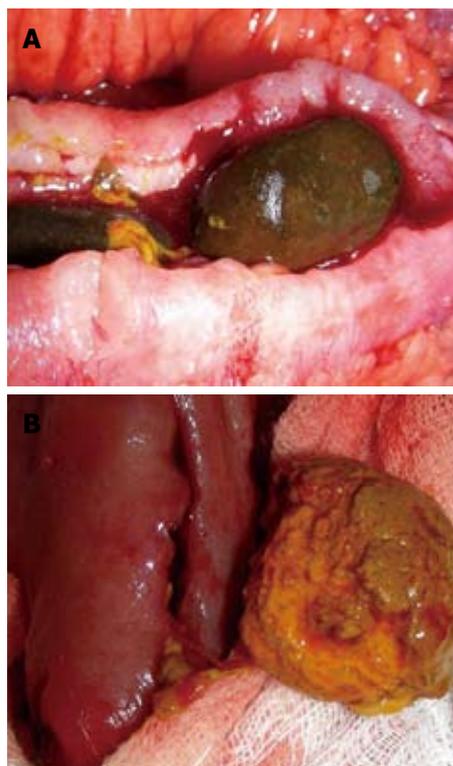


Figure 5 Typical images of enteroliths. A: Long-standing enterolith with blank-looking polished surface; B: Emerging enterolith with a rough and fragile surface.

bowel segment. Additionally, the enteroliths stood out with a very hard consistency and blank-looking, polished surface (Figure 5A). About 20 cm proximal of the stricture another enterolith was detected in the resected specimen with completely different characteristics: this enterolith was morbid, fragile, rough and easily movable, potentially because there was no stricture (Figure 5B). We hypothesize that this additional enterolith represents the typical status nascendi of an enterolith which is usually not observable, since it is asymptomatic and potentially excreted.

The radiological diagnosis of enteroliths depends on their calcium content^[13,14]. In our case, two radiopaque enteroliths were clearly visible on abdominal X-ray, whereas the third mobile enterolith was not detectable. The differential diagnosis of radiopaque enterolith-like alterations includes gallstones, urolithiasis-calcified lymph nodes or pancreatic calcifications. Of note, enteroliths may change their localization on radiographs due to the mobility of the small bowel^[9].

Due to its typical strictures impairing the intestinal flow, Crohn's disease offers favorable conditions for the development of enteroliths. Nevertheless, enteroliths are a very rare condition in Crohn's disease, since most patients never become symptomatic or because symptoms are not attributable to enteroliths. Studies have proven that enterolithiasis in Crohn's disease occurs only in patients with a long history of the disorder and long duration of symptoms of between 7 and 40 years (median

15.7 years)^[9]. Additionally, a few cases of enterolithiasis-associated adenocarcinomata have been reported in the literature^[15]. In summary, there is no evidence for prophylactic treatment of asymptomatic enterolithiasis in Crohn's disease, although enteroliths represent clear indicators of a stenotic condition. Patients with intestinal obstruction require laparotomy with stricturoplasty or segmental bowel resection.

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An aortoduodenal fistula as a complication of immunoglobulin G4-related disease

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Author contributions: Sarac M, Marjanovic I and Bezmarevic M operated on the patient; Bezmarevic M took the photos and researched sources for the references; Bezmarevic M, Petrovic S, Sarac M and Marjanovic I were engaged in the postoperative treatment; Sarac M, Bezmarevic M, Zoranovic U and Petrovic S participated in the follow up; Mihajlovic M and Petrovic S helped in the interpretation of photos and helped draft the final version of the manuscript; all authors participated in writing the case report and revising the draft.

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sociated with IgG4-related sclerosing disease as a possible complication of IgG4-related inflammatory aortic aneurysm. Endovascular grafting of a primary aortoduodenal fistula is an effective and minimally invasive alternative to standard surgical repair.

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Key words: Abdominal aortic aneurysm; Aortoduodenal fistula; Endovascular repair; Immunoglobulin G4-related disease

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Abstract

Most primary aortoduodenal fistulas occur in the presence of an aortic aneurysm, which can be part of immunoglobulin G4 (IgG4)-related sclerosing disease. We present a case who underwent endovascular grafting of an aortoduodenal fistula associated with a high serum IgG4 level. A 56-year-old male underwent urgent endovascular reconstruction of an aortoduodenal fistula. The patient received antibiotics and other supportive therapy, and the postoperative course was uneventful, however, elevated levels of serum IgG, IgG4 and C-reactive protein were noted, which normalized after the introduction of steroid therapy. Control computed tomography angiography showed no endoleaks. The primary aortoduodenal fistula may have been as-

INTRODUCTION

A primary aortoenteric fistula (AEF) is a communication between the aorta and the intestines, and occurs in a setting without prior aortic surgery. It is a rare but potentially lethal condition with an incidence of 0.04% to 0.07%^[1]. Primary fistulas are in most cases (90%), the result of erosion of the bowel wall, caused by an abdominal aortic aneurysm (AAA)^[2]. The majority occur between the aorta and the duodenum^[3]. Primary aortoduodenal fistulas (ADF) have been reported in the presence of various conditions, including underlying atherosclerotic aortic aneurysm disease, gallstone erosion, foreign body ingestion, and invasive intra-abdominal malignancies^[4]. However, it has been reported that an aortic

aneurysm can be a part of a spectrum of immunoglobulin G4 (IgG4)-related sclerosing disease^[5]. Most reported cases of IgG4-related inflammatory aortic aneurysm of abdominal aorta (IAAA) have no association with other IgG4-related sclerosing diseases. Without apparent pathological conditions in other organs, confirmation of the existence of IgG4-related IAAA requires histopathological evidence^[6-10]. An ADF as a complication of IgG4-related disease has not yet been reported. Endovascular aortic repair is an effective alternative to standard open surgical management of primary aortoenteric fistulae, especially in situations where an open surgical procedure is either difficult or contraindicated^[11]. In this article we present the urgent endovascular repair of an ADF in a patient with high serum IgG4 levels.

CASE REPORT

A 56-year-old Caucasian male was admitted to our institution due to severe anemia caused by gastrointestinal hemorrhage. He had chronic fatigue and at least one episode of melena every day of one month duration. At admission he had anemia with a red blood cell count (RBC) of 2.8 million per μL , hematocrit (HCT) of 25% and blood pressure of 90/70 mmHg with a heart rate of 110 beats/min. No abdominal pulsating mass was found on physical examination. A digital rectal examination showed signs of melena.

The patient had undergone previous abdominal surgery in another medical institution (9 years before), due to acute generalized abdominal pain. He had no medical documentation, and we were unable to obtain more information about his previous condition.

Urgent upper endoscopy (EGDS) did not show any signs of bleeding. Abdominal ultrasound showed an infrarenal AAA that was verified on computed tomography (CT) angiography (Figure 1). During the same CT evaluation a gas collection in a suspected thrombotic mass in an aneurysmatic sac was noted, suggesting an AEF. The CT findings excluded the presence of autoimmune pancreatitis.

Because there was a suspicion of Meckel's diverticulum, we performed abdominal scintigraphy, without any pathological findings. Colonoscopy findings were normal. In addition to administering blood and blood product substitution therapy, standard therapy was also administered, however, melena persisted. Control EGDS findings were normal. Due to worsening of the patient's general condition [persistent melena followed by anemia; RBC 3.1 million per μL , HCT 27% and C-reactive protein (CRP) 82 mg/L], the case was presented at a meeting of gastroenterologists, vascular and abdominal surgeons, radiologists and anesthesiologists, and it was decided that operative treatment was necessary. We performed an emergency endovascular repair of the AAA using an Excluder Stent Graft (Excluder[®], WL Gore and Associates, Flagstaff, Arizona, United States) (Figure 2).

We initiated empiric broad spectrum intravenous



Figure 1 An infrarenal aneurysm (arrow) of the abdominal aorta with gas collection in a suspected thrombotic mass in the aneurysmatic sac, suggesting an aortoenteric fistula.

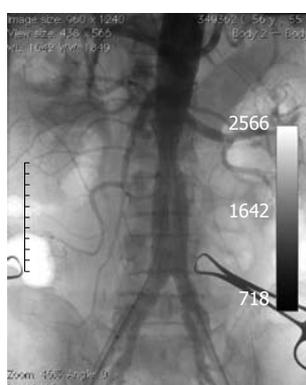


Figure 2 Intraoperative computed tomography angiography after endovascular repair of abdominal aortic aneurysm with excluder stent graft.

antibiotics (imipenem in combination with vancomycin) preoperatively, and continued with the same medication for 4 wk after endovascular repair. The patient then continued taking an oral antibiotic for the next two months (ciprofloxacin 1000 mg per day for 4 wk then doxycycline 100 mg per day for another 4 wk). The post-operative course was without complications and without further blood loss. After the patient was introduced to *per os* food intake, control CT angiography showed no endoleaks and a reduction in thrombotic mass volume in the aneurysm with regression of the aneurysm diameter, however, the gas collection persisted (Figure 3A).

On the tenth day after endovascular repair, RBC and HCT values were normal (RBC 4.2 million per μL and HCT 35%). The patient had improved, but the laboratory tests showed a CRP serum level of 56 mg/L and a high serum IgG level of 20.2 g/L and IgG4 of 4.24 g/L, respectively. Autoantibodies against lactoferrin and carbonic anhydrase II were negative, and autoantibodies including anti-nuclear antibodies, antineutrophil cytoplasmic antibodies and rheumatoid factor were not found. Markers for viral hepatitis Bs infection, hepatitis C virus, hepatitis A virus, human immunodeficiency viruses 1 and 2 were negative. Due to lack of histopathological findings, but elevated serum levels of IgG, it could not

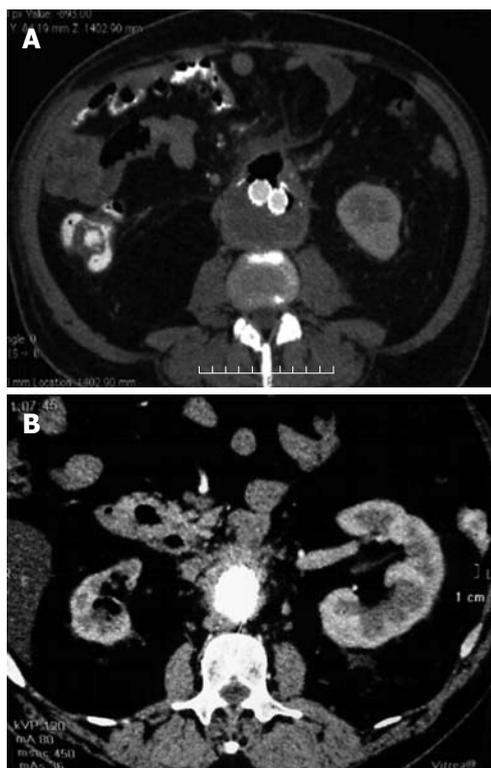


Figure 3 Control computed tomography angiography. A: Control computed tomography (CT) angiography shows no endoleaks and reduction of thrombotic mass volume in the aneurysmatic sac with persistence of gas collection; B: Control CT angiography after 6 mo showed no endoleaks and near total reduction of thrombotic mass in the aneurysmatic sac, without gas collection.

be determined with confidence that this was a systemic disease. It was decided that the patient should start steroid therapy with oral prednisolone at 40 mg per day for 4 wk, and then decrease the dosage by 5 mg every week. Four wk after initiation of steroid therapy, serum levels of IgG (16 g/L) and IgG4 (0.7 g/L) as well as CRP were normal. In the control examination conducted 6 mo after the patient's discharge from hospital (almost 3 mo after the end of steroid therapy), the levels of serum immunoglobulins and CRP were in the normal ranges and control CT angiography showed no endoleaks, near total reduction of the thrombotic mass in the aneurysmatic sac, and no gas collection (Figure 3B).

DISCUSSION

Primary ADF represents a serious condition and is much less frequent than a secondary ADF which occurs as a result of previous aortic aneurysm grafting. The first described case of primary ADF was more than a century ago, and since then about 250 cases have been reported. In the absence of treatment, the mortality rate is almost 100%. With surgical intervention, survival ranges from 18%-93%, but 40% of operated cases develop complications with an overall postoperative mortality rate greater than 30%^[12]. Diagnosis of primary ADF is also difficult. Only 33% to 50% of AEF are diagnosed preoperatively^[3]. Gastrointestinal bleeding and abdominal pain

have been described in all cases, but the classic trio of abdominal pain, palpable mass and gastrointestinal bleeding was found in only 6% to 27.8% of patients^[2,13,14]. Our patient only had gastrointestinal bleeding presenting as melena. Diagnostic procedures include EGDS, CT scan, and arteriography. Endoscopy is considered the modality of choice for initial evaluation of upper gastrointestinal bleeding, but has the potential risk of inducing massive hemorrhage by dislodging fresh thrombus in the fistula^[15,16]. In our case, EGDS findings were without signs of gastrointestinal hemorrhage. CT angiography was reliable in diagnosing AEF but may be somewhat challenging in unstable patients.

The surgical treatment of ADF consists of repairing the duodenal defect and performing a prosthetic repair of the aorta with graft. Where contamination is present, or in the case of mycotic aneurysm, an extraanatomical aortic graft is preferred and extensive debridement is required^[17]. Although the endovascular approach is increasingly utilized in the repair of secondary AEF, only a few primary AEF treated using this method have been reported^[4,11,12,18,19]. In the case of any applied vascular procedure, long-term broad spectrum antibiotic therapy is necessary^[11,12]. We believe that patients with persistent anemia who undergo laparotomy without diagnosis is hazardous.

Primary ADF is the result of inflammatory destruction of an aortic aneurysm arising from an atherosclerotic AAA. This is an etiological factor in 73% of all primary ADFs, while 26% are caused by traumatic or mycotic aneurysms^[2,17,20]. Rarer causes such as radiation, tumors, ulcers and ingestion of foreign bodies account of 1% or less^[4,12]. A very small number of AEF occur in the absence of an aortic aneurysm. In comparison to atherosclerotic AAA, IAAA occurs in only 2% to 10% of all AAA, and has different clinical characteristics, such as younger age of patients, non-specific symptoms, and large aneurysm diameter. Recent reports showed a close relationship between IgG4 and sclerosing lesions of various organs^[21]. IgG4-related sclerosing disease, which are also called IgG4-related sclerosing disease, was first reported with regard to autoimmune pancreatitis^[22]. The clinical characteristics of IgG4-related sclerosing disease are a frequent occurrence in adult male patients, and include elevation of serum IgG4 levels, and steroid sensitivity. In addition to affected organs in IgG4-related disease, elevated IgG4 levels have been described in atopic dermatitis, asthma, some parasitic diseases, pemphigus vulgaris, pemphigus foliaceus and pancreatic cancer^[23]. Our patient had no clinical signs of these diseases. The correct diagnosis of IAAA as part of IgG4-related disease is based on histopathological findings^[21]. According to these recommendations, the diagnosis of IAAA as part of IgG4-related disease in our patient could not be established due to lack of histopathological findings, as endovascular repair of AAA, was successful. If the AAA in our patient was an IAAA, this would be the first case with ADF in IgG4-related sclerosing disease. In addition, the patient had a positive response to steroid therapy. The

question remains as to how it is possible to make a diagnosis of IgG4-related IAAA in such cases where urgent minor surgery is the treatment of choice. In our case, endovascular repair of ADF was successful, and this may be an alternative treatment, especially in patients with high operative risk. Broad spectrum antibiotic therapy is as important as the endovascular treatment in ADF.

In conclusion, primary ADF is a rare complication of aortic aneurysms and is a rare cause of gastrointestinal bleeding. It can be associated with IgG4-related sclerosing disease, whether it occurs as a complication of IAAA or not. CT angiography is a good diagnostic test for an ADF. This case demonstrates that endovascular grafting of primary ADF as an effective, minimally invasive alternative to standard surgical repair. Other supportive therapy such as antibiotics are very important in the treatment of this condition. High serum IgG4 levels and a positive response to steroid therapy suggest the existence of an ADF in IgG4-related IAAA, without histopathological examination.

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Should anticoagulants be administered for portal vein thrombosis associated with acute pancreatitis?

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Abstract

Venous complications in patients with acute pancreatitis typically occur as a form of splenic, portal, or superior mesenteric vein thrombosis and have been detected more frequently in recent reports. Although a well-organized protocol for the treatment of venous thrombosis has not been established, anticoagulation therapy is commonly recommended. A 73-year-old man was diagnosed with acute progressive portal vein thrombosis associated with acute pancreatitis. After one month of anticoagulation therapy, the patient developed severe hematemesis. With endoscopy and an abdominal computed tomography scan, hemorrhages in the pancreatic pseudocyst, which was ruptured into the duodenal bulb, were confirmed. After conservative treatment, the patient was stabilized. While the rupture of a pseudocyst into the surrounding viscera is a well-known phenomenon, spontaneous rupture into the duodenum is rare. Moreover, no reports of upper gastrointestinal bleeding caused by pseudocyst rupture

in patients under anticoagulation therapy for venous thrombosis associated with acute pancreatitis have been published. Herein, we report a unique case of massive upper gastrointestinal bleeding due to pancreatic pseudocyst rupture into the duodenum, which developed during anticoagulation therapy for portal vein thrombosis associated with acute pancreatitis.

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Key words: Pancreatitis; Pancreatic pseudocyst; Portal vein; Venous thrombosis; Warfarin

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INTRODUCTION

The vascular complications of pancreatitis are major causes of morbidity and mortality and are typically related to hemorrhage. Venous complications generally occur as a form of thrombosis in the splenic vein and less commonly in the portal or superior mesenteric vein^[1]. Although no randomized controlled trial regarding the use of anticoagulants in acute portal vein thrombosis has been conducted, the use of unfractionated heparin, with subsequent transition to oral warfarin, is the most common approach to anticoagulation^[2,3].

Pancreatic pseudocysts are common findings, but spontaneous rupture occurs infrequently. Pseudocyst rupture can occur in the free peritoneal cavity, stomach,

duodenum, colon, portal vein, pleural cavity, and abdominal wall. However, rupture into the duodenum is very rare^[4]. Furthermore, no reports of upper gastrointestinal bleeding caused by pseudocyst rupture in patients under anticoagulation therapy for venous thrombosis associated with acute pancreatitis have been published.

We describe herein a very rare and unique case of massive upper gastrointestinal bleeding due to pancreatic pseudocyst rupture into the duodenum, which developed during anticoagulation therapy for portal vein thrombosis associated with acute pancreatitis.

CASE REPORT

A 73-year-old man was transferred to our emergency department for the management of massive hematemesis. On arrival, his systolic blood pressure was 70 mmHg, and his blood hemoglobin level was 6.8 g/dL. He received a transfusion of two units of packed red blood cells. Seven weeks prior, he had initially developed acute alcoholic pancreatitis, and one month prior, he had been diagnosed with acute progressive portal vein thrombosis associated with non-necrotizing acute alcoholic pancreatitis. Since then, he had been receiving anticoagulation therapy. Computed tomography (CT) scans of the abdomen revealed not only thrombosis of the portal vein trunk but also thrombosis of the right and left portal veins (Figure 1A), and low perfusion areas were diffusely observed on the liver parenchyma. A 12 mm × 10 mm cystic lesion was found, suggesting the presence of a pancreatic pseudocyst and extensive fluid collection around the pancreatic head (Figure 1B). Endoscopic ultrasonography also demonstrated the absence of a color Doppler signal and thrombosis of the portal vein, superior mesenteric vein, and distal splenic vein. Arterial portography confirmed an occlusion and thrombosis of the portal vein, superior mesenteric vein, and distal splenic vein. The factors indicating a coagulation defect and tumor markers were normal. We decided to perform anticoagulation therapy because the patient complained of abdominal pain, and serial CT scans showed the progression of the acute portal vein thrombosis. He was treated with intravenous heparin and then switched to warfarin.

Endoscopy and abdominal CT scans were performed to evaluate the hematemesis. Emergency endoscopy (Figure 2A) revealed a 2 cm submucosal tumor-like lesion located just distal to the pylorus. The mucosa showed a central ulceration covered with dark blood clots, which was suggested as a cause of the upper gastrointestinal bleeding. Abdominal CT scans (Figure 1C) revealed a large, walled-in fluid collection of high attenuation around the pancreatic head, indicating a hemorrhagic pseudocyst of the pancreatic head that caused the displacement of the duodenum.

The patient was treated with a high dose of pantoprazole, a proton pump inhibitor, and the warfarin medication was stopped. The patient was hemodynamically

stabilized, and no recurrent bleeding occurred. On the seventh day following admission, follow-up endoscopy (Figure 2B) revealed a reduction of the bulging lesion and a fistula opening with clear discharge, which was suspected to be pancreatic fluid. A follow-up abdominal CT scan (Figure 1D) showed a small collapsed cyst without internal hematoma, and a fistulous tract was also observed between the duodenum and the pseudocyst, resulting in air pocket in the cyst. After only conservative treatment, the patient recovered without recurrent bleeding and was discharged without warfarin medication.

DISCUSSION

Pancreatitis may cause a spectrum of venous and arterial vascular complications. Of these complications, venous thromboses are typically reported in the splenic vein and less commonly in the superior mesenteric vein or portal vein^[5]. Thrombotic complications have been known to be more common in alcohol-induced, necrotizing, and chronic pancreatitis^[6,7]. It has been suggested that the pathogenesis of venous thromboses involves stasis, spasm, and mass effects from the surrounding inflamed pancreas and direct damage of the venous wall by liberated enzymes^[5]. Our case was associated with non-necrotizing, alcoholic pancreatitis. The portal vein was mainly involved, although venous thromboses also occurred in the distal splenic vein and superior mesenteric vein.

Specific therapeutic management for portal vein thrombosis seems to be mandatory to resolve portal vein obstruction, thereby preventing the development of chronic portal vein thrombosis and avoiding serious complications, such as portal vein hypertension, mesenteric ischemia, and infarction^[2,3]. However, randomized controlled studies on the efficacies of most forms of therapy for portal vein thrombosis are lacking. The use of unfractionated heparin, with subsequent transition to oral warfarin is the most common approach to anticoagulation^[2], while Gonzelez *et al*^[8] recently reported that recanalization is observed in almost one third of patients, irrespective of whether they receive systemic anticoagulation. The issue of warfarin dose has not been addressed in randomized trials, and the optimal duration of anticoagulation is controversial^[2]. What is certain is that the sooner the treatment is given, the better the outcome will be; the rate of recanalization is approximately 69% if anticoagulation is initiated within the first week after diagnosis, while it falls to 25% when initiated during the second week^[3].

In our case, anticoagulation therapy was not started at an earlier stage of portal venous thrombosis but began after the confirmation of thrombotic progression, which could explain why the patient achieved only partial portal vein recanalization. Fortunately, the patient did not develop mesenteric ischemia or infarction, and the portal vein thrombosis did not worsen further.

Gastrointestinal bleeding in the setting of pancreatitis arises from vascular complications or coexisting

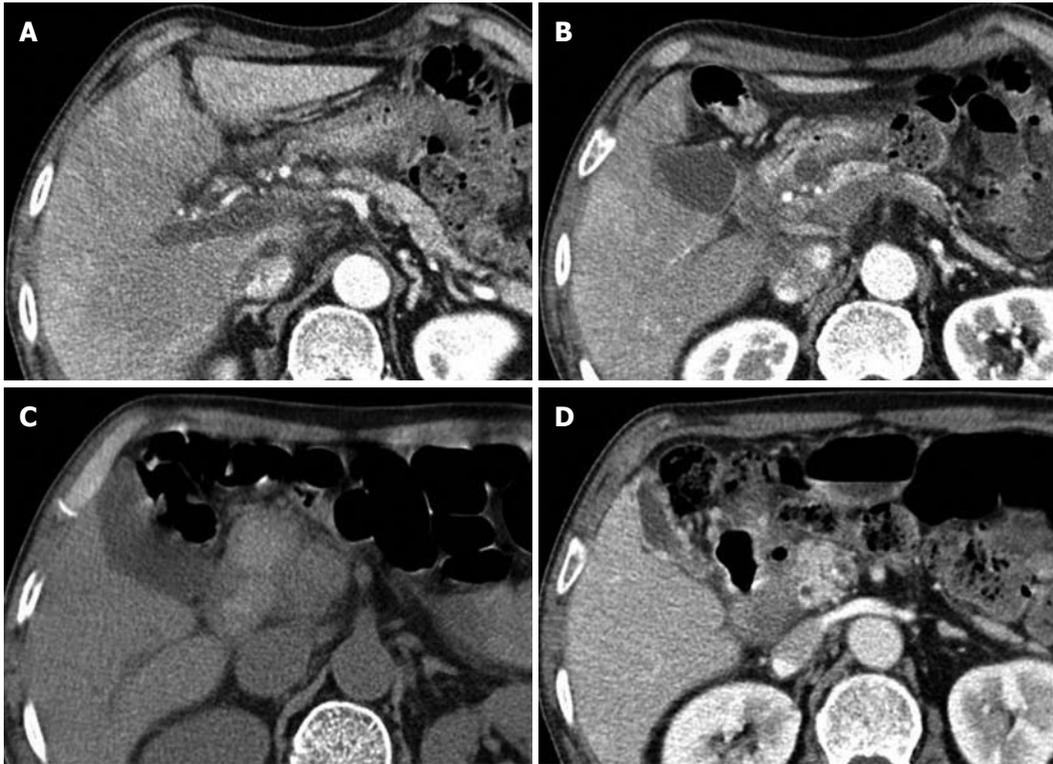


Figure 1 Abdominal computed tomography images. A: A filling defect and intra-luminal hypoattenuation in the portal vein consistent with acute portal vein thrombosis; B: A 12 mm × 10 mm cystic lesion suggestive of a pseudocyst and extensive inflammatory fluid collections around the pancreatic head; C: A walled-in fluid collection with high attenuation, indicative findings of a hemorrhagic pseudocyst around the pancreatic head and compressed duodenum; D: Reduced hemorrhagic fluid collections and an air-filled cyst, indicative of a fistula between the duodenum and the pseudocyst.

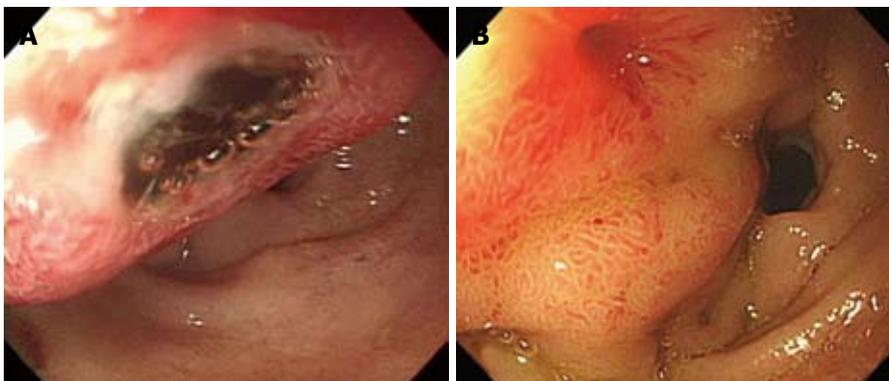


Figure 2 Upper gastrointestinal endoscopy. A: Emergency endoscopy shows a 2 cm submucosal tumor-like bulging mass with central ulceration; B: A follow-up endoscopy image showing a decreased mass size and fistula orifice.

lesions^[9,10]. Major gastrointestinal bleeding is considered to be rare, but its exact incidence is not well established. According to a recent report of 1356 acute pancreatitis patients, spontaneous bleeding occurred in 10 patients, and 6 patients had gastrointestinal bleeding^[10]. The most frequent cause of severe bleeding in pancreatitis is a ruptured pseudoaneurysm, which accounts for approximately 60% of cases, and a hemorrhagic pseudocyst without a pseudoaneurysm and a capillary, venous, or small vessel hemorrhage each account for approximately 20% of cases^[7,11]. In a review of cases from 1987 to 1996, 31 patients were found to have developed vascular lesions either in

the form of hemorrhage into a pseudocyst (12 patients) or pseudoaneurysms (19 patients)^[12].

In the management of any hemorrhage, the early recognition and investigation of hemorrhagic episodes is imperative because accurate diagnosis and timely radiological interventional procedures can reduce mortality^[7]. Dynamic bolus CT and angiography are considered to be the most useful means of finding a hemorrhage^[13]. In particular, angiography can play an invaluable role both in locating the source of bleeding and in the embolization of the bleeding vessel^[14]. In the present case, the diagnosis of a ruptured bleeding pseudocyst was made

with CT scans and endoscopy, and the ruptured bleeding pseudocyst confirmed the presence of the fistula tract and the walled-in fluid collection with high attenuation around the pancreatic head, providing evidence supportive of a bleeding pseudocyst. Due to the hemodynamically stable clinical conditions and no evidence of pseudoaneurysm formation on CT scans, we did not perform angiography or surgical therapy and decided to pursue a “watchful waiting” policy.

The rupture of a pseudocyst into the gastrointestinal tract either results in no symptoms or leads to melena or hematemesis that typically requires urgent measures^[15]. Although rupture into the surrounding viscera is a well-known phenomenon, the spontaneous rupture of a pseudocyst into the upper gastrointestinal tract is very rarely reported^[4,16-21], and the spontaneous rupture of a pseudocyst into the duodenum most frequently occurs in the second portion of the duodenum^[21]. In the presented case, the first part of the duodenum was involved, and rupture into the duodenum led to hematemesis requiring transfusion.

In our case, the exact pathogenic mechanisms of the bleeding and rupture of the pancreatic pseudocysts were unclear. However, we can suggest some possibilities as to why massive bleeding due to pseudocyst rupture occurred. First, the pseudocyst may have been tensely distended because of intra-cystic bleeding caused by warfarin use and eventually ruptured into the duodenum; Second, the rupture of the pancreatic pseudocyst could have developed first, and severe bleeding of the ruptured duodenal mucosa followed due to the anticoagulation therapy. Consequently, we considered that the warfarin either initiated or aggravated the bleeding into the duodenum. Finally, in this case, it was not clear whether portal hypertension by portal vein thrombosis affected the bleeding risk itself and its severity.

In conclusion, we report a rare case of massive upper gastrointestinal bleeding due to pancreatic pseudocyst rupture into the duodenum, which developed during anticoagulation therapy for acute pancreatitis associated with portal vein thrombosis. Gastroenterologists should consider a pseudocyst rupture into the gastrointestinal tract as a bleeding source in patients with pancreatitis and should also keep in mind that anticoagulants used to manage portal vein thrombosis associated with acute pancreatitis can lead to serious bleeding.

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Postoperative retroperitoneal desmoid tumor mimics recurrent gastrointestinal stromal tumor: A case report

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and surgical resection is the treatment option depending on the anatomic location.

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Abstract

Desmoid tumor is a locally invasive, myofibroblastic, nonmetastatic tumor. Its pathogenesis remains unclear and it may involve genetic abnormalities, sex hormones and traumatic injury, including surgery. Postoperative intra-abdominal desmoid tumor is rare, especially in the retroperitoneum. We report a case of postoperative retroperitoneal desmoid tumor that developed 29 mo after the first excision of a gastrointestinal stromal tumor. Sporadic trauma-related intra-abdominal desmoid tumors reported in the English literature are also reviewed. Despite an extremely low incidence, postoperative desmoid tumor should be considered in the differential diagnosis when a recurrent neoplasm is found at least one year after operation. However, it is a clinical challenge to distinguish recurrent malignant neoplasms from desmoid tumors,

INTRODUCTION

Desmoid tumor (also known as aggressive fibromatosis or fibromatosis) is an infrequently occurring, locally invasive, nonmetastatic tumor. Desmoid tumors account for 0.03% of all newly diagnosed neoplasms and 3% of all soft tissue neoplasms^[1]. These tumors originate from musculoaponeurotic planes and are found intra- and extra-abdominally. The cause of desmoid tumor is unclear and it may be related to trauma, hormonal factors, or genetic associations^[2]. The occurrence of a surgery-related retroperitoneal desmoid tumor after the excision of gastrointestinal stromal tumor (GIST) is rare. However, despite its scarcity, desmoid tumor should be differentiated from recurrent neoplasm in diagnosis, especially those occurring near the previous operative site.

CASE REPORT

A 56-year-old man complained of epigastralgia for three weeks without symptomatic improvement after receiving medical treatment at another hospital in 2009, where a deep-seated gastric ulcer had been found by panendoscopy. Because there had been no symptomatic relief, he was referred to the in-patient department of our hospital. On physical examination, abdominal fullness with obvious rebounding pain was noticed. A perforated peptic ulcer was suspected and an abdominal computed tomography (CT) scan was performed. The abdominal CT scan revealed a 10-cm gastric mass with perforation (Figure 1A). In May 2009, the patient underwent an emergent explorative laparotomy with debulking of the intraperitoneal tumor and irrigation. A ruptured GIST, of intermediate risk category, with spreading intra-abdominal tumors was diagnosed pathologically (Figure 1B and C). He had no family history of familial adenomatous polyposis (FAP) or colorectal diseases among his close relatives. Because the abdominal CT scan had shown no signs of FAP, colonoscopy was not performed. His clinical course was uneventful and he was discharged on day 8 after admission. After the operation, imatinib (Glivec) 200 mg *b.i.d.* was prescribed. However, due to intractable diarrhea, the target therapy was discontinued. The follow-up abdominal CT scan showed no recurrent tumor two months after the operation. He was then lost to follow-up. The patient revisited our out-patient clinic 12 mo later after the previous operation. The abdominal CT scan showed a 4.7-cm hepatic tumor in segments 6 and 7 and an intra-abdominal 2.5-cm mass located in the upper greater curvature of the stomach (Figure 1D). The impression was metastatic GISTs. He received a second operation with a smooth course in June 2010 and metastatic GISTs were confirmed pathologically (Figure 1E). However, a 1.9-cm tumor between the pancreatic tail and splenic hilum was found in the follow-up abdominal CT scan 3 mo after the second operation. The tumor was located in the incision area of the first debulking operation. The patient refused another operation, and he was managed with target therapy with imatinib (Glivec) 200 mg *b.i.d.*, which was well tolerated. A series of follow-up abdominal CT scans were performed. Fifteen months after the second operation, CT scan revealed progressive enlargement of the splenic hilar tumor from 1.9 cm to 3.2 cm (Figure 1F). The patient continued to take imatinib (Glivec) regularly. Under the impression of a second recurrent GIST, he underwent a third operation in October 2011. Intraoperatively, the tumor was found in the retroperitoneum adhering to the peri-tumor vessels, nerves and the pancreatic tail. This tumor was resected *en bloc* with sacrifice of adjacent vessels and nerves. Grossly, the tumor measured 3.6 cm × 2.5 cm × 1.1 cm and was elastic with grayish-brown cut surface. Histologically, the tumor demonstrated proliferative spindle cells in fibrotic background with keloid-like, glassy, hyalinized collagen fibers, nodular fasciitis-like erythrocyte extravasation and infiltrative growth pattern.

Immunohistochemical staining of the tumor cells revealed positive nuclear stains for beta-catenin, but negative stains for CD117 and CD34 (Figure 1G). A desmoid tumor was diagnosed pathologically. The patient's clinical course was uneventful and no recurrent tumor was found 6 mo after the third operation.

DISCUSSION

Although desmoid tumor is locally aggressive with infiltrative growth behavior, it is considered to be a benign neoplasm for its bland cellular appearance, scant mitosis and lack of metastasis. The term "desmoid" originates from the Greek word "demos", meaning band or tendon like, and was first named by Müller^[3] in 1838. Desmoid tumor accounts for 0.03% of all neoplasms and 3.0% of all soft tissue tumors^[1].

Although the actual cause of desmoid tumor is still being debated, its likely pathogenesis includes genetic abnormalities, sex hormones and trauma, including surgical trauma. Kulaylat *et al.*^[2] found that 10%-30% of all sporadic abdominal wall desmoid tumors occurred following surgical intervention and half of these tumors developed within 4 years after the surgery. Warren^[4] described the criteria for post-traumatic neoplasm, including prior integrity of the tumor site, injury severe enough to initiate reparative cell proliferation, reasonable latent period, and tumor compatible with the scar tissue and anatomic location of the injury. In the present case, the splenic hilar region was incised during the debulking operation for the ruptured GIST of the stomach, and the desmoid tumor occurred 29 mo after the surgery. The present case of postoperative retroperitoneal desmoid tumor was compatible with Warren's criteria^[4] and occurred within an appropriate latent period.

The English literature includes 12 cases, including the present case, of sporadic postoperative intra-abdominal desmoid tumor^[5-15] (Table 1). The male-to-female ratio was 8:3 (one case showed no gender datum) and the mean age was 49.6 years (range, 27-79 years). Five of the 12 cases were located on the mesentery, which is the most frequent site of this type of desmoid tumor to date. Tumor sizes ranged from 2.8 cm to 18 cm and the mean duration from previous operative insult to excision of desmoid tumor was 2.3 years (range, 11 mo to 7 years). It indicates that a reasonable latent period for this type of desmoid tumor is at least one year. None of the desmoid tumors was diagnosed preoperatively. This denotes the diagnostic challenge of this type of desmoid tumor. Ten of the 12 desmoid tumors were widely excised under the impression of recurrent malignant neoplasm. No recurrence was found in 8 cases followed up between 6 mo to 2 years. Up till now, only 3 postoperative desmoid tumors after resection of gastric GIST have been reported in the literature. Whether GIST is a risk factor for the development of desmoid tumors or just a coincidence should be further elucidated.

Recently, the Wnt/Wingless/Wnt signaling pathway was hypothesized to be involved in the tumorigenesis of

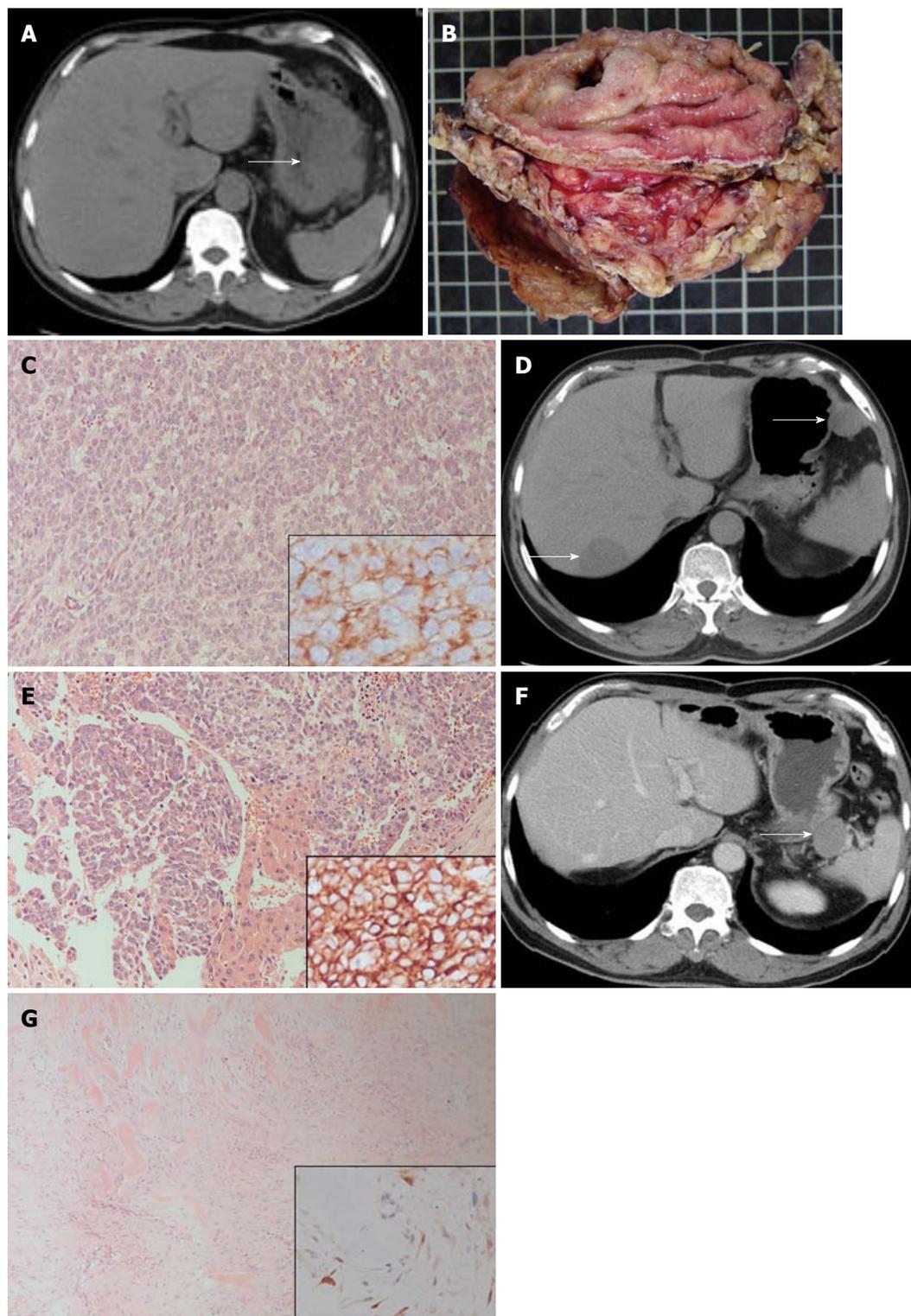


Figure 1 Abdominal computed tomography images and pathologic features of gastrointestinal stromal tumor, metastatic hepatic gastrointestinal stromal tumor and retroperitoneal desmoid tumor. A: Abdominal computed tomography (CT) scan reveals a 10-cm tumor (arrow) located at the greater curvature of the stomach; B: The excised specimen discloses a patch of gastric mucosa with a central deep-seated ulcer and the gastrointestinal stromal tumor adhering to the red yellow omental tissues; C: Histologically, the 10-cm gastric tumor demonstrated sheets of CD117-positive epithelioid cells after immunohistochemical (IHC) staining (right lower inset), hematoxylin and eosin (HE) stain, $\times 200$; D: The follow-up abdominal CT scan shows metastatic tumors in the liver (left lower arrow) and upper greater curvature of the stomach (right upper arrow); E: The histology of metastatic liver tumor demonstrated nests and sheets of CD117-positive epithelioid tumor cells (right lower inset, IHC stain). Entrapped hepatocytes are seen in the middle lower portion, HE stain, $\times 200$; F: Abdominal CT scan discloses a tumor neogrowth (arrow) located in the retroperitoneum between the pancreatic tail and splenic hilar region; G: Histologically, the retroperitoneal tumor demonstrated proliferative spindle cells with keloid-like bundles and erythrocyte extravasation, HE stain, $\times 200$. IHC staining reveals a positive nuclear beta-catenin in spindle cells (right lower inset).

Table 1 Sporadic postoperative intra-abdominal desmoid tumors reported in the literature

No.	Authors	Sex/age	Location	Tumor size(cm)	Diagnosis of previous operation	Type of primary trauma	Duration	Treatment	R/F
1	Mizuno <i>et al</i> ^[5]	M/61	Mesentery near anastomosis	2.8	Ascending colon cancer (pT3N1M0)	Right hemicolectomy	16 mo	Excision with ileum and colon	N/2 yr
2	Liao <i>et al</i> ^[6]	F/79	Left lower abdomen	17 × 14 × 13	Encapsulated lipoma	Resection	7 yr	Excision	N/6 mo
3	Khan <i>et al</i> ^[7]	-/37	Mesentery	6 × 4.5 × 4	Gastric GIST	Total gastrectomy	11 mo	Excision	N/8 mo
4	Vendrell <i>et al</i> ^[8]	M/58	Posterior side of stomach	8 × 6 × 4	Gastric GIST	Laparoscopic tumorectomy	2 yr	Wide excision with splenectomy and total gastrectomy	-
5	Komatsu <i>et al</i> ^[9]	M/50	Mesentery in left upper abdominal cavity	10 × 6	Gastric adenocarcinoma (pT3N1M0)	Total gastrectomy	1 yr	Excision with jejunum	N/9 mo
6	Tamura <i>et al</i> ^[10]	F/73	Mesentery of jejunal pouch	6.3 × 5 × 5	Gastric cancer (pT1N1M0)	Total gastrectomy	1 yr	Excision and reconstruction	N/4 yr
7	Lawatsch <i>et al</i> ^[11]	M/27	Retroperitoneum	17 × 13.5 × 8.5	Mixed germ cell tumor of right testis	Retroperitoneal lymph node dissection	2 yr	Excision with ileum and colon	N/10 mo
8	Lai <i>et al</i> ^[12]	M/45	Anterior lower abdomen	-	Mesenteric injury and bowel gangrene	Abdominal surgery	1 yr	Excision with ileum	-
9	Firoozmand <i>et al</i> ^[13]	F/27	Pelvic	17 × 14 × 10	-	Colectomy with ileoanal J pouch anastomosis	4 yr	En bloc resection	-
10	Little <i>et al</i> ^[14]	M/31	Left upper abdomen	12 to 14	Mixed germ cell tumor of right testis	Retroperitoneal lymph node dissection	2 yr	Excision with jejunum	N/18 mo
11	Pasciak <i>et al</i> ^[15]	M/51	Mesentery	18 × 12 × 11	Transitional cell carcinoma of urinary bladder	Radical cystectomy	3 yr	Excision with small bowel	-
12	Shih <i>et al</i> , present	M/56	Retroperitoneum	3.6 × 2.5 × 1.1	Gastric GIST	Exploratory debulking	29 mo	En bloc excision	N/6 mo

R: Recurrency; F: Follow-up; N: No; -: Not mentioned; GIST: Gastrointestinal stromal tumor.

desmoid tumors, especially the two key proteins, adenomatous polyposis coli (APC) and beta-catenin^[16]. APC is considered to be a tumor suppressor gene and beta-catenin an oncogene. Sporadic desmoid tumors typically present oncogenic mutation in beta-catenin. However, FAP-associated desmoid tumors are associated with germline APC mutation followed by somatic inactivation of the wild-type APC allele^[17,18]. Beta-catenin protein level is upregulated in desmoid tumors, due to either APC mutations and subsequent ineffective regulation of beta-catenin activation, or beta-catenin gene mutations that led to stabilization and constitutive activation of the beta-catenin. These pathways indicated that the expression of nuclear beta-catenin may play a role in the differential diagnosis of desmoid tumors from fibroblastic or smooth muscle neoplasms^[16].

Primary wide surgical resection with tumor-free margins is the treatment of choice for desmoid tumor. This surgical strategy made it essential to sometimes sacrifice the adhered normal vital tissues such as vessels and nerves. Resections with tumor-positive margins indicate a high risk of recurrence, and secondary resection, chemotherapy or radiotherapy may be performed consequently according to the patient's condition^[19]. Garbay *et al*^[20] treated 62 patients with cytotoxic chemotherapy for progressive or recurrent desmoid tumors, and 80%

of the patients had a clinical benefit (objective response plus stable disease) from the cytotoxic chemotherapy. Anthracycline-containing regimens appeared to be associated with a higher response rate. Radiotherapy for unresectable cases or local tumor control after surgery has been empirically applied in some instances^[21]. However, the efficacy of the adjuvant treatment still needs to be further elucidated. Recently, three discrete mutations (ACC41GCC, TCT45TTT and TCT45CCT) in two codons of CTNNB1 exon 3 were reported^[22]. Target therapy for desmoid tumor may be feasible and become another treatment option in the future.

Postoperative intra-abdominal desmoid tumor is exceptionally rare and is often overlooked by clinicians. Most clinicians believe that a tumor with locally infiltrative growth behavior in patients with a history of malignancy is a recurrent malignant tumor, and a wide excision with sacrifice of adjacent organs is usually done. However, despite an extremely low incidence, a postoperative desmoid tumor should be included in the differential diagnoses.

In conclusion, postoperative intra-abdominal desmoid tumor is rare and a correct preoperative diagnosis is a clinical challenge. Physicians should keep in mind the possibility that a postoperative desmoid tumor may appear as a so-called "recurrent" neoplasm, especially when the tumor presents at the previous surgical site.

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Events Calendar 2012

January 13-15, 2012
 Asian Pacific *Helicobacter pylori*
 Meeting 2012
 Kuala Lumpur, Malaysia

January 19-21, 2012
 American Society of Clinical
 Oncology 2012 Gastrointestinal
 Cancers Symposium
 San Francisco, CA 3000,
 United States

January 19-21, 2012
 2012 Gastrointestinal Cancers
 Symposium
 San Francisco, CA 94103,
 United States

January 20-21, 2012
 American Gastroenterological
 Association Clinical Congress of
 Gastroenterology and Hepatology
 Miami Beach, FL 33141,
 United States

February 3, 2012
 The Future of Obesity Treatment
 London, United Kingdom

February 16-17, 2012
 4th United Kingdom Swallowing
 Research Group Conference
 London, United Kingdom

February 23, 2012
 Management of Barretts
 Oesophagus: Everything you need
 to know
 Cambridge, United Kingdom

February 24-27, 2012
 Canadian Digestive Diseases Week
 2012
 Montreal, Canada

March 1-3, 2012
 International Conference on
 Nutrition and Growth 2012
 Paris, France

March 7-10, 2012
 Society of American Gastrointestinal
 and Endoscopic Surgeons Annual
 Meeting
 San Diego, CA 92121, United States

March 12-14, 2012
 World Congress on
 Gastroenterology and Urology
 Omaha, NE 68197, United States

March 17-20, 2012
 Mayo Clinic Gastroenterology and
 Hepatology
 Orlando, FL 32808, United States

March 26-27, 2012
 26th Annual New Treatments in
 Chronic Liver Disease
 San Diego, CA 92121, United States

March 30-April 2, 2012
 Mayo Clinic Gastroenterology and
 Hepatology
 San Antonio, TX 78249,
 United States

March 31-April 1, 2012
 27th Annual New Treatments in
 Chronic Liver Disease
 San Diego, CA 92121, United States

April 8-10, 2012
 9th International Symposium on
 Functional GI Disorders
 Milwaukee, WI 53202, United States

April 13-15, 2012
 Asian Oncology Summit 2012
 Singapore, Singapore

April 15-17, 2012
 European Multidisciplinary
 Colorectal Cancer Congress 2012
 Prague, Czech

April 18-20, 2012
 The International Liver Congress
 2012
 Barcelona, Spain

April 19-21, 2012
 Internal Medicine 2012
 New Orleans, LA 70166,
 United States

April 20-22, 2012
 Diffuse Small Bowel and Liver
 Diseases
 Melbourne, Australia

April 22-24, 2012
 EUROSON 2012 EFSUMB Annual

Meeting
 Madrid, Spain

April 28, 2012
 Issues in Pediatric Oncology
 Kiev, Ukraine

May 3-5, 2012
 9th Congress of The Jordanian
 Society of Gastroenterology
 Amman, Jordan

May 7-10, 2012
 Digestive Diseases Week
 Chicago, IL 60601, United States

May 17-21, 2012
 2012 ASCRS Annual Meeting-
 American Society of Colon and
 Rectal Surgeons
 Hollywood, FL 1300, United States

May 18-19, 2012
 Pancreas Club Meeting
 San Diego, CA 92101, United States

May 18-23, 2012
 SGNA: Society of Gastroenterology
 Nurses and Associates Annual
 Course
 Phoenix, AZ 85001, United States

May 19-22, 2012
 2012-Digestive Disease Week
 San Diego, CA 92121, United States

June 2-6, 2012
 American Society of Colon and
 Rectal Surgeons Annual Meeting
 San Antonio, TX 78249,
 United States

June 18-21, 2012
 Pancreatic Cancer: Progress and
 Challenges
 Lake Tahoe, NV 89101, United States

July 25-26, 2012
 PancreasFest 2012
 Pittsburgh, PA 15260, United States

September 1-4, 2012
 OESO 11th World Conference
 Como, Italy

September 6-8, 2012
 2012 Joint International

Neurogastroenterology and Motility
 Meeting
 Bologna, Italy

September 7-9, 2012
 The Viral Hepatitis Congress
 Frankfurt, Germany

September 8-9, 2012
 New Advances in Inflammatory
 Bowel Disease
 La Jolla, CA 92093, United States

September 8-9, 2012
 Florida Gastroenterologic Society
 2012 Annual Meeting
 Boca Raton, FL 33498, United States

September 15-16, 2012
 Current Problems of
 Gastroenterology and Abdominal
 Surgery
 Kiev, Ukraine

September 20-22, 2012
 1st World Congress on Controversies
 in the Management of Viral Hepatitis
 Prague, Czech

October 19-24, 2012
 American College of
 Gastroenterology 77th Annual
 Scientific Meeting and Postgraduate
 Course
 Las Vegas, NV 89085, United States

November 3-4, 2012
 Modern Technologies in
 Diagnosis and Treatment of
 Gastroenterological Patients
 Dnepropetrovsk, Ukraine

November 4-8, 2012
 The Liver Meeting
 San Francisco, CA 94101,
 United States

November 9-13, 2012
 American Association for the Study
 of Liver Diseases
 Boston, MA 02298, United States

December 1-4, 2012
 Advances in Inflammatory Bowel
 Diseases
 Hollywood, FL 33028, United States

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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 PMCID: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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- 9 Outreach: Bringing HIV-positive individuals into care. *HRS A Careaction* 2002; 1-6 [PMID: 12154804]

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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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- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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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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Portal biliopathy

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Abstract

Biliary ductal changes are a common radiological finding in patients with portal hypertension, however only a small percentage of patients (5%-30%) develop symptomatic bile duct obstruction. The exact pathogenesis is not clear, but an involvement of factors such as bile duct compression by venous collaterals, ischemia, and infection is accepted by most authors. Although endoscopic retrograde cholangiopancreatography was used to define and diagnose this condition, magnetic resonance cholangiopancreatography is currently the investigation of choice for diagnosing this condition. Treatment is indicated only for symptomatic cases. Portosystemic shunts are the treatment of choice for symptomatic portal biliopathy. In the majority of patients, the changes caused by biliopathy resolve after shunt surgery, however, 15%-20% patients require a subsequent bilio-enteric bypass or endoscopic management for persistent biliopathy. There is a role for endoscopic therapy in patients with bile duct stones, cholangitis or when portosystemic shunt surgery is not feasible.

INTRODUCTION

The term portal biliopathy (syn. pseudosclerosing cholangitis) was first coined in the 1990s^[1], where it was used to describe abnormalities in the intrahepatic and extrahepatic biliary tract, gallbladder and cystic duct secondary to portal hypertension. However, jaundice and common bile duct (CBD) compression associated with portal hypertension had been, in fact, described by Fraser *et al*^[2] in 1944 and by Gibson *et al*^[3] in 1965. Hunt^[4], also in 1965, described the treatment of CBD obstruction secondary to distended venous collaterals.

Portal biliopathy is predominantly associated with extrahepatic portal venous obstruction (EHPVO). Studies in these patients using endoscopic retrograde cholangiopancreatography (ERCP) have shown that changes in the bile ducts occur in 81%-100% of them, although only 5%-30% have symptoms of biliary obstruction^[5-10]. The condition has also been described in patients who have non-cirrhotic portal fibrosis (NCPF) and cirrhosis, albeit in smaller numbers.

Portosystemic shunting as a decompressive treatment for portal biliopathy was first described in 1989

by Choudhuri *et al*^[11]. However, subsequent studies have shown that in a subset of patients the biliary obstruction is not relieved by portosystemic shunts alone and requires an additional biliary drainage procedure. The characteristics of patients in whom the biliary obstruction is not reversed after a portosystemic shunt and the role of endoscopic management of the condition is still not clear.

In this article, we review the current data on portal biliopathy and outline the various controversies associated with its effective treatment.

The anatomical basis for the condition was suggested by the works of Petren^[12] and Saint^[13] who described the venous anatomy of the bile ducts in 1932 and 1971 respectively. An epicholedochal plexus (of Saint) forms a reticular network of veins (maximum size 1 mm) on the outer surface of the bile ducts. The paracholedochal network of Petren courses parallel to the CBD and is connected to the gastric, pancreaticoduodenal and portal veins below, and to the liver above.

PATHOGENESIS

There are three main theories for the pathogenesis of portal biliopathy; that it is the result of compression of the bile ducts, ischemia, or infection.

Compression theory

The first cholangiographic evidence of CBD varices compressing the bile duct was published by Williams *et al*^[14] in 1982. In EHPVO, long standing obstruction of the portal vein leads to replacement of the portal vein by large collaterals along the CBD - the so-called cavernomatous transformation of the portal vein. These large collaterals compress the pliable CBD, leading to the changes seen on ERCP^[5-7]. Also, with increased duration of portal thrombosis, there is vascular neogenesis and formation of tumor-like connective tissue, which can encase the CBD or cause angulation of the bile ducts^[9]. In a study by Dilawari *et al*^[5], 18 out of 20 patients had indentations suggestive of external compressions on ERCP. The reversibility of biliary tract changes after portal decompressive surgery^[11,15-17] or transjugular intrahepatic portosystemic shunts (TIPS)^[18,19], shown in various studies, further corroborates this mechanism.

Ischaemic theory

According to this theory, longstanding portal thrombosis leads to sclerosis of the veins draining the bile ducts, which in turn can lead to damage to the capillaries and arterioles. This interruption of the vascular supply can, in turn, lead to the development of ischaemic strictures in the bile duct which are not reversed after a portosystemic shunt or TIPS. In a study by Khuroo *et al*^[7], strictures in the CBD, both short segment and long confluent, were the most common findings seen on ERCP suggestive of an ischemic pathology. Dhiman

et al^[20] studied bile duct changes after shunt surgery in 5 patients by performing ERCP pre- and post-surgery and reported complete reversal in one patient, partial reversal in three and no reversal in one patient, postulating that ischemia or scarring may be the etiology behind persistence of bile duct changes.

Infective theory

Infection or cholangitis was postulated by some authors in earlier studies to be the cause of jaundice in patients with portal vein thrombosis^[15,21,22]. However, later cholangiographic studies have shown that changes in the biliary tract are seen even in asymptomatic patients, and cholangitis occurs late in its natural history. Cholangitis, once present, may lead to inflammation, neogenesis and deposition of fibrous tissue, along with persistence of strictures following shunt surgeries.

All the above mentioned mechanisms may be present simultaneously, resulting in the characteristic changes of portal biliopathy.

EPIDEMIOLOGY

Portal biliopathy is an uncommon presentation in patients with portal hypertension. In 1992, Dilawari *et al*^[5] published a series of 20 patients with EHPVO in which ERCP was done prospectively, and found that changes in the biliary tract were seen in all of them. The left hepatic ducts were always involved, the right ductal system was involved in 56% and there were changes in the common bile duct in 90%. Sarin *et al*^[6] found the incidence on ERCP to be 80%. Studies published by various authors utilising ERCP/magnetic resonance cholangiopancreatography (MRCP) found the frequency to be between 81%-100%^[5-10]. Portal biliopathy is also seen in patients with cirrhosis of the liver (0%-30%)^[1,23,24] and NCPF (9%-40%)^[1,24]. The natural history of portal biliopathy is not known. The majority of patients (70%-95%) do not manifest with any symptoms of biliary obstruction. However, patients with symptomatic portal biliopathy are normally older than patients presenting with EHPVO^[7,10], which is suggestive of long term obstruction. Patients with long term obstruction, or inadequate endoscopic or surgical management, may develop secondary biliary cirrhosis (2%-4%)^[1,9]. No evidence of malignant potential on long term follow-up exists in the literature.

CLINICAL FEATURES

In patients with EHPVO, 5%-38% develop symptomatic portal biliopathy^[5-10,25]. These symptoms may be secondary to bile duct obstruction like jaundice and pruritus, or to ductal stones like fever with chills and biliary colic. Dilawari *et al*^[5] reported a 5% incidence of symptoms. Khuroo *et al*^[7] reported a 38% incidence of symptoms ranging from jaundice, recurrent cholangitis

and biliary colic. All symptomatic patients in his study were adults and almost a decade older than the patients presenting with variceal bleeding. Sezgin *et al*^[10] studied 10 patients with portal biliopathy who presented with jaundice, cholangitis, pruritus and abdominal pain and their mean age at presentation was 36 years, whereas other patients with EHPVO generally presented with bleeding or splenomegaly during childhood. Their studies also suggest that portal biliopathy is a progressive condition which develops late in the course of portal hypertension and may progress to secondary biliary cirrhosis characterised by decreased serum albumin levels, ascites and a deranged coagulation time^[1,9].

INVESTIGATIONS

Most patients with EHPVO with biliary changes detected on ERCP are asymptomatic. However, because ERCP is an invasive procedure, its routine use in all patients with either EHPVO or NCPF is not justified. Liver function tests are the best initial investigations to identify patients who might benefit from imaging studies. A raised serum bilirubin level with a predominant increase in its direct component and an elevated serum alkaline phosphatase is an indication for performing biliary imaging. Serum albumin levels and prothrombin time abnormalities become abnormal only after prolonged biliary obstruction when secondary biliary cirrhosis develops.

Abdomen ultrasound with Doppler

Ultrasonography (USG) has a poor sensitivity in identifying the CBD in EHPVO as it is usually obscured by the portal vein collaterals. However, there is a role for USG in outlining the splenoportal axis before surgery and to identify gallbladder varices, which are seen in about 35% patients, if a cholecystectomy is planned^[26,27].

Endoscopic retrograde cholangiopancreatography

ERCP has been used by various authors to define portal biliopathy. The changes seen in the bile ducts include single or multiple smooth strictures of varying length and degree, saccular dilatations, indentations, dilated intrahepatic bile duct radicles, displacement of bile ducts, clustering and pruning of intrahepatic ducts, and filling defects in the CBD which may be due to stones or varices^[5-10,25]. These changes occur most commonly in the CBD and the left hepatic ducts. Although seen predominantly in EHPVO, patients with NCPF and cirrhosis also show these changes in 40% and 25% respectively^[1,23,24].

The differential diagnoses on cholangiography include sclerosing cholangitis, recurrent pyogenic cholangitis, CBD stones with stricture, and biliary ascariasis. Patients' history, clinical examination and ultrasound findings showing normal liver, portal cavernoma and echogenic shadowing suggestive of ascariasis may help in reaching a diagnosis^[1].

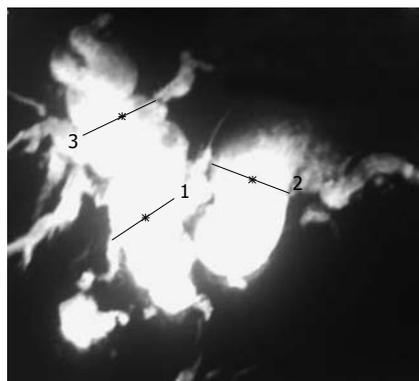


Figure 1 Magnetic resonance cholangiopancreatography showing common bile duct stricture with upstream dilatation of intra- and extrahepatic bile ducts. 1: Distance: 1.85 cm; 2: Distance: 2.57 cm; 3: Distance: 2.44 cm.

ERCP also has a therapeutic role in portal biliopathy. This includes removal of CBD stones, relief of cholangitis, and dilatation of dominant strictures with stenting. The latter is indicated only in patients not fit for surgery, or in whom shunt surgery is not feasible or has not reversed the biliopathy.

Presently, ERCP is indicated only if a therapeutic intervention is required and not for diagnosis.

Magnetic resonance cholangiopancreatography

Due to the invasive nature of ERCP and its attendant risks, magnetic resonance cholangiopancreatography (MRCP) with or without magnetic resonance (MR) portography has become the investigation of choice for portal biliopathy. The sensitivity of MRCP has been found to be similar to ERCP by various authors^[9,28]. Additional benefits of MR portography are that it differentiates choledochal varices from stones, images the splenoportal axis in surgical planning, and identifies portal collaterals (Figure 1).

Endoscopic ultrasonography

The additional information obtained from endoscopic ultrasonography (EUS) pertains to the differentiation between CBD varices^[29,30], stones, and tumours when other imaging modalities are not clear. EUS is not routinely recommended in the workup of a patient with portal biliopathy.

MANAGEMENT

Asymptomatic patients do not require any treatment if their liver function tests are within normal limits. Patients with persistently raised serum bilirubin and alkaline phosphatase levels need to be investigated by imaging (MRCP/ERCP/USG) to look for biliary tract changes^[1].

There is no consensus on the optimal treatment for symptomatic portal biliopathy. Endoscopic treatment is preferred in patients with CBD stones, cholangitis or if shunt surgery is not feasible^[1,17,31-34]. This includes use

of a balloon catheter or Dormia basket to clear CBD stones. Mechanical lithotripsy may be required for large stones. Endoscopic papillotomy with stenting or nasobiliary tube drainage may be necessary in patients with cholangitis^[1,34-37]. Balloon dilatation of dominant CBD strictures with stone extraction has also been described by various authors^[1,10,34].

The problems with endoscopic management are: (1) Filling defects seen on imaging may be due to varices and may lead to bleeding during attempted clearance. Stones usually move with a balloon catheter and varices appear as longitudinal defects on MRCP. Some authors prefer a balloon catheter over a Dormia basket for stone clearance as it is less traumatic^[34]; (2) Venous collaterals in the region of the ampulla of Vater may lead to bleeding during papillotomy, so this procedure should only be attempted in experienced centres which have a good surgical backup^[38,39]; (3) Balloon dilatation with stenting of dominant strictures may help to relieve biliary obstruction. However these stents become blocked frequently requiring multiple changes with their inherent risk of bleeding^[40]. Sezgin *et al.*^[10] reported long-term relief of portal biliopathy in only 3 out of 10 patients with endoscopic dilatation with stenting or nasobiliary drainage, whereas 7 out of 10 patients required repeated stent changes every 6 mo or earlier if cholangitis developed. Vibert *et al.*^[41] also reported long-term relief of biliary obstruction with percutaneous transhepatic biliary drainage with endobiliary stenting in only 3 out of 19 patients with symptomatic portal biliopathy. Furthermore, recurrent stent blockade with cholangitis may further decrease the chances of reversibility of the CBD obstruction following portosystemic shunt surgery; and (4) Multiple sessions require the patients to be compliant and have ready access to endoscopic expertise.

Therefore, although endoscopic extraction remains the preferred treatment in patients with CBD stones, most centres consider shunt surgery to be the first line of management for biliary obstruction secondary to bile duct strictures, unless complications like cholangitis or absence of a shuntable vein exist.

The role of ursodeoxycholic acid (UDCA) has not been evaluated for treatment or prophylaxis in portal biliopathy in any of the large studies. In a study by Condat *et al.*^[9], UDCA was effective in 3 out of 4 patients with portal biliopathy in relieving biliary symptoms and preventing recurrence. Most centres, however, rely on endoscopic extraction of biliary stones and experience in use of UDCA is limited.

Surgical management

The first published report of surgical treatment of portal biliopathy was by Hunt^[4] in 1965. He described separating the collaterals from the bile duct wall by dividing the fibrous adhesions between them and reported a relief from jaundice in the early postoperative period. However this technique has a high risk of intraoperative

hemorrhage as the collaterals are closely related to the wall of the CBD and attempting to separate them may cause torrential bleeding.

Choudhuri *et al.*^[11] in 1988 published the first case report of relief of CBD obstruction secondary to portal cavernoma after a proximal lienorenal shunt, in a patient with symptomatic portal biliopathy with EHPVO. Chaudhary *et al.*^[15] then published a series of 9 patients, out of whom 7 underwent proximal lienorenal shunts for symptomatic portal biliopathy. Five patients experienced reversal of their portal biliopathy with two patients requiring a biliary bypass (Roux-en-Y hepaticojejunostomy) for refractory bile duct strictures. Other authors have published similar results^[16,41].

In patients with non cirrhotic portal hypertension (EHPVO and NCPF) with portal biliopathy, portosystemic shunt surgery is the treatment of choice. The advantages of this approach are: (1) A successful portosystemic shunt not only prevents variceal bleeding but in a large number of patients may be the only treatment required for portal biliopathy^[11,15,16,41-43]; (2) Primary biliary bypass for portal biliopathy is associated with a risk of severe intraoperative bleeding due to the presence of large collaterals in the bile duct wall^[7,15,16,41,44,45]; and (3) Even if a portosystemic shunt fails to completely revert the bile duct obstruction, a patent shunt decompresses the collaterals present in the region of the bile duct enough to render a later biliary bypass possible^[15,16].

Portosystemic shunt surgery is also the procedure of choice in patients with imaging features of portal biliopathy who are otherwise asymptomatic and are being operated on for other complications of portal hypertension like variceal bleeding or symptomatic hypersplenism, as these patients may develop significant bile duct obstruction later, even after splenectomy and devascularisation^[7,46]. A suggested algorithm for the management of portal biliopathy is shown in Figure 2.

In conclusion, although abnormalities in the biliary tract on imaging are seen in the majority of patients with EHPVO, symptomatic portal biliopathy is a late and uncommon presentation in the natural history of the condition. The exact pathogenesis of portal biliopathy is not clear, but compression by dilated collaterals, ischemia resulting from venous thrombosis, and infection may all have a role to play in its development. The diagnostic investigation of choice is an MRCP which is done based on clinical suspicion and biochemical abnormalities. Endoscopic management is indicated in the presence of CBD stones, cholangitis and dominant strictures without a shuntable vein. Portosystemic shunt surgery, if feasible, is indicated in most patients, as it causes reversal of portal biliopathy and also renders a subsequent biliary bypass easier if the biliary obstruction persists. Thus, in patients with portal biliopathy, both endoscopic and surgical management are complementary and should be used appropriately according to individual situations.

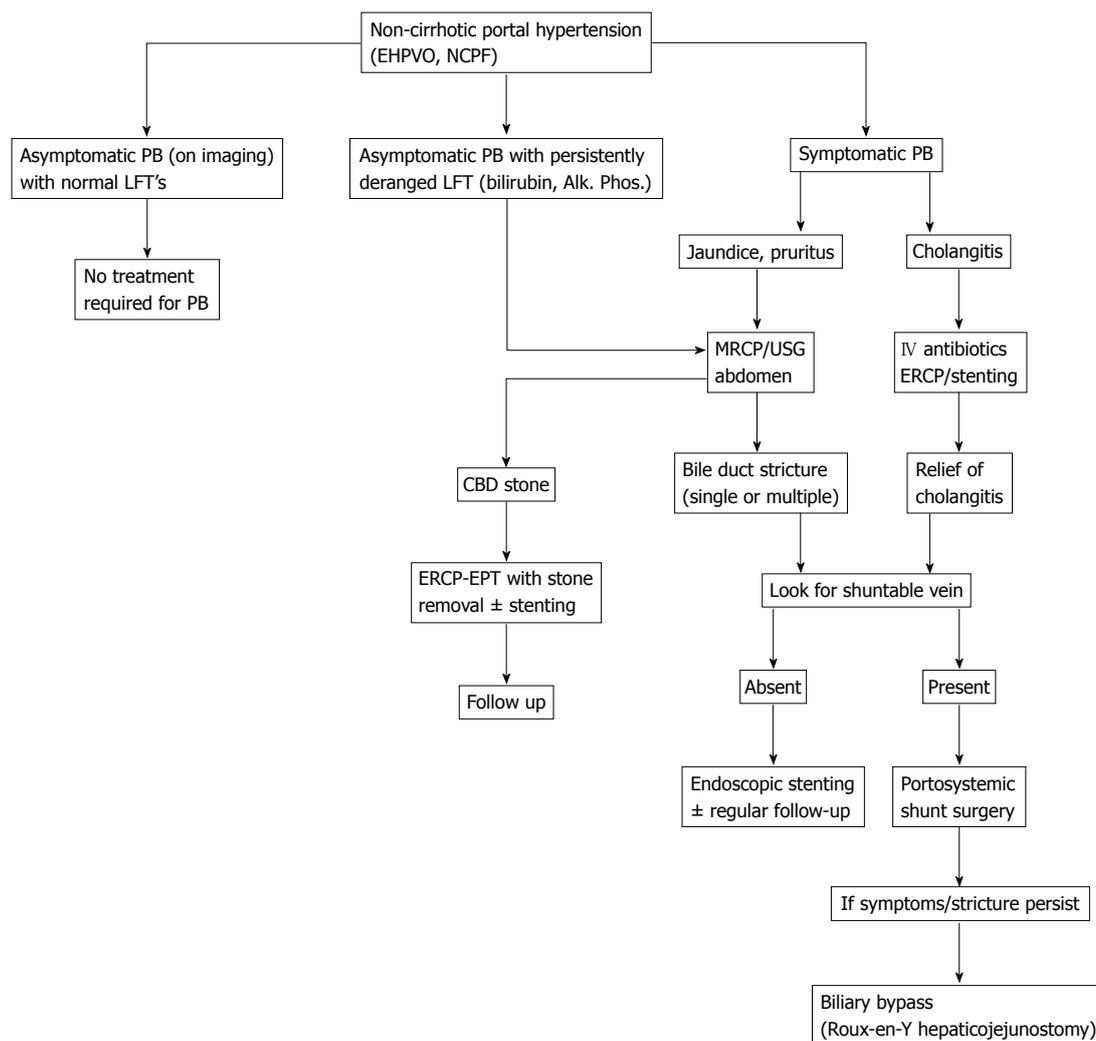


Figure 2 Algorithmic approach to management of portal biliopathy in patients with non-cirrhotic portal hypertension. EHPVO: Extrahepatic portal venous obstruction; NCPF: Non-cirrhotic portal fibrosis; LFT: Liver function test; PB: Portal biliopathy; ERCP: Endoscopic retrograde cholangiopancreatography; EPT: Endoscopic papillotomy; CBD: Common bile duct; MRCP: Magnetic resonance cholangiopancreatography; USG: Ultrasonography; Alk.: Alkaline; Phos.: Phosphatase.

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Telaprevir/boceprevir era: From bench to bed and back

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Abstract

Hepatitis C virus (HCV) infects approximately 200 million people worldwide. Interferon-based therapies have dominated over the past two decades. However, the overall response rates remain suboptimal. Thanks to the tremendous effort from both academia and industry, two serine protease inhibitors telaprevir and boceprevir for treating chronic hepatitis C have finally reached the clinic. Although these compounds are only approved for combination use with interferon and ribavirin in genotype 1 HCV infected chronic patients, the management of HCV patients however is now evolving incredibly. Here, we overviewed a series of landmark studies, regarding the clinical development of telaprevir and boceprevir. We discussed the mechanism-of-action of telaprevir/boceprevir and their potential application in HCV-positive liver transplantation patients. We further emphasized some emerging concerns with perspective of further development in this field.

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INVITED COMMENTARY ON HOT ARTICLES

Since the discovery of hepatitis C virus (HCV) in 1989^[1], the lack of good model systems has hampered the research at the early stages. Even though still little is known about the virus, interferon was found to be effective for treating chronic HCV and the first interferon- α (IFN- α) is approved by the United States Food and Drug Administration (FDA) to treat HCV in 1991^[2]. Interferons have a vital role for immune modulation of the host cells and involve the induction of a multitude of interferon-stimulated genes to establish an antiviral status^[3]. Through a series of optimizations, the current pegylated interferon (peg-IFN) in combination with ribavirin has dramatically improved the outcome, but still only approximately 50% of the patients can develop a sustained virologic response (SVR)^[4,5].

Thanks to the development of various cell culture models and the important involvement of biotechnology^[6], research in the HCV field has been flourishing both in academy and industry, over the past decade. This progress has enabled further improvement on the current interferon-based standard antiviral therapy and

the development of novel antivirals with distinct mechanism of action. A range of directly acting antiviral agents (DAAs), including protease and polymerase inhibitors, have been in various stages of clinical development^[7]. The serine protease inhibitors telaprevir and boceprevir are the most advanced in clinic. Both had been approved by FDA in May 2011 for treating chronic genotype 1 HCV infection and later were also approved by the European Medicines Agency^[7].

Mechanism-of-action of telaprevir and boceprevir

The HCV genome composes of a single open reading frame, encoding a polyprotein precursor of approximately 3000 amino acids, flanked by 5' and 3' non-coding region (NCR). Translation of the viral polyprotein is mediated by an internal ribosome entry site located within the highly conserved 5' NCR. The synthesized polyprotein is subsequently cleaved into four structural (core, E1, E2 and p7) and six non-structural (NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins^[8]. Processing of structural proteins, including the p7/NS2 junction, is carried out by at least two host signal peptidases and non-structural proteins and is matured by two viral proteases, NS2 and NS3/4A^[8,9].

The NS3/4A serine protease is a non-covalent, heterodimer complex formed by the catalytic subunit of the N-terminal serine protease domain of NS3 and the activation subunit of the NS4A cofactor. Unique to the NS3 protease is an extended polydentate substrate binding cleft, which ensures substrate specificity^[10]. It is responsible for the proteolytic cleavage of NS3/NS4A self cleavage, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B. The multi-functional property of NS3/4A protease has attracted for targeted drug development. Current protease inhibitors can be divided into covalent and non-covalent peptidomimetic inhibitors. Telaprevir and boceprevir are covalent linear inhibitors, discovered by using structure-based drug design approach^[11,12]. Both act *via* formation of a reversible covalent interaction with serine-139. The assailment of electrophilic carbonyl of the ketoamide by the catalytic serine leads to tetrahedral formation which mimics the transition state of peptide bond cleavage. This is further stabilized by additional ionic interactions with the active site^[11,12]. However, telaprevir and boceprevir were designed to target the protease of genotype 1 HCV and therefore their activity varies among genotypes. Experimental data has shown that both were active against genotypes 2, 5 and 6, but not 3^[13]. Clinical evidence suggested that telaprevir was effective against genotype 2 but not genotype 3 and uncertain for genotype 4^[14,15].

Clinical efficacy

Two weeks monotherapy with telaprevir has demonstrated substantial antiviral activity in patients with genotype 1 HCV in a phase 1 trial (3 log drop in viral load in majority of patients). However, some patients showed resistance to telaprevir can be seen from viral break-

through during dosing and were associated with selection of the variants^[16]. Therefore, combination therapy with peg-IFN- α /ribavirin was required for the subsequent larger multicentre phase 2, PROVE 1^[17], PROVE 2^[18] and PROVE 3^[19] trials. Overall, telaprevir increased 20%-30% SVR rates in both naïve and genotype 1 treated patients (Figure 1). Ribavirin was an essential component of this triple therapy (Figure 1).

Phase 3 trials in the treatment of naïve or experienced patients were carried out in the ADVANCE^[20] and REALIZE^[21] studies, respectively. Superior SVR rates with treatment of telaprevir-based regimens were confirmed in both naïve and experienced genotype 1 patients. The illuminate phase 3 study supported the concept of response-guided telaprevir combination treatment (RGT) in naïve patients. This study summarized that treatment of telaprevir in combination with peg-IFN/ribavirin in the first 12 and 24 wk was noninferior to the same regimen for 48 wk in patients with undetectable HCV RNA at weeks 4 and 12^[22].

One week monotherapy with boceprevir resulted in an average HCV RNA load reduction of about 1.0 and 1.6 log₁₀ IU/mL in nonresponders of peg-IFN/ribavirin at dose of 200 mg and 400 mg, 3 times a day, respectively^[23]. However, during boceprevir monotherapy, resistance mutations at six positions within the NS3 protease were detected. All mutations were associated with reduced replicative fitness estimated by mathematical modeling with cross-resistance to telaprevir^[24]. Similarly, the successful clinical development of boceprevir treatment was used in combination with peg-IFN/ribavirin both in naïve and experienced genotype 1 patients, as demonstrated in the phase 2b SPRINT-1 trial (Figure 2)^[25], the phase 3 SPRINT-2 trial (Figure 2)^[26] and the phase 3 RESPOND-2 trial (Figure 2)^[27].

The most serious adverse effects of telaprevir were rash and anemia and of boceprevir were anemia, dyspnea, neutropenia and anal discomfort.

The use of DAAs in liver transplant patients?

End-stage liver disease caused by chronic HCV infection is currently the leading indication for liver transplantation^[28]. However, HCV re-infection of the graft occurs universally and often results in accelerated recurrence of liver fibrosis and early development of cirrhosis^[29]. In general, peg-IFN/ribavirin is much less effective in transplant patients with approximate SVR rates of only 20%^[30]. An aggravated course of infection and more resistance to antiviral therapy have been attributed to several host and viral factors, in particular the application of specific immunosuppressive medication^[31].

The suppression of HCV viraemia in pre-transplant patients may attenuate the risk of viral recurrence. However, the tolerability and efficacy of peg-IFN/ribavirin antiviral therapy is compromised in decompensated cirrhosis patients^[32]. For telaprevir or boceprevir, a few patients with advanced fibrosis or compensated cirrhosis have been included in these phase 3 trials^[20-21,26-27]. How-

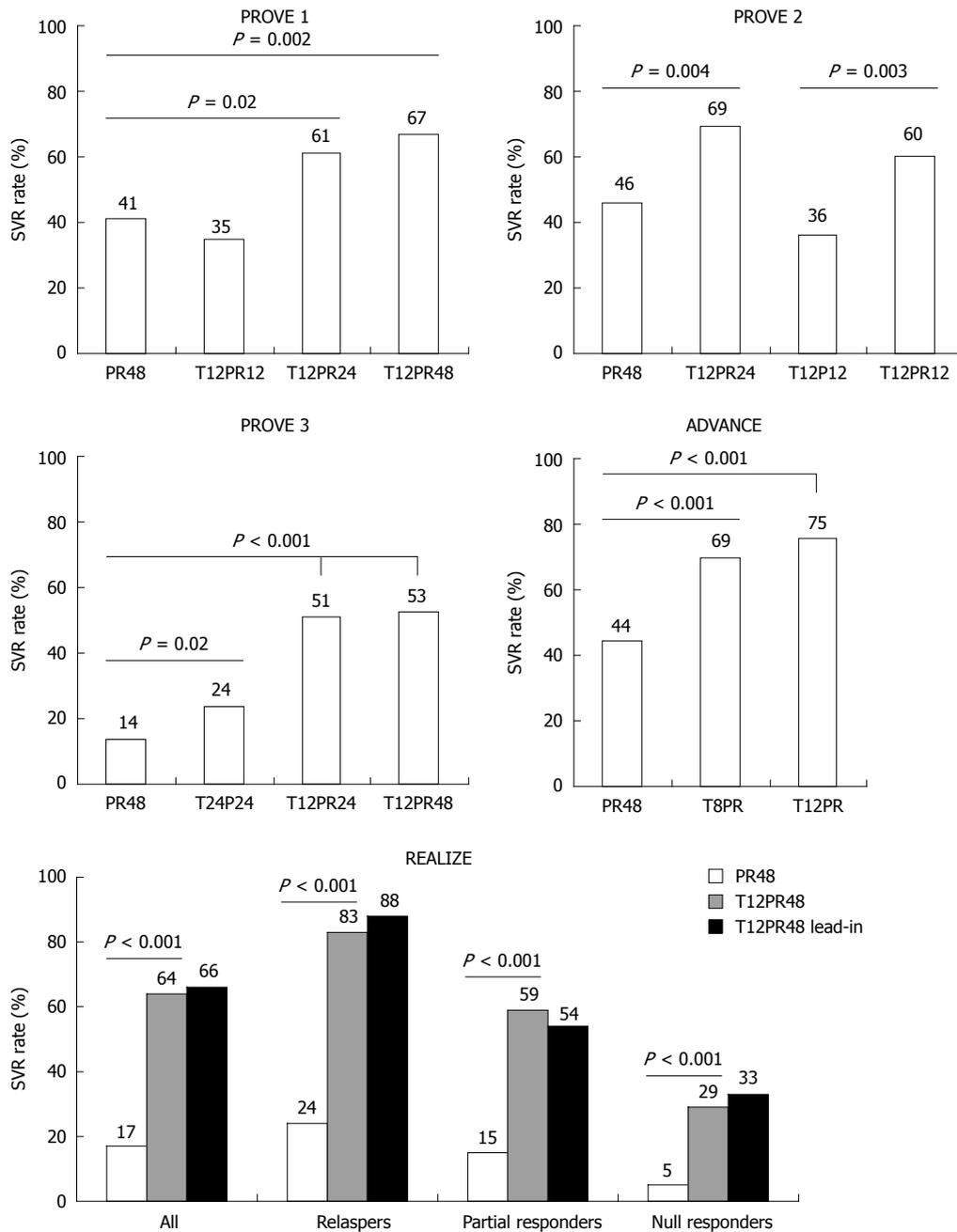


Figure 1 The outcome of the five landmark trials for telaprevir-based triple therapy in hepatitis C virus genotype 1 patients. Sustained virologic response rates are compared between groups. PROVE 1, PROVE 2 and ADVANCE trials were conducted in treatment-naïve patients. PROVE 3 and REALIZE trials were conducted in treatment-experienced patients. T: Telaprevir; P: Peg-IFN; R: Ribavirin; The numbers (8, 12, 24 or 48): Week of treatment; SVR: Sustained virologic response.

ever, the response rates in patients with advanced fibrosis were significantly lower than in patients with mild to moderate fibrosis but there is no experience in patients with decompensated cirrhosis so far.

Although prophylactic treatment, preemptive treatment or treatment of established HCV recurrence after transplantation with peg-IFN/ribavirin is common, its tolerability and efficacy remain unsatisfactory^[33]. Therefore, there is much expectation of telaprevir and boceprevir for improving the outcome in liver transplant patients. In the 18th annual congress of the International Liver Transplantation Society this year in San Francisco, United States, the first series of clinical data

of treating HCV recurrence using telaprevir or boceprevir in liver transplant patients were revealed, although these were rather small clinical studies. In a multicenter study of 7 patients, 2 patients had an undetectable viral load and 2 had viral load below 2 log at week 12 after treatment with boceprevir in combination with peg-IFN/ribavirin. However, all patients had severe anemia requiring erythropoietin and 4 patients required granulocyte colony stimulating factor. The dosage of peg-IFN and/or ribavirin was reduced in 4 patients and treatment was stopped prematurely in 3 patients^[34]. In one of the telaprevir-based triple therapy studies, 2 out of 9 patients achieved rapid viral response, however hematological ad-

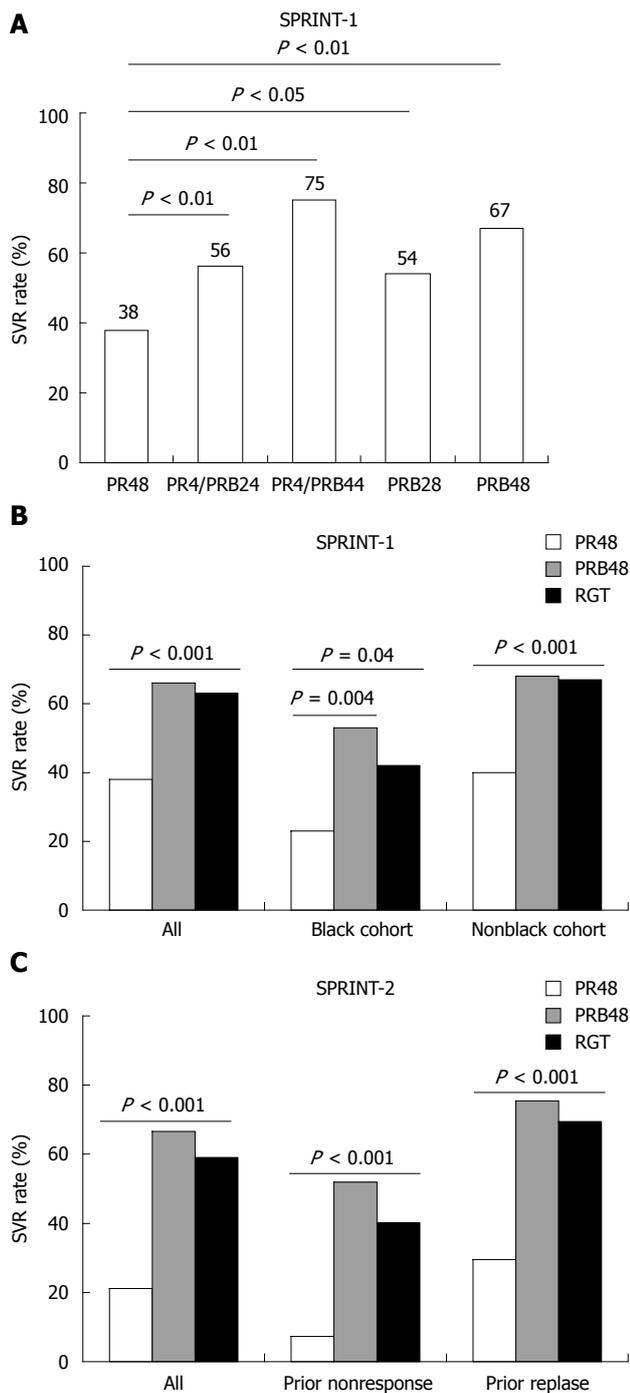


Figure 2 The outcome of the three landmark trials for boceprevir-based triple therapy in hepatitis C virus genotype 1 patients. Sustained virologic response rates are compared between groups. The SPRINT-1 and SPRINT-2 trials were conducted in treatment-naïve patients. The RESPOND-2 trial was conducted in treatment-experienced patients. B: Boceprevir; P: Peg-interferon; R: Ribavirin; The numbers (4, 24, 28 or 48): Week of treatment; SVR: Sustained virologic response; RGT: Response-guided telaprevir combination treatment.

verse events were common, including anemia and leucopenia^[35]. In another telaprevir-based triple therapy study, 4 out of 5 patients were telaprevir tolerated but 1 patient stopped the treatment due to complications. Two out of these 4 patients obtained SVR after 5 wk of therapy. Another 2 patients achieved 2-log and 3-log viral reduc-

tions in the 1st week follow-up, respectively^[36]. Triple combination therapy has shown substantial antiviral efficacy but larger properly designed trials are definitely required to further evaluate the use of telaprevir and boceprevir in this setting. However, these studies have consistently raised the concerns of poor tolerability of these compounds in transplant patients. Clinical studies of additional DAAs peri-transplant are in progress and should be monitored closely.

In addition to the adverse events, potential interactions between telaprevir/boceprevir with immunosuppressive drugs are other concerns. As reported in a phase 1 study, telaprevir interferes the metabolism of both cyclosporin A (CsA) and tacrolimus by inhibition of cytochrome P450 3A enzymatic activity^[37]. Telaprevir caused a significant increase in blood concentrations of both immunosuppressants and could potentially lead to serious or even life-threatening adverse effects. McCashland *et al*^[35] has specifically evaluated the drug level profile of CsA during telaprevir therapy in liver transplant patients, however no negative impact in achieving CsA target levels was observed. In fact, different immunosuppressive drugs can also have distinct effects on peg-IFN/ribavirin, such as CsA and MPA can promote the antiviral effects of interferon whereas tacrolimus does not^[31,38,39]. Thus, more attention from both basic and clinical perspective is needed, regarding drug to drug interaction with telaprevir/boceprevir.

The role of interferon and ribavirin?

Monotherapy with telaprevir or boceprevir not only been associated with resistance but also the antiviral potency is not sufficient for complete eradication of the virus^[16,24]. Therefore, combination treatment with peg-IFN/ribavirin has emerged as the ultimate solution to resolve these limitations. A specific issue which would be concerned is that whether the *IL28B* genetic variation as one of the important SVR prediction factors to peg-IFN/ribavirin, is still relevant to telaprevir/boceprevir-based triple combination therapy or not. In treatment of naïve patients, the *IL28B rs12979860-CC* genotype was a positive predictor to select appropriate candidates for RGT in the ADVANCE and SPRINT-2 trials^[20,26]. In treatment of experienced patients, the *CC* genotype however is less frequent than in general population, *IL28B* therefore is expected to be less important for response prediction. However, data remain limited to address the exact prediction value of *IL28B* genotype in telaprevir/boceprevir-based regimens. Another clear evidence from the PROVE and SPRINT trials suggested that the treatment regimens with low-dose or without ribavirin were associated with lower SVR rates, probably due to viral breakthrough and viral relapse after therapy^[18-19,25].

An emerging concept is the development of interferon-free regimens, which aims to limit the contraindications and adverse effects of peg-IFN. The combination of different reagents offers the potential for interferon-

free therapy including the development of various DAAs such as protease, NS4B, NS5A, polymerase inhibitors and host-targeting agents including cyclophilin inhibitors and anti-miR-122 oligonucleotides^[40]. In current clinical trials, different DAA combinations with or without ribavirin are the main scenario. It is not surprising that with or without interferon, a study of combining a non-nucleoside polymerase inhibitor with a protease inhibitor clearly showed the benefit of ribavirin^[41]. In spite of the mechanism of how ribavirin can synergize interferon that has been extensively extrapolated^[42-44], the mechanism of how ribavirin works with DAAs are poorly investigated. Notably, a recent study has shown that high SVR rate could be achieved with two DAAs (the NS5A inhibitor daclatasvir and the NS3 inhibitor asunaprevir) only, although higher SVR rates were achieved when combined with peg-IFN/ribavirin^[45]. Therefore, it remains an open question regarding the future of ribavirin in interferon-free-based DAA combination therapy. Further basic and clinical research will conceivably help to figure out this issue.

Summary and perspectives

The approval of telaprevir and boceprevir by FDA and the European Medicines Agency in last year (2011) has indeed changed the management of chronic HCV. From these series of clinical landmark studies, a 20%-30% increase of SVR rates were obtained with the additional telaprevir/boceprevir to the conventional standard peg-IFN/ribavirin therapy. Despite these exciting results, certainly there are several remaining concerns. An apparent limitation of telaprevir/boceprevir as well as most of the pipeline DDAs (such as protease inhibitors asunaprevir, BI 201335 and ABT-450) is genotype 1 specificity, whereas other HCV genotypes also prevail all over the world. Secondly, these compounds have very low genetic barrier to the development of resistant viral variants. Thirdly, although telaprevir/boceprevir are generally well tolerated, their adverse effects are still hampering the successful application in specific populations, particularly for liver transplant patients.

Perspectively, these issues could likely be minimized by further optimizing the clinical protocol. In addition, by the development of other compounds, more optimal combinations will be available. As being expected, the dream of achieving high SVR rates with all-oral interferon-free regimens will be no longer far from clinical reality.

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MicroRNAs in biliary diseases

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Abstract

Cholangiopathies are a group of diseases primarily or secondarily affecting bile duct cells, and result in cholangiocyte proliferation, regression, and/or transformation. Their etiopathogenesis may be associated with a broad variety of causes of different nature, which includes genetic, neoplastic, immune-associated, infectious, vascular, and drug-induced alterations, or being idiopathic. miRNAs, small non-coding endogenous RNAs that post-transcriptionally regulate gene expres-

sion, have been associated with pathophysiological processes in different organs and cell types, and are postulated as potential targets for diagnosis and therapy. In the current manuscript, knowledge regarding the role of miRNAs in the development and/or progression of cholangiopathies has been reviewed and the most relevant findings in this promising field of hepatology have been highlighted.

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Key words: miRNAs; Cholangiopathies; Cholangiocarcinoma; Polycystic liver diseases; Primary biliary cirrhosis

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INTRODUCTION

Cholangiocytes are the epithelial cells lining the bile ducts, and are key players in normal liver physiology^[1]. They participate in fundamental secretory processes by modifying the composition and flow of primary bile generated at the canaliculi of hepatocytes upstream of the biliary tree. Thus, transport of water, ions or solutes between the blood and the bile-duct lumen finally result in fluidization and alkalization of bile^[1-4]. Although cholangiocytes only represent 3%-5% of the total liver cell population, they may account for up to 30% of to-

tal bile flow^[1]. Cholangiocytes contain a single primary cilium extending from the apical membrane into the bile-duct lumen^[5]. This antenna-like organelle possesses sensory properties that detect physical and chemical changes in bile that are transmitted into the cell to activate signaling pathways and finally modify cellular functions. The primary cilium of cholangiocytes functions as a mechano- (detecting changes in bile flow)^[6], chemo- (interacting with different molecules^[7] and vesicles^[8]), and osmo- (identifying changes in osmolarity: hypo- and hyper-tonicity)^[9] sensor.

In addition, cholangiocytes are primarily or secondarily affected in a group of diseases termed cholangiopathies, whose etiopathogenesis is of diverse nature (genetic, neoplastic, immune-associated, idiopathic, infectious, vascular or drug-induced)^[10]. These biliary diseases may result in cholangiocyte proliferation, regression and/or transformation. Important advances are being achieved in understanding the molecular mechanisms involved in the development and progression of these disorders. In this regard, there is increasing evidence suggesting the role of miRNAs in the etiopathogenesis of cholangiopathies, which are postulated as potential targets for diagnosis and therapy.

miRNAs are small non-coding RNAs (approximately 22 nucleotides) that regulate the expression of multiple genes by binding to complementary sites of targeted mRNAs, causing translational repression (imperfect target duplexes) or degradation (perfect matches)^[11,12]. They participate in the regulation of multiple cell types under physiological and pathological conditions, and are fundamental in different cellular processes such as development, proliferation, apoptosis, metabolism, morphogenesis, and in diseases^[13,14]. In the current article, knowledge regarding the role of miRNAs in the development and/or progression of cholangiopathies has been highlighted.

CHOLANGIOCARCINOMA

Cholangiocarcinoma (CCA) is a malignant tumor affecting the biliary tree^[15,16]. The incidence of CCA (1:50 000) is increasing worldwide and the therapeutic options are very limited because of high chemoresistance. CCA accounts for up to 10%-15% of primary hepatobiliary malignancies and 3% of all gastrointestinal tumors, affecting individuals of both sexes. Owing to slow growth and late metastasis, this cancer is usually diagnosed in patients older than 65 years when it is at an advanced stage, thus reducing the success of surgical procedures. Complications of CCA include bile-duct obstruction, liver failure, metastasis to other organs, infections, and uncontrolled vomiting. Although the pathogenesis of CCA is poorly understood, the presence of primary sclerosing cholangitis, chronic biliary irritation, or choledochal cysts appears to predispose to the development of this cancer.

CCAs can be anatomically classified into extrahepatic or intrahepatic^[15,16]. Extrahepatic is more common than

intrahepatic CCA, accounting for up to 80%-95% of all CCAs. In addition, depending on the tumor location in the extrahepatic biliary system, extrahepatic CCA can be divided into proximal or perihilar (also frequently referred to as Klatskin tumor), and distal. On the other hand, intrahepatic CCA originates within the liver and accounts for up to 5%-20% of all CCAs.

Increasing evidence suggests the importance of miRNAs in the pathogenesis of CCA. Thus, global changes in the miRNA profile of malignant cholangiocytes has been reported^[17-21], which may alter different cholangiocyte features such as cell cycle, proliferation, migration and apoptosis (Table 1).

Cell cycle, proliferation and migration

Different miRNAs are able to act as onco-miRNAs by repressing the expression of tumor suppressor genes. In this regard, miR-421, upregulated in human CCA, was reported to modulate the expression of farnesoid X receptor (FXR), an event associated with cell proliferation, colony formation, and migration *in vitro*^[22]. FXR is a member of the nuclear receptor superfamily that plays crucial roles in bile acid, cholesterol, lipid and glucose metabolism^[23,24]. Likewise, FXR was shown to act as a tumor suppressor for hepatocellular carcinoma and breast cancer^[23,24]. Aberrant bile-acid secretion has been linked to CCA, therefore, further investigations will be needed to test the interplay between bile acids, FXR and miRNAs on biliary tract tumorigenesis.

Another miRNA involved in tumor proliferation is miR-21, which is reported to be overexpressed in human CCA due to upregulation of arsenic resistance protein 2 (Ars2)^[25]. This protein plays an important role in miRNA biogenesis, and hence its depletion reduces the levels of different miRNAs, including miR-21. Ars2 knock-down in CCA cells decreases miR-21 levels, inhibits cell proliferation, and prevents tumor formation in immunodeficient mice. It is suggested that miRNA-21 may negatively control the expression of tumor suppressor phosphatase and tension homolog (PTEN)^[18,21,25] leading to cell proliferation. Moreover, miR-21 is suggested to regulate the expression of the tissue inhibitor of metalloproteinase (TIMP)-3^[20], an inhibitor of cell-matrix TIMP activity downregulated in CCA, and could result in increased migration properties.

In addition, it has been recently reported that miRNA-26a is overexpressed in human CCA promoting cell proliferation and migration *in vitro*, and tumor growth *in vivo*^[26]. This miRNA was demonstrated to downregulate directly the expression of glycogen synthase kinase (GSK)-3 β ^[26]. This enzyme phosphorylates the serine and threonine residues of β -catenin, leading to its degradation. Thus, miR-26a/GSK-3 β targeting results in intracellular accumulation of β -catenin that activates the expression of c-Myc, cyclin D1, and peroxisome proliferator-activated receptor δ ; three proteins involved in dedifferentiation, tumor growth, and migration^[26].

Induction of chronic cholestasis in a new murine model accelerates the progression of CCA by alter-

Table 1 Classification of miRNAs differentially expressed in cholangiocarcinoma regarding their action, expression and targets

miRNA	Expression	Disease	Target	Altered function	Ref.
miR-320	Down	Human-ICC	Mcl-1		[17]
miR-29b	Down	Human-CCA	Mcl-1		[21,30]
miR-204	Down	Human-ICC	Bcl-2	Apoptosis	[17]
miR-25	Up	Human-ICC	DR4		[29]
miR-21	Up	Human-CCA	PDCD4		[20,25]
miR-421	Up	Human-ICC	FXR		[22]
miR-34a	Down	Mouse-CCA	c-Myc		[27]
miR-210	Up	Mouse-CCA	Mnt	Proliferation	[27]
miR-21	Up	Human-CCA	PTEN		[18,21,25]
miR-26a	Up	Human-CCA	GSK-3 β		[26]
miR-421	Up	Human-ICC	FXR		[22]
miR-21	Up	Human-CCA	TIMP-3	Migration	[20]
miR-26a	Up	Human-CCA	GSK-3 β		[26]
miR-494	Down	Human-CCA	CDK-6	Cell cycle	[19]
miR-141	Up	Human-CCA	Gen CLOCK	Circadian rhythm	[18]
miR-370	Down	Human-CCA	MAP3K8, DNMT-1		[38]
miR-373	Down	Human-ECC	MBD2	Epigenetics	[36,37]
miR-148a	Down	Human-CCA	DNMT-1		[39]
miR-152	Down	Human-CCA	DNMT-1		[39]
miR-let7a	Up	Human-CCA	NF2		[41]
miR-21	Up	Human-CCA	PTEN	Chemoresistance	[18]
miR-200b	Up	Human-CCA	PTPN12		[18]

miRNA-15a/Cdc25A interplay participates in hepatic cystogenesis of polycystic liver diseases. miRNA-15a directly targets the cell cycle regulator Cdc25a. In polycystic rat and human cholangiocytes, miR15a is downregulated, thus resulting in Cdc25a upregulation, cell proliferation and cystogenesis. The Cdc25a inhibitor vitamin K3 was demonstrated to target Cdc25a, blocking the hepatorenal cystogenesis of several rodent models of polycystic kidney and liver disease. This suggests the therapeutic potential of vitamin K3 for polycystic kidney and liver diseases. CCA: Cholangiocarcinoma; ICC: Intrahepatic cholangiocarcinoma; ECC: Extrahepatic cholangiocarcinoma; CDK: Cell division kinase; Mcl-1: Myeloid cell leukemia sequence 1; Bcl-2: B-cell lymphoma 2; CDK: Cyclin-dependent kinase; DR4: Death receptor 4; PDCD4: Programmed cell death protein 4; FXR: Farnesoid X receptor; TIMP: Tissue inhibitor of metalloproteinase; GSK: Glycogen synthase kinase; MBD: Methyl-CpG-binding domain; MAP3K8: Mitogen-activated protein kinase 8; DNMT: DNA methyltransferases; NF2: Neurofibromatosis 2; PTEN: Phosphatase and tension homolog; PTPN12: Protein tyrosine phosphatase non-receptor type 12.

ing miRNA-34a, let-7a and miRNA-210 expression^[27]. Downregulation of miRNA-34a results in overexpression of its target c-Myc that mediates the upregulation of cyclin D1. In this regard, knockdown of c-Myc reduces progression of CCA. Moreover, in this animal model, the suppressor of miRNAs biogenesis LIN28B is found to be upregulated. LIN28B specifically binds to the family of let-7 miRNA precursors, inhibiting their processing, and inducing their degradation^[28]. These experimental data suggest that LIN28B upregulation in humans might also be associated with the inhibition of let-7a, a miRNA involved in the development of cystic hyperplasia, cystic atypical hyperplasia, cholangioma and CCA. On the other hand, the hypoxia inducible factor-2 α was shown to mediate miRNA-210 upregulation, which further inhibits Mnt (transcriptional repressor and antagonist of c-Myc-dependent transcriptional activation and cell growth)^[27].

Among those miRNAs reported to be downregulated in CCA based on miRNA arrays, miR-494 is involved in the control of the cell cycle by direct targeting cyclin-dependent kinase-6^[19]. Thus, it is suggested that miRNA-494 could be a potential therapeutic target for CCA because its overexpression decreases the growth of bile-duct cancer cells *in vitro* and *in vivo*^[19].

Apoptosis

Activation of anti-apoptotic pathways is a general event

involved in carcinogenesis. In this regard, CCA is often associated with alterations in the expression profile of miRNAs that regulate apoptosis. Thus, miRNA-25, which is overexpressed in CCA, may protect cholangiocytes from tumour necrosis factor-related apoptosis-inducing ligand-induced apoptosis through the inhibition of death receptor 4^[29]. Likewise, the overexpression of miRNA-21 in CCA is also able to downregulate the programmed cell death protein 4^[20,25].

On the other hand, several miRNAs that modulate proapoptotic mechanisms are downregulated in CCA. Among those downregulated miRNAs, both miR-320^[17] and miR-29b^[21,30] play a role in the expression control of the antiapoptotic effector myeloid cell leukemia sequence 1, thus promoting cell survival and proliferation. Moreover, miR-204, which is also downregulated in CCA, targets the antiapoptotic protein B-cell lymphoma 2^[17].

Epigenetics

Although genetic alterations have been widely associated with CCA, epigenetic modifications are poorly understood in this type of cancer. However, CCA has been associated with aberrant DNA methylation, which is an essential mechanism for gene expression regulation^[31,32]. This mechanism is mostly regulated by at least three DNA methyltransferases (DNMT-1, DNMT-3A and DNMT-3B). Once a DNA sequence becomes methyl-

ated, it can repress transcription by blocking the recognition of transcriptional activators to DNA sequences, or by recruiting methyl-CpG-binding domain (MBD) proteins to modify chromatin compaction. MBD proteins are transcription repressors that bind to methylated gene promoters, resulting in gene expression silencing. Interestingly, aberrant DNA methylation has been found in many types of cancers, indicating that hypomethylation or hypermethylation of gene promoter CpG islands may result in tumor cell genomic instability or tumor suppressor gene silencing, respectively^[33,34]. Moreover, both miRNAs and methylation are reversible regulators that can interact with each other^[35]. In this regard, miRNA-373, which was recently reported to be downregulated in hilar CCA, negatively regulates MBD2 protein expression through specific binding to its 3' untranslated region (3'-UTR)^[36]. As a result of miRNA-373 downregulation in CCA, MBD2 expression is upregulated, leading to hypermethylation and silencing of the tumor suppressor gene *RASSF1A*. Hence, miRNA-373 downregulation is associated with poor cell differentiation, advanced clinical stage, and shorter survival in hilar CCA. Interestingly, miRNA-373 expression can also be regulated by MBD2 in CCA^[37].

In addition, it has been demonstrated that interleukin-6 (IL-6) may epigenetically regulate the expression of selected miRNAs and contribute to CCA growth^[38]. IL-6, an inflammation-associated cytokine that induces mitogen and survival features in cholangiocytes, is overexpressed in human CCA and presumably contributes to tumor cell growth. In this regard, IL-6 overexpression in CCA cells stimulates the expression of different methyltransferases (DNMT-1 and HASJ4442) that further downregulate the expression of seven miRNAs; one of these downregulated miRNAs, miRNA-370, directly targets the oncogene mitogen-activated protein kinase mitogen-activated protein kinase 8 in both *in vitro* and in tumor cell xenografts *in vivo*^[38]. Moreover, IL-6 may also regulate the expression of other miRNAs, such as miR-148a, miRNA-152 and miRNA-301^[39]. These three miRNAs are downregulated in CCA cells and possess sequence complementarity to the 3'-UTR region of the DNMT-1 mRNA transcript. However, only miRNA-148a and miRNA-152 directly regulate DNMT-1 expression. Interestingly, DNMT-1 modulates the expression of the tumor suppressor genes *Rassf1a* and *p16INK4a* by promoter hypermethylation, leading to malignant cell transformation.

Chemoresistance

CCAs are tumors with a marked multidrug resistance phenotype that includes changes in the expression of genes involved in the apoptosis/survival balance^[40].

As mentioned above, miRNA-21 and miRNA-200b are highly overexpressed in malignant cholangiocytes^[18]. These two miRNAs are suggested to contribute to chemoresistance in CCA by modulating the chemotherapy-induced apoptosis. Thus, overexpression of oligonucle-

otides anti-miR21 and anti-miR200b in malignant cholangiocytes cell lines satisfactorily increases gemcitabine-induced cytotoxicity. Moreover, miRNA-21 targets the tumor suppressor PTEN, which through its phosphatase activity inhibits phosphatidylinositol 3-kinase-dependent growth and survival. In addition, miRNA-200b targets the protein tyrosine phosphatase non-receptor type 12 that is involved in cell growth, cell dedifferentiation and oncogenic transformation.

In contrast to the aforementioned data obtained using an experimental animal model of chemically induced CCA, other authors have reported that miRNA-let7a is overexpressed in human CCA^[41]. Thus, miRNA-let7a overexpression is able to target the tumor suppressor neurofibromatosis 2 (NF2), which modulates the signal transducer and activator of transcription 3 (STAT3)-activated survival mechanism^[41]. Interestingly, intratumor administration of oligonucleotides anti-miRNA-let7a increases NF2 and decreases p-STAT3 expression in CCA xenografts *in vivo*. Moreover, these effects increase gemcitabine toxicity, resulting in decreased tumor growth^[41].

Circadian rhythm

The circadian rhythm controls on a 24-h range many fundamental physiological features such as behavior, metabolism or cell proliferation. The suprachiasmatic nuclei located in the hypothalamus synchronizes the molecular clocks in most mammalian cells through different circadian physiological rhythms including rest-activity, body temperature, feeding patterns, and hormonal secretions. Therefore, circadian alterations have been suggested to affect carcinogenesis risk also in humans^[42]. In this regard, miRNA-141, overexpressed in intrahepatic CCA, was predicted by bioinformatics approaches to target CLOCK directly; a tumor suppressor that controls the cellular circadian rhythm^[18]. Indeed, CLOCK may inhibit cell cycle, both directly and indirectly, and increases apoptosis. Administration of oligonucleotides anti-miRNA-141 in CCA cells results in CLOCK protein downregulation. These data indicate that circadian rhythm and miRNAs may interact in physiological and pathological conditions.

POLYCYSTIC LIVER DISEASES

Polycystic liver diseases (PCLDs) are genetic disorders characterized by bile-duct dilatation and/or cyst development, which become progressively more severe and require liver transplantation as the only therapeutic option^[43]. The large volume of hepatic cysts causes symptoms such as abdominal distension, local pressure with back pain, gastroesophageal reflux, and dyspnea. The estimated prevalence of PCLDs is around 1:1000, and these patients often develop polycystic kidney disease. Different genetic mutations trigger the appearance and growth of cysts in PCLDs, and in some cases this phenomenon is also associated with hepatic fibrosis. Most of the proteins encoded by these genes are located in

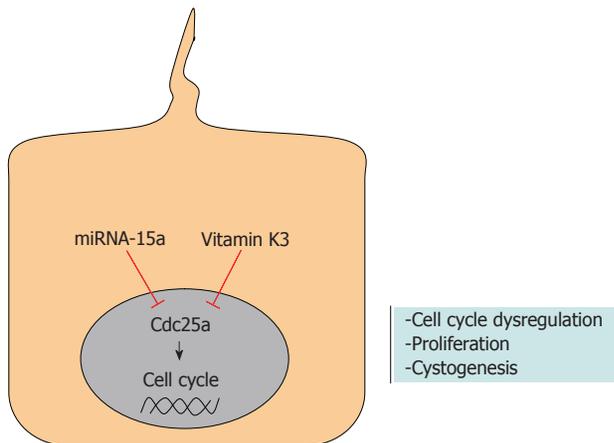


Figure 1 miRNA-15a/Cdc25a interplay participates in hepatic cystogenesis of polycystic liver diseases. miRNA-15a directly targets the cell cycle regulator Cdc25a. In polycystic rat and human cholangiocytes, miR15a is down-regulated, thus resulting in Cdc25a upregulation, cell proliferation and cystogenesis. The Cdc25a inhibitor vitamin K3 was demonstrated to target Cdc25a, blocking the hepatorenal cystogenesis of several rodent models of polycystic kidney and liver disease. This suggests the therapeutic potential of vitamin K3 for polycystic kidney and liver diseases.

the primary cilium of cholangiocytes^[44]. Many of these proteins interact with each other forming complexes that signal through common pathways. Mutations in genes encoding proteins located in the primary cilium of cholangiocytes result in physical and functional defects of this organelle, and outcome in the development of several forms of PCLD classified as ciliopathies. For this reason, and because the only cells in the liver that have cilia are cholangiocytes, the PCLDs affecting genes encoding proteins which are localized in the cilia are called cholangiociliopathies^[44]. It has been recently demonstrated that hepatic cystogenesis in PCLDs is the result of hyperproliferation^[45], hypersecretion^[46], and alteration in the pattern of miRNAs in the bile duct cells^[47], and that these alterations are intracellularly associated with an increase in the levels of cAMP and a decrease in calcium^[45,48,49]. In this regard, global changes in the expression pattern of miRNAs were observed between cultured cholangiocytes from normal and PCK rats (animal model of hepatorenal polycystic disease, i.e., autosomal recessive polycystic kidney disease)^[47,50]. In total, 121 and 148 miRNAs were detected in normal and PCK rat cholangiocytes, respectively. Twelve miRNAs were expressed in normal rat cholangiocytes and not in PCK rat cholangiocytes, and 39 were present only in PCK rat cholangiocytes. Moreover, there were changes in the expression of 109 common miRNAs between both cell lines. Interestingly, 97 of these common miRNAs (i.e., 87%) were found to be downregulated in PCK rat cholangiocytes compared to normal rat cholangiocytes, and 12 miRNAs (11%) were overexpressed in cystic cholangiocytes. Among those highly downregulated miRNAs in cultured PCK rat cholangiocytes, miRNA-15a is also downregulated *in vivo* in cholangiocytes from PCK rats and PCLD patients^[47]. This miRNA-15a downregulation

runs in parallel with the upregulation of its predicted target, the cell-cycle regulator cell division cycle 25A (Cdc25A). Experimental overexpression of miRNA-15a in PCK rat cholangiocytes results in decreased Cdc25A protein levels, inhibition of cell proliferation, and cyst growth reduction. Interestingly, experimental suppression of miRNA-15a in normal rat cholangiocytes accelerates cell proliferation, increases Cdc25A expression, and promotes cyst growth^[47]. All these data suggest that the miRNA-15a/Cdc25A interplay could be a potential target to inhibit hepatic cystogenesis (Figure 1). Importantly, inhibition of Cdc25A with its inhibitor vitamin K3 suppresses hepatorenal cystogenesis in rodent models of polycystic kidney and liver disease, being a valuable potential pharmacological approach to test in clinical trials^[51] (Figure 1). Moreover, it will be interesting to demonstrate in future studies the role of many other altered miRNAs in the pathogenesis of PCLDs.

PRIMARY BILIARY CIRRHOSIS

Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease that mainly affects middle-aged women^[52]. It is a rare disease, with an estimated prevalence of 1:8000 in countries such as the United Kingdom. Four stages of this disorder have been characterized by progressive bile-duct loss that runs in parallel with an increase in cholestasis and fibrosis^[52]. The etiology of PBC remains unknown, and the pathogenesis is poorly understood. The serological hallmark of PBC is the development of high titers of antimitochondrial autoantibodies (AMAs) in serum associated with an immunological attack on the small intrahepatic bile ducts^[52]. Interestingly, PBC patients usually have a poor response to immunosuppressants and the only therapeutic option that improves the outcome of the disease in two thirds of PBC patients is daily administration of ursodeoxycholic acid (UDCA), a hypercholeretic bile acid^[52]. In addition, and importantly, PBC patients show alterations in the biliary secretion of bicarbonate. Thus, PBC patients show a failure in the secretin-stimulated biliary secretion of bicarbonate^[53], as well as a reduction in the hepatobiliary expression of anion exchanger 2 (AE2/SLC4A2)^[54,55]. AE2 is a Cl⁻/HCO₃⁻ exchanger located in the canalicular membrane of hepatocytes and in the apical membrane of cholangiocytes that controls the intracellular pH (pHi) and promotes the alkalization and fluidization of bile^[1,2,56]. An interesting hypothesis suggests that, in PBC patients, long-term maintained alteration of pHi by AE2 downregulation might modify cholangiocyte and lymphocyte function^[57]. To test the etiopathogenic role of AE2 downregulation in PBC, an Ae2^{a,b1,b2} mouse knockout was generated. Interestingly, these animals spontaneously reproduce over time many PBC features^[57], such as: (1) portal infiltration of T lymphocytes and bile duct damage; (2) increased oxidative stress in cholangiocytes; (3) elevated production of interferon- γ and IL-12; (4) periductular hepatic fibrosis; (5) increased levels of

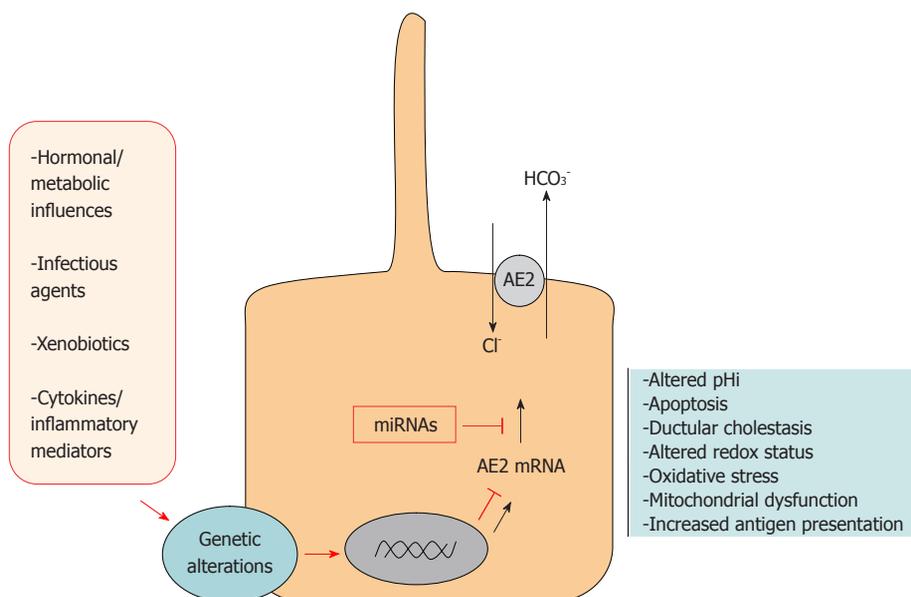


Figure 2 Role of miRNA-506 in the etiopathogenesis of primary biliary cirrhosis. Different risk factors could induce genetic alterations leading to anion exchange 2 (AE2) downregulation in primary biliary cirrhosis (PBC) cholangiocytes. miRNA-506 is overexpressed in PBC cholangiocytes, thus resulting in the inhibition of both AE2 protein translation and subsequent $\text{Cl}^-/\text{HCO}_3^-$ activity, and impairing the biliary secretory functions.

IgM, IgG, and hepatic alkaline phosphatase; and (6) the presence of PBC-specific AMAs. Different agents such as hormones, metabolic influences, infectious agents, xenobiotics, cytokines/inflammatory mediators, and/or miRNAs could be involved in the AE2 downregulation present in PBC patients. Regarding miRNAs, a differentially expressed miRNA profile in livers from PBC patients compared with normal controls has been recently reported^[58]. The change consists of 35 differentially expressed miRNAs (11 upregulated and 24 downregulated), which were predicted to target genetic transcripts involved in cell proliferation, apoptosis, inflammation, oxidative stress and metabolism^[58]. Regarding those miRNAs that were overexpressed in the miRNA array, we observed by using bioinformatics approaches that miRNA-506 could be a potential direct AE2 regulator. Therefore, we tested if AE2 downregulation in PBC cholangiocytes is dependent on miRNAs, and specifically on miRNA-506. Our data showed that miR-506 is upregulated in cholangiocytes from PBC patients, binds to the 3'-UTR of AE2 mRNA, and prevents protein translation, leading to diminished AE2 activity and impaired biliary secretory functions^[59]. These data suggest the etiopathogenic role of miR-506 in the AE2 downregulation characteristic of PBC cholangiocytes (Figure 2). On the other hand, the reported AE2 downregulation in PBC lymphocytes was not associated with changes in the expression of miRNA-506^[59]. This disease preferentially affects women, therefore, it is interesting to remark that the gene encoding miRNA-506 is located in the X chromosome. Further investigations are needed to characterize fully the role of miRNA-506 in the regulation of other targets and cellular features, as well as the analysis of its expression in other tissues. Moreover, the role of

UDCA and estrogens on miRNA-506 expression will be valuable information to expand the etiopathogenic role of this miRNA in PBC.

CONCLUSION

In summary, the available information clearly indicates that miRNAs represent a new research area in the field of biliary pathophysiology. As stated in this review, they may modify different features in cholangiocytes, such as secretion, apoptosis, proliferation, and migration, and can be regulated by different mechanisms and conditions, such as epigenetics, hypoxia, or circadian rhythms. There is still limited information about the role of miRNAs in biliary diseases, but future investigations will provide more evidence regarding their role in the development and progression of cholangiopathies, as well as in their potential use for diagnosis and therapy. Finally, it is important to remark upon the increasing information about the role of stem cells in the pathophysiology of biliary diseases^[60]; in this regard, the potential role of miRNAs in the regulation of biliary stem cells during cholangiocyte injury, as well as the role of miRNAs in the development of cancer stem cells in CCA^[61] need to be elucidated.

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Current endoscopic approach to indeterminate biliary strictures

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Abstract

Biliary strictures are considered indeterminate when basic work-up, including transabdominal imaging and endoscopic retrograde cholangiopancreatography with routine cytologic brushing, are non-diagnostic. Indeterminate biliary strictures can easily be mischaracterized which may dramatically affect patient's outcome. Early and accurate diagnosis of malignancy impacts not only a patient's candidacy for surgery, but also potential timely targeted chemotherapies. A significant portion of patients with indeterminate biliary strictures have benign disease and accurate diagnosis is, thus, paramount to avoid unnecessary surgery. Current sampling strategies have suboptimal accuracy for the diagnosis of malignancy. Emerging data on other diagnostic modalities, such as ancillary cytology techniques, single operator cholangioscopy, and endoscopic ultrasonography-guided fine needle aspiration, revealed promising results with much improved sensitivity.

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INTRODUCTION

Cholangiocarcinoma is usually diagnosed at an advanced stage, which is the main reason for the poor prognosis of this tumor. Patients with T1 stage tumor who undergo resection have an excellent prognosis with a cumulative 5-year survival rate of about 100%^[1]. T1 stage tumors are confined to the bile-duct wall and are limited to the mucosa or fibromuscular layer of the bile duct and do not usually present with lymph node metastases. Therefore, a diagnosis of bile duct carcinoma in T1 stage is crucial for long term survival. Serum alkaline phosphatase and gamma-glutamyltransferase are elevated in 40% of these patients and 40% are non-icteric^[1]. Cholangiocarcinoma typically presents as biliary strictures. These strictures remain a diagnostic dilemma since a

significant proportion of them remain indeterminate for malignancy despite radiologic, endoscopic, and laboratory testing. Early and accurate diagnosis impacts not only patients' outcome and patients' possible surgical candidacy, but also potential targeted chemotherapies. Since 13% to 24% of patients with presumed hilar cholangiocarcinoma are found to have benign disease^[2,3], accurate diagnosis is paramount to avoid unnecessary surgery for patients with benign strictures. The difficulty is amplified when attempting to discern malignant from non-malignant strictures in patients with primary sclerosing cholangitis (PSC) as this affects transplantation decision. This review explores strategies that can be employed by the endoscopist to improve the diagnostic yield of endoscopic work-up in patients with indeterminate strictures. Biliary strictures are considered indeterminate when basic work-up, including transabdominal imaging and endoscopic retrograde cholangiopancreatography (ERCP) with routine cytologic brushing, are non-diagnostic.

RADIOLOGIC WORK-UP

Transabdominal ultrasound (TUS) is usually the initial diagnostic modality used to investigate suspected biliary pathology. TUS is non-invasive, relatively cheap, widely available, and allows visualization of the biliary tree. However, TUS does not reliably examine the distal part of the common bile duct because of the interference of bowel gas^[4]. Abdominal computed tomography (CT) is useful for work-up of patients with suspected cholangiocarcinoma. However, it has suboptimal sensitivity for the detection of early tumors^[5]. Since its introduction in 1991^[6], magnetic resonance cholangiopancreatography (MRCP) has emerged as an accurate noninvasive modality for biliary imaging^[7]. MRCP has a high sensitivity for bile-duct stenosis and filling defects associated with bile duct carcinoma; however, its specificity and positive predictive values are suboptimal as it cannot reliably distinguish malignant strictures from other strictures due to benign etiologies^[8,9]. Still, some ductal features on MRCP and ERCP may suggest malignant or benign etiology of biliary strictures. Malignancy is suggested by long (> 10 mm), asymmetric, and irregular strictures. However, these criteria are not particularly sensitive or specific^[10]. Therefore, unless abdominal imaging detects biliary mass lesions, further endoscopic work-up is warranted to determine the etiology of biliary strictures.

ENDOSCOPIC RETROGRADE CHOLANGIOPANCREATOGRAPHY

Intraductal brushing during ERCP (Figure 1) remains the first-line approach for tissue sampling of biliary strictures because of its wide availability and technical ease in most cases. However, most studies report a poor sensitivity of 27% to 56%^[11-14]. Multiple strategies have been employed to improve the sensitivity without sacrificing the specificity. These have included novel brushes^[15], biliary stricture



Figure 1 Fluoroscopic image during endoscopic retrograde cholangiopancreatography showing a hilar stricture with left intrahepatic biliary dilation. Endoscopic brushing was performed and routine cytology confirmed hilar cholangiocarcinoma.

dilation with subsequent brushings^[16], repeated brushings, immunohistochemistry testing, mutational analysis, digital image analysis (DIA), and fluorescence *in situ* hybridization (FISH)^[17].

Fogel *et al.*^[15] studied the use of a longer biliary cytology brush with stiffer bristles. Despite improved cellularity, cancer detection rates were not improved by using the new brush design. The low sensitivity of biliary brushings is attributed to the submucosal pattern of tumor growth or extrinsic malignancy. Interrupting the mucosa with endoscopic dilation may theoretically improve the cytologic yield. de Bellis *et al.*^[16] studied this strategy by obtaining brushing cytology pre and post dilation. Sensitivity did not improve after dilation (35% and 31%, respectively); however, importantly, the combined sensitivity was improved at 44%. The authors concluded that stricture dilation was not helpful but that repeat biliary brushings increase the diagnostic yield. Inadequate biliary cytology specimens are the main reason for non-diagnostic samples. This may be overcome by the presence of an onsite cytopathologist or technician, which allows real time assessment of cytology samples and may decrease the likelihood of inadequate samples and improper sample preparation [same as widely practiced with endoscopic ultrasound-guided fine needle aspiration (EUS-FNA)]^[18]. If this is not possible, other practiced strategies include cutting the entire brush and submitting it to pathology in a fixative solution or creation of slides by the endoscopy team and placing them in a fixative solution prior to submission to pathology^[18].

Ancillary cytology techniques

Chromosomal abnormalities are typically seen in biliary tract malignancies. Flow cytometric analysis for DNA content has been used with moderate gains in sensitivity at 42%, but at the expense of a lower specificity of 77%^[19]. This technique is also limited by its requirement of a relatively large amount of tissue for examination. New ancillary cytologic techniques, such as FISH and DIA, have recently been used to improve the sensitivity of routine cytology for the diagnosis of malignancy in

pancreatobiliary strictures. FISH analysis detects chromosomal polysomy using fluorescent probes, whereas DIA technique quantifies nuclear DNA *via* special stains to assess for the presence of aneuploidy^[17,20,21]. Only 80% of pancreaticobiliary malignancies manifest these cellular alterations. Therefore, the sensitivity of these advanced techniques is still not optimal. Levy *et al*^[20] found that FISH improves sensitivity 14% to 24% when routine cytology is negative. Fritcher *et al*^[17] found that patients with abnormal FISH results were 77 times more likely to have carcinoma than those with normal FISH. They also found that DIA had a higher sensitivity (44.8%) than cytology. However, specificity was significantly lower at 89.1%. In addition, DIA was not found to be a significant independent predictor of malignancy^[17]. Therefore, FISH seems to be a more valuable ancillary cytologic technique for the evaluation of indeterminate biliary strictures. It is particularly useful in biliary malignancy as it requires fewer cells for analysis than routine cytology or flow cytometry. A recent report studied the additional value of including deletion of 9p21 (p16) in the diagnostic criteria of FISH for malignant biliary strictures^[22]. This significantly improved the sensitivity of FISH from 47% to 84%.

It is crucial to realize that benign strictures in patients with PSC may manifest chromosomal abnormalities and, thus, the specificity of FISH in this setting (67%-88%) is lower than routine cytology^[23]. However, the sensitivity of FISH for malignancy in this setting is still higher than that of routine cytology at 72%^[23]. In short, FISH increases the sensitivity of brush cytology of indeterminate biliary strictures at the expense of a lower specificity. Therefore, FISH should be reserved for patients with high pre-test probability for malignant strictures (e.g., PSC patients with dominant stricture, patients with persistent elevation of carbohydrate antigen 19-9 after biliary decompression, *etc.*).

Endobiliary forceps biopsy

Endobiliary forceps biopsy of biliary strictures during ERCP is another endoscopic technique used in routine clinical practice for sampling biliary strictures. In general, forceps biopsies have had the highest yield when compared to brush cytology and fluoroscopically-guided FNA. Cancer detection rates using endobiliary forceps range from 44% to 89 % for cholangiocarcinoma and 33% to 71 % for pancreatic cancer^[24-27]. Wright *et al*^[28] studied the “smash protocol” for handling biopsies of biliary strictures obtained using endobiliary forceps. Biopsies were smashed between two glass slides, stained by rapid Papanicolaou, and immediately read by on-site pathologists in the ERCP suite. The authors found that immediate cytopathologic diagnosis at ERCP was established in 72 % of cases and concluded that this approach permits immediate diagnosis and avoids the need for subsequent procedures, adds little cost and time, and is safe to perform. External validation of these results is warranted. Jailwala *et al*^[29] showed that endobiliary

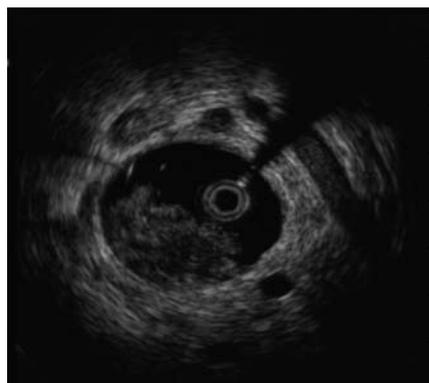


Figure 2 Intraductal ultrasound showing bile duct mass and surrounding lymph nodes.

forceps biopsy of biliary strictures had an incremental diagnostic yield for diagnosis of bile duct malignancy as compared to cytology alone. Triple sampling with brushing, transpapillary biopsy and endoluminal FNA had the highest sensitivity. The authors recommended the use of at least two sampling methods. However, endobiliary biopsy remains technically challenging (especially for proximal biliary strictures) and complications, including bleeding and biliary perforation, have been infrequently described. Jailwala *et al*^[29] remark that biopsy sampling added 10 to 15 min to the procedure time. Currently most biopsies are done using standard biopsy forceps alongside a guidewire which makes positioning the forceps more difficult^[30]. While adapted biopsy forceps^[31,32] are used they are not widely available or utilized. Widely available dedicated biliary forceps are needed to improve the yield and safety of biliary biopsies.

Intraductal ultrasound

ERCP with intraductal ultrasound (IDUS) has also been utilized to improve the diagnostic yield of biliary strictures^[33-35]. IDUS is accomplished by over-the-wire insertion of a small and high-frequency ultrasound probe into the biliary system through a standard duodenoscope under fluoroscopic guidance^[36]. Advantages of these probes include ease of biliary cannulation obviating the need for sphincterotomy in most cases, and the provision of high resolution, detailed images of ductal and periductal tissues without significantly lengthening the ERCP procedure. Moreover, although distant metastases and lymph node involvement may fall outside the imaging field of the device, IDUS can provide local staging required to select patients who would benefit from surgical resection when a malignancy is identified^[37]. IDUS has consequently emerged as an adjunct to ERCP in the evaluation of biliary strictures (Figure 2). Sonographic features seen during IDUS that are suggestive of malignancy include eccentric wall thickening with an irregular surface, a hypoechoic mass, heterogeneity of the internal echo pattern, a papillary surface, disruption of the normal three-layer sonographic structure of the bile duct, presence of lymph nodes, and vascular invasion^[38]. Find-

ings suggestive of benign lesions include preservation of the normal three-layered sonographic appearance of the bile duct wall, homogeneous echo-rich masses with smooth margins, and the absence of a mass lesion^[38]. The accuracy of these criteria in patients with biliary strictures ranges from 83% to 90%^[36,39,40]. IDUS has been shown to improve the diagnostic accuracy of ERCP (with routine cytology) to 58% to 90%^[35,39,41]. The benefit of IDUS, however, is limited in the repeated evaluation of strictures, as the presence of a previously placed biliary stent affects its diagnostic yield^[42]. Lee *et al*^[42] favored EUS to IDUS given that their patients typically had prior stents placed for the treatment of indeterminate strictures. However, IDUS may have a role in concert with EUS, especially in patients without prior stent or in those with proximal biliary (e.g., hilar strictures) lesions, where EUS has shown suboptimal accuracy (see below)^[35,43].

ENDOSCOPIC ULTRASOUND

Endoscopic ultrasound (EUS) has become a valuable tool in the evaluation of lesions in the gastrointestinal tract as well as pancreaticobiliary system. It has the advantage of being able to both provide real time imaging of the GI tract and adjacent organs as well as obtain tissue through FNA. EUS-FNA has a sensitivity of about 85% and a specificity approaching 100% for the diagnosis of pancreatic tumors^[44,45]. The role of EUS in indeterminate biliary strictures is still not well defined. EUS has a sensitivity and specificity comparable to MRCP in the diagnosis of pancreaticobiliary disease^[46]. Sai *et al*^[9] studied 123 non-icteric patients with elevated alkaline phosphatase and common bile duct dilation on TUS. MRCP followed by EUS had a sensitivity of 90% and specificity of 98% for the diagnosis of cholangiocarcinoma. The positive predictive value was also dramatically increased from 35% to 70% when EUS was added to MRCP^[9].

The advantages of EUS-FNA in the diagnostic work-up of patients with indeterminate biliary strictures are multiple. EUS may visualize a biliary mass missed by other imaging modalities in a significant proportion of patients (Figure 3). Eloubeidi *et al*^[47] reported visualizing a mass in 33% of patients following previously non-diagnostic imaging. Similarly, a more recent study reported a mass visualized on EUS in 94% of patients (Figure 4), while CT and magnetic resonance imaging (MRI) revealed a mass in only 30% and 42% of these patients, respectively^[43]. EUS allows performance of FNA of visualized masses with reported sensitivity for malignancy of 43% to 86%^[42,47-49]. It is noteworthy mentioning that the sensitivity of EUS-FNA is significantly higher in distal than in proximal cholangiocarcinoma. Mohamadnejad *et al*^[43] studied 81 patients with cholangiocarcinoma who underwent EUS. Sensitivity of EUS-FNA was significantly higher in distal compared with proximal cholangiocarcinoma (81% *vs* 59%, respectively; $P = 0.04$). Another advantage of EUS is ability to define

unresectable tumors in some patients where CT and/or MRI failed to detect unresectability^[43]. In addition, EUS-FNA permits identification of extraductal tumors and allows triage of patients to potential non-operative management (e.g., lymphoma, metastatic lesions)^[50,51]. Therefore, EUS-FNA is an important diagnostic modality in patients with a distal indeterminate biliary stricture. ERCP remains the preferred initial approach in patients with proximal (defined as < 2 cm from the hilum) strictures. In symptomatic (i.e. icteric) patients, ERCP should still be the first-line approach because drainage can be accomplished concomitantly with tissue sampling. EUS-FNA can be performed subsequently if tissue samples obtained during ERCP are non-diagnostic.

CHOLANGIOSCOPY

Direct visualization of biliary strictures through cholangioscopy (percutaneous or endoscopic) may improve the diagnostic yield of cholangiography and routine cytology^[52]. Percutaneous cholangioscopy is effective in visualizing the biliary tree; however, it requires a percutaneous biliary access and repeated dilations to accept the cholangioscope. The use of “mother-baby” scopes have fallen out of favor due to requirement of two operators, fragility, suboptimal irrigation systems, and lack of 4 way tip deflection (Figure 5)^[53]. The Spyglass direct visualization system (Boston Scientific, Natick, MA, United States)^[54-56] allows for single operator cholangioscopy (SOC)^[54-56]. The components of the SOC system include the disposable SpyScope (Boston Scientific), a 10Fr access and delivery catheter with a 1.2 mm diameter working channel and 2 dedicated irrigation channels (Figure 6). It is introduced through a duodenoscope with a minimum working channel diameter of 4.2 mm. The catheter is capable of tip deflection of at least 30 degrees in 4 directions^[54,56]. The reusable SpyGlass Fiber Optic Probe (Boston Scientific) provides 6000 pixel images. The disposable SpyBite Biopsy Forceps (Boston Scientific) incorporates jaws at the tip designed to excise and retrieve visually targeted tissue.

An initial prospective observational feasibility study at 2 tertiary medical centers demonstrated that the SOC system can provide adequate samples for histologic diagnosis and to successfully guide stone therapy^[54]. Subsequently, Chen *et al*^[57] conducted a larger scale multicenter prospective observational study of SOC procedures in 297 patients with biliary strictures and/or stones and aimed to provide confirmatory evidence that direct visualization using the SOC system can aid in the diagnosis of biliary disease and facilitate stone therapy. The overall procedure success rate was 89%. SOC visual impression had a sensitivity, specificity, positive predictive value and negative predictive value for diagnosing malignancy of 78%, 82%, 80% and 80%, respectively (Figure 7). For SOC-directed biopsy, the respective results were 49%, 98%, 100% and 72%. Sensitivity was higher (84% and 66%, respectively) for intrinsic bile duct malignancies

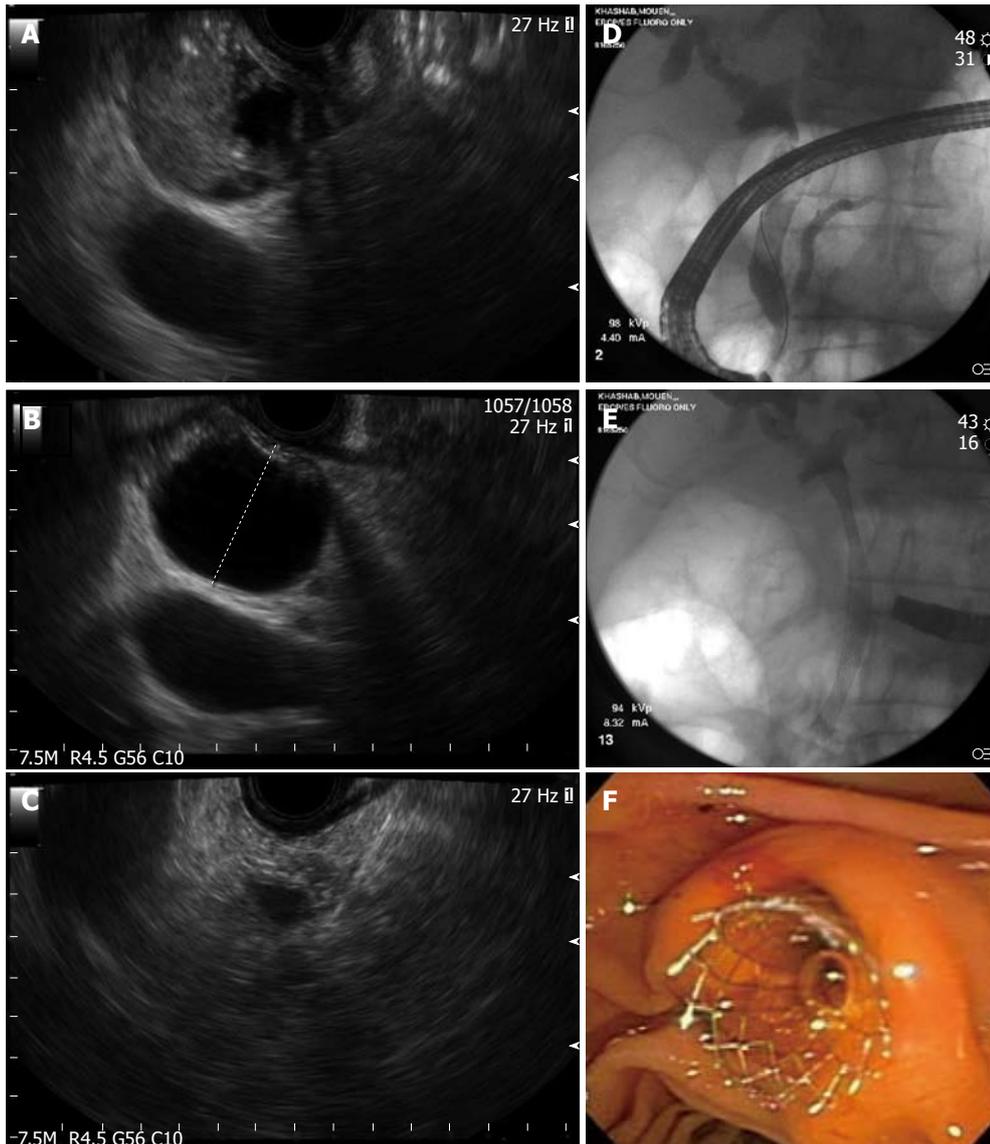


Figure 3 Endoscopic diagnosis and therapy for a bile duct mass missed on transabdominal imaging. A: Endoscopic ultrasound showing a bile duct mass that was missed by computed tomography and magnetic resonance imaging; B: Biliary dilation was present proximal to the stenosis; C: Endoscopic ultrasound-guided fine needle aspiration was performed and was diagnostic of cholangiocarcinoma; D: Endoscopic retrograde cholangioscopy was performed during the same session and cholangiography revealed distal biliary stricture; E, F: A fully-covered metal biliary stent was placed.

as compared to non-intrinsic malignancies. Diagnostic SOC procedures altered clinical management in 64% of patients. The incidence of serious procedure-related adverse events was 7.5% for diagnostic SOC. Ramchandani *et al*^[58] recently described a sensitivity of 95% and specificity of 79% for visual impression during Spyglass cholangioscopy in 36 patients with indeterminate biliary strictures. Both sensitivity and specificity were 82% after utilizing Spybite cholangioscopic biopsies^[58]. Draganov *et al*^[59] reported that the SOC guided biopsies had significantly higher accuracy of 84.6% as compared to standard transpapillary biopsies and cytology with accuracies of 53.9% and 35.5%, respectively. The authors were careful to point out that SOC guided biopsies had suboptimal negative predictive value of 69.2% over the mean 22 mo follow up^[59]. These results suggest a benefit of SOC in patients with indeterminate biliary strictures.

Visual impression of malignancy is an integral part of cholangioscopy, especially that the yield of SpyBite biopsies is suboptimal. Presence of “tumor vessels” within biliary strictures during cholangioscopy is indicative of biliary malignancy^[60]. These irregular, dilated vessels are due to neovascularization at the site of the stricture due to tumor growth. Their presence has sensitivity up to 100% for malignancy^[61]. Intraductal nodules and masses (Figure 8) can be visualized during cholangioscopy and are indicative of malignancy^[60]. However, these ductal findings are only visualized in a fraction of patients with cholangiocarcinoma. Biliary mucosal changes can be further delineated using methylene blue-aided cholangioscopy. In a feasibility study, Hoffman *et al*^[62] showed that normal and non-dysplastic mucosa was characterized by a homogenous light blue staining pattern, where as inflamed and dysplastic mucosa was characterized by in-

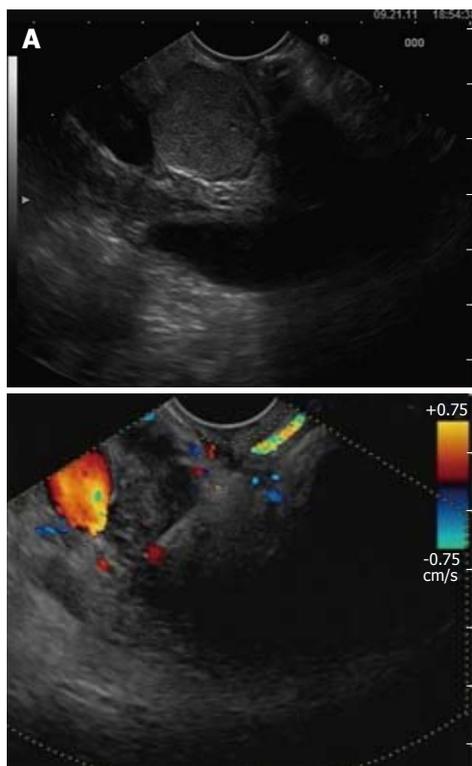


Figure 4 Endoscopic ultrasound evaluation of bile duct mass not seen on transabdominal imaging. A: Endoscopic ultrasound demonstrating the presence of bile duct mass; B: Endoscopic ultrasound-guided fine needle aspiration was diagnostic of cholangiocarcinoma



Figure 5 The “mother-baby” scope cholangioscopy system. The main disadvantage of this system is the requirement for two endoscopists to perform the procedure.

tense and inhomogeneous dark blue staining. More studies are needed to depict the utility of chromoendoscopy during cholangioscopy.

SOC is a technically feasible but is currently not a first line modality for evaluation of biliary strictures. It has some limitations. The 10 French catheter size typically requires sphincterotomy to advance the cholangioscope into the bile duct. At times due to the location and diameter of the stricture, the system cannot be advanced to the desired location. Complications for ERCP with cholangioscopy are reported by Chen *et al*^[57] as 7.5% for

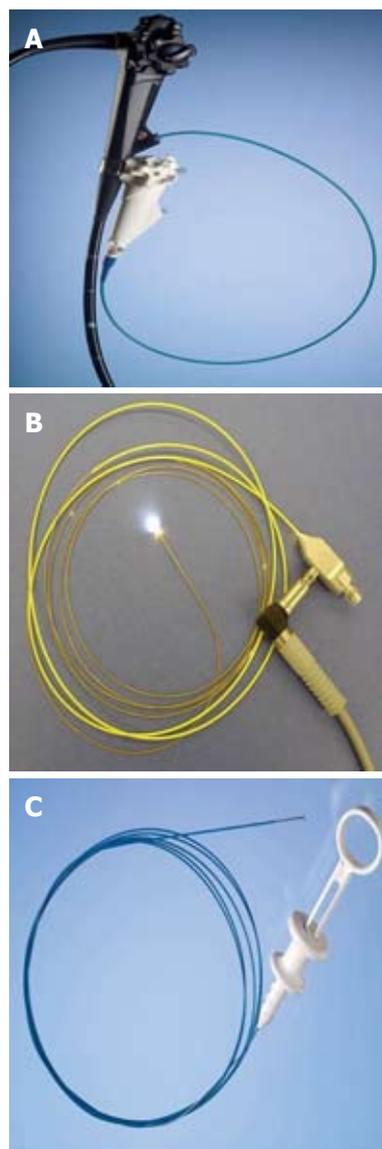


Figure 6 Spyglass single operator cholangioscopy system. A: SpyScope 10Fr access and delivery catheter; B: SpyGlass fiber optic probe; C: SpyBite biopsy forceps.

the diagnostic SOC. Sethi *et al*^[63] report that ERCP with cholangioscopy had complication rate of 7.0% as compared to the ERCP only rate of 2.9%. Subgroup analysis revealed a high proportion of cholangitis in the cholangioscopy group. They postulate that this may be due to the saline infusion used in cholangioscopy^[63]. Draganov *et al*^[59] did not report any episodes of cholangitis in 26 patients who underwent SOC for indeterminate stricture. A recent editorial from Gaidhane *et al*^[64] suggests that this lack of cholangitis may be due to aggressive biliary drainage in these patients.

Probe-based confocal laser endomicroscopy

Confocal laser endomicroscopy permits real time histologic evaluation during endoscopy. Probe-based confocal laser endomicroscopy (p-CLE) can be used to generate microscopic information during ERCP^[65]. The Cholan-

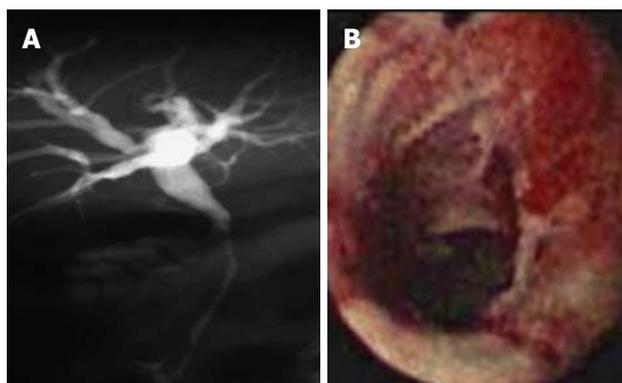


Figure 7 Single operator cholangioscopy used to obtain a diagnosis in a stricture with nondiagnostic cytology. A: Magnetic resonance cholangiopancreatography showing a long distal biliary stricture with proximal biliary dilation. Endoscopic retrograde cholangiopancreatography with brushing was non-diagnostic; B: SpyGlass cholangioscopy revealed a malignant-appearing ulcerated biliary stricture. Spybite biopsies confirmed cholangiocarcinoma.

gioFlex probe (Maunakea Tech, Paris, France) is specially designed for p-CLE during ERCP procedures and has been miniaturized to have an external diameter of 0.94 mm. The probe fits in the 1.2 mm diameter working channel of a cholangioscope. The probe can be inserted as a standard ERCP accessory device. The radio opacity of the probe tip allows for fluoroscopic guidance and probe positioning, and the optical penetration of the confocal plane provides subsurface information with no interference from bile or solid residues. In a feasibility prospective study on 14 patients with indeterminate biliary strictures, the investigators predicted neoplasia with a sensitivity of 83%, specificity of 88%, and accuracy of 86%^[66]. In a larger study of 102 patients with indeterminate pancreaticobiliary strictures, the overall diagnostic accuracy of pCLE was 81%^[67]. p-CLE resulted in a significant increase in overall diagnostic accuracy of ERCP with tissue acquisition (90% *vs* 73%, $P = 0.001$). Biliary p-CLE is still in its infancy and requires further study before its routine use in the work-up of indeterminate biliary strictures is recommended.

Direct peroral video cholangioscopy

Direct peroral video cholangioscopy (D-PVCS) involves direct insertion of an ultra slim endoscope into the bile duct. This is advantageous as it requires one operator, provides high quality digital images (including narrow band imaging), provides separate water and air channels, and allows a larger working channel for diagnosis and therapeutics^[68,69]. D-PVCS has been accomplished using a variety of methods including direct insertion, wire-guided insertion, overtube-balloon assisted insertion, occlusion balloon-assisted insertion, and anchoring balloon-assisted insertion^[70]. D-PVCS requires an adequate biliary sphincterotomy to facilitate insertion of the ultra slim scope. Insertion rates have improved with intraductal anchoring balloons. Moon *et al*^[71] reported insertion success of 95.2% using an intraductal balloon catheter. A dedicated anchoring balloon system was sub-

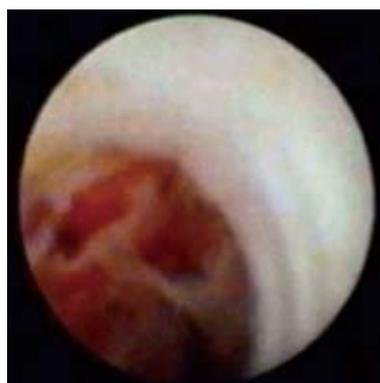


Figure 8 SpyGlass cholangioscopy revealing a bile duct mass. This is indicative of cholangiocarcinoma.

sequently developed by Cook Medical (Winston-Salem, NC) to facilitate D-PVCS^[62]. However, few episodes of cardiac and cerebral air embolisms have been reported with this system and were thought to be due to biliovenous fistula^[64]. Carbon dioxide insufflation during D-PVCS may decrease or eliminate embolization risk. However, this has to be further studied in animal models before embarking on further human studies.

In conclusion, indeterminate biliary strictures remain elusive. ERCP with routine cytology and transpapillary biopsy is the first diagnostic test of choice, especially in that these strictures often require treatment with dilation and/or stenting. Advances in endoscopic and cytopathologic techniques and accessories have improved the diagnostic yield of endoscopic work-up. These advances include EUS +/-FNA, IDUS, cholangioscopy, ancillary cytology techniques, among others. If initial ERCP with routine cytology and biopsy is non-diagnostic, one or a combination of these diagnostic techniques is warranted. The choice of what next diagnostic modality should be used should be individualized and depends on local expertise, patients' anatomy, comorbidities (e.g., PSC), and preferences.

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Microscopic colitis

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Abstract

Microscopic colitis may be defined as a clinical syndrome, of unknown etiology, consisting of chronic watery diarrhea, with no alterations in the large bowel at the endoscopic and radiologic evaluation. Therefore, a definitive diagnosis is only possible by histological analysis. The epidemiological impact of this disease has become increasingly clear in the last years, with most data coming from Western countries. Microscopic colitis includes two histological subtypes [collagenous colitis (CC) and lymphocytic colitis (LC)] with no differences in clinical presentation and management. Collagenous colitis is characterized by a thickening of the subepithelial collagen layer that is absent in LC. The main feature of LC is an increase of the density of intra-epithelial lymphocytes in the surface epithel-

ium. A number of pathogenetic theories have been proposed over the years, involving the role of luminal agents, autoimmunity, eosinophils, genetics (human leukocyte antigen), biliary acids, infections, alterations of pericryptal fibroblasts, and drug intake; drugs like ticlopidine, carbamazepine or ranitidine are especially associated with the development of LC, while CC is more frequently linked to cimetidine, non-steroidal antiinflammatory drugs and lansoprazole. Microscopic colitis typically presents as chronic or intermittent watery diarrhea, that may be accompanied by symptoms such as abdominal pain, weight loss and incontinence. Recent evidence has added new pharmacological options for the treatment of microscopic colitis: the role of steroidal therapy, especially oral budesonide, has gained relevance, as well as immunosuppressive agents such as azathioprine and 6-mercaptopurine. The use of anti-tumor necrosis factor- α agents, infliximab and adalimumab, constitutes a new, interesting tool for the treatment of microscopic colitis, but larger, adequately designed studies are needed to confirm existing data.

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Key words: Microscopic colitis; Lymphocytic colitis; Collagenous colitis; Watery diarrhea; Immunosuppressive agents; Anti-tumor necrosis factor- α agents

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INTRODUCTION

Microscopic colitis is a clinical syndrome of unknown etiology, characterized by chronic watery diarrhea in the absence of macroscopic changes in the large bowel. Once a rare diagnosis, its prevalence is now increasing, also because of the reduction of misdiagnoses, as it is included more and more often in the differential diagnosis of watery diarrhea.

As a consequence, knowledge about microscopic colitis has significantly improved in the last decade, with breakthroughs such as the recent reports of endoscopic findings or the use of anti-tumor necrosis factor (TNF)- α agents (infliximab and adalimumab) for refractory forms of the disease.

This paper will review the state of the art of epidemiology, theories on pathogenesis, diagnostic opportunities and, in particular, therapeutic improvements for microscopic colitis.

EPIDEMIOLOGY

Epidemiological data on microscopic colitis (MC)^[1] mainly originate from the Western world. MC accounts for 4%-13% of cases of chronic diarrhea. In Europe, collagenous colitis (CC)^[2] has an incidence of 0.6-2.3/100 000 per year, and a prevalence of 10-15.7/100 000. Lymphocytic colitis (LC)^[3], on the other hand, has an incidence of 3.1/100 000 per year, and a prevalence of 14.4/100 000. In particular, three European epidemiological studies have shown data on the incidence of MC in, respectively, France (0.6/100 000 per year), Sweden (1.8/100 000 per year) and Spain (1.1/100 000 per year). Recent data from North America report an incidence of 3.1-4.6/100 000 per year for CC and 5.4/100 000 per year for LC^[4-7]. A Spanish study has devoted particular attention to the epidemiological differences between CC and LC: patients with CC seem to be younger than patients with LC in a statistically significant way; moreover, in these patients diagnosis occurs later than in those with LC^[5]. Both pathologies are more frequent among females, but more markedly so in CC^[8], though this finding is not statistically significant^[9].

Actually, it may be difficult to determine the real epidemiological impact of MC for at least two reasons. First, the low specificity of signs and symptoms may cause the diagnosis to be missed or at least delayed, so that the incidence of the condition is underestimated. Secondly, until recently there were no epidemiological population studies, the scientific literature on MC mainly consisting of sporadic case reports. It is therefore unclear whether the increased incidence of MC is real or a simple consequence of the increased attention drawn to the disease^[10].

PATHOGENESIS

The aetiology of MC is still unknown: a number of

pathogenetic theories have been however proposed over the years, often in small or conflicting reports.

Abnormal response of luminal antigens

This group of theories is supported by multiple pieces of evidence. It has been shown that an ileostomy with a diversion of the faecal flow determines the clinical and histological resolution of the disease^[11-13]; in a case in which a Hartmann's intervention had been performed, the disease was still present in the intact proximal colon, whereas it had disappeared in the distal colon, that had undergone diversion^[14].

Moreover, the benefits of drugs such as colestyramine in affected patients could be partly related to the removal of luminal toxins^[15]. However, most of the patients with CC do not improve with cholestyramine; if toxins do play a role, it could therefore be less important in CC than in LC^[16].

Chronic inflammation/autoimmunity

One of the most plausible pathogenetic hypotheses identifies the cause of MC in autoimmunity: a chronic inflammatory process against self antigens unleashed by an initial stimulation (infectious, chemical, or of other nature) in a predisposed individual.

This theory rests on diverse, solid items of evidence: (1), MC is more common among females, like most other autoimmune diseases; (2), in MC various markers of autoimmunity have been found, such as the antinuclear antibodies (ANAs) and antiwall antibodies^[17], and, in one case, antineutrophilic cytoplasmic antibodies^[18]; moreover, a recent controlled study by Holstein and colleagues has found a statistically significant association of IgG ANA, antigliadin IgA, anti-Saccharomyces cerevisiae antibodies IgA and IgG with with CC, but not with LC^[19]; and (3), there is association between MC and various autoimmune diseases, such as thyroid diseases, rheumatoid arthritis, diabetes mellitus 1, disorders of the connective tissue, and coeliac disease^[17]; this latter association has a particular importance: in about 30% of untreated celiac patients, colon biopsies show lesions identical to those of LC, and these lesions are even more evident in the cases of refractory coeliac disease^[20-22]. Conversely, another study has evaluated the frequency of coeliac disease in the patients with LC: of 46 patients with MC who had undergone the screening for coeliac disease, 19 had CC and 27 had LC; of these, 4 were coeliac, and all had LC, an incidence much higher than in the general population^[23]. Moreover, another study shows that the CC can be a way of presentation of the coeliac disease^[24]. The possibility that the autoimmune process could be mediated by an abnormal response to luminal antigens (a notion included in the above described theory) would strengthen both theories and pave the way to linking them to each other or even merging them.

Malabsorption of biliary acids

There is conflicting evidence on the role of biliary acids.

The colic infusion of biliary acids in animal models may determine a colitis^[25,26], and patients with ileal resection causing malabsorption of biliary acids may have diarrhea. An association between atrophy of ileal villi and MC has also been described^[27,28].

However, small studies conducted with the bile acid breath test have shown little or no evidence of malabsorption of biliary acids in patients with MC^[29,31]. Other studies investigating the retention of selenium homocholyltaurine have found variable degrees of malabsorption^[27,28,32], but this method is still considered uncertain and of dubious validity.

Brainerd diarrhea and other forms of infection

The term “brainerd diarrhea” indicates a watery diarrhea related to the exposure of an unknown etiologic agent (the agent that initially gave its name to the syndrome is raw milk)^[33].

Brainerd diarrhea is indistinguishable from MC both clinically^[34] and histologically, as it displays the same mucosal lymphocytosis, epithelial damage and collagen deposits^[33,34]. Moreover, a subpopulation of patients with MC responds to antibiotic therapy^[32,34-36], in particular to metronidazole. Finally, a study on an animal model has shown that human leukocyte antigen (HLA) B27/ β 2 microglobulin transgenic mice, exposed to colon bacteria, develop a damage similar to that of LC^[37].

A role has also been suggested of infectious agents such as *Yersinia*^[38] and *Clostridium difficile* (*C. difficile*)^[39,40]. However, this body of evidence has not yet led to identifying a specific etiologic agent.

Correlation with HLA

In general, studies on HLA haplotypes provide insubstantial information: LC has been found to be positively associated with haplotypes A1 and DRW53, DQ2 e DQ1.3, and negatively associated with A3; CC has been found to be both positively and negatively associated with DQ2^[17,41,42]; other studies have found no statistically significant association between HLA haplotypes and MC^[43].

Role of eosinophils

It has been shown that histamine may cause a chloride hypersecretion in the colon, and this mechanism might contribute to cause the secretory diarrhea associated with CC, through increased blood flow and a reduced sphincter activity. Moreover, a case has been reported of a patient with LC and a significant intraepithelial eosinophil infiltrate that improved with antihistaminic therapy^[44]. Also, mast cells could mediate the abnormal deposition of collagen^[45], as patients with systemic mastocytosis often display fibrosis^[46].

Abnormalities of pericryptal fibroblasts

Physiologic production of collagen at the level of the lamina propria is ensured by a sheath of fibroblasts that surrounds lieberkuhn crypts (pericryptal fibroblasts): the

collagen they produce deposits around the crypts in the basal lamina^[47-50]. These fibroblasts have a myofibroblast-like behaviour: they form in the deepest part of the crypts and later climb them. During this migration they mature into collagen producing fibrocytes^[50]. It has been hypothesized that abnormalities of this process could contribute to the excess deposition of collagen in CC, where collagen deposits below the basal lamina in a subepithelial position^[47]. Studies have been conducted with the purpose characterize the subepithelial collagen, but findings are heterogeneous: some reports show that the subepithelial band mostly consists of collagen type VI^[51,52], which finding would have pointed to a primary alteration of collagen synthesis^[44]; other studies have demonstrated the presence of collagen I and III - the latter probably representing an attempt to repair after a chronic inflammatory damage^[53] - and the absence of collagen type IV^[44]; Günther *et al*^[54] have shown the presence of collagen VI along with I and III.

Ischemic processes are an insufficient explanation for the differences between LC and CC: while ischemia does not result in alterations of collagen deposition, it also does not account for the inflammatory infiltrate, and no sign of ischemia has been found in LC. Moreover, abnormalities of pericryptal fibroblast similar to those of CC have been also observed in the fibrotic form of ulcerative colitis^[49]. Therefore, no clear relationship has been defined so far between MC and dysfunctions of pericryptal fibroblasts.

Drug intake

Use of several drugs has been associated with the development of MC. LC has been suggested to be associated with ticlopidine^[55], carbamazepine^[56], ranitidine^[57], vinburine^[58], tardyferon^[59], acarbose^[60] and Cyclo 3 Fort^[61]. In particular, Berrebi *et al*^[55] have shown that ticlopidine-induced LC is accompanied by increased epithelial apoptosis - which could be either a second effect of the drug-associated damage or a consequence of the colitis itself.

The list of drugs suggested as causes of CC includes cimetidine^[62], non-steroid anti-inflammatory drugs^[63,64], and, more recently, lansoprazole^[65].

Recently, Beaugerie *et al*^[66] have proposed a scoring system specific for drug-induced MC, based on chronological and causality criteria. Chronological criteria are based on the time between exposure to a drug and an adverse event, the evolution of the event if the therapy is interrupted, and the evolution if the therapy is restored; causality criteria are based on the exclusion of other causes of diarrhea and on specific histological findings. By combining the two sets of criteria, a score is obtained that defines the probability that a drug has caused MC: 0, not related; 1, doubtful; 2, possible; 3, likely; 4, almost definite.

HISTOPATHOLOGY

Definitive diagnosis of MC rests on histological exami-

nation, which is necessary both to rule out other possible causes of chronic diarrhea and to distinguish between LC and CC.

The diagnosis of LC is based on the following features. First, an increase of the density of intra-epithelial lymphocytes (IEL) in the surface epithelium. Physiologically, IEL of the colonic mucosa are less than 5 per 100 epithelial cells, while in LC they are at least 25 per 100 surface epithelial cells^[3,15,67], typically CD8⁺, carrying the $\alpha\beta$ T-cell receptor^[68-70] and expressing the human mucosal lymphocyte antigen, specific for intestinal lymphocytes^[68,71,72]; lymphocytosis in the crypt epithelium is also seen, but is less constant^[3].

Rarely, an infiltrate of eosinophils in the epithelium can also be found, and in a few reports neutrophils have been described, considered as a sign of acute stage of colitis^[73].

Second, an inflammatory infiltrate in the lamina propria, with prevalence of mononuclear cells such as lymphocytes and plasma cells, but with the sporadic presence of eosinophils, mast cells, macrophages and neutrophils (very rare); unlike those in the surface epithelium, lymphocytes of the lamina propria are mostly CD4⁺^[68,69]. It has been argued that lymphocytosis in the epithelium and in the lamina propria could be a histological response to a *primum movens* inflammatory process, rather than a primitive immunological dysfunction^[16].

Third, surface epithelial damage, manifested with flattening and degeneration of the epithelial cells (with features such as vacuolization of cytoplasm, nuclear irregularity, karyorrhexis and pyknosis) and focal loss and detachment of the epithelium - these features being more common in CC^[3,74,75]. There is also a minimal distortion of the structure of the crypts, but no crypt abscesses and granulomas^[76]. Moreover, active cryptitis has been reported by Gledhill *et al*^[49] in 41% of subjects with LC and in 29% with CC.

CC is characterized by a thickening of the subepithelial collagen layer that is absent in LC. The collagen band appears extremely eosinophilic in routine hematoxylin-eosin staining, but is better recognizable with Masson's trichrome staining; tenascin immunohistochemical stain appears to further improve sensitivity^[77,78].

In the healthy colon, the subepithelial collagen band is thinner than 3 μm ^[48]. The diagnostic criterion for CC has been proposed to be a thickness of at least 10 μm by some authors^[15,32,74], at least 7 μm by others^[29,49,76]. However, it is plausible that in most cases the collagen band reaches even 100 μm ^[15]. According to Lazenby *et al*^[3], the thickness of the collagen band alone is neither sufficient nor necessary for the diagnosis of CC: there are also some qualitative abnormalities, such as entrapment of red blood cells and cells of inflammation in the collagen band, and an irregular appearance of the inferior edge of the basement membrane, because of collagen bundles extending into the lamina propria. Some studies report a decreasing gradient of presence of intraepithelial lymphocytes and thickness of collagen band from the

cecum to the rectum^[69,76], others suggest that biopsies of the transverse colon give the best chance of diagnosis^[79], but as a general rule left-sided colonic biopsies, easily carried out with a flexible rectosigmoidoscope, are considered sufficient for the diagnosis of MC; if descending colon biopsies are not diagnostic and clinical suspicion is strong, a colonoscopy with random biopsies can be performed.

Studies of immunohistochemistry have shown that the collagen band consists basically of type III collagen - the subtype produced with repair functions - pointing to a reactive origin (the normal basement membrane mainly consists of fibronectin, laminin and type IV collagen)^[80].

The histological features of MC are not specific: CC-like findings have been reported in colon cancer, carcinoid lesions, hyperplastic polyps, *C. difficile* infection, Crohn's colitis, constipation and healthy people^[48,76,80-87], while features resembling LC have been described in human immunodeficiency virus, Crohn's disease, healthy people^[67,81,88,89].

CLINICAL MANIFESTATIONS

MC typically presents as chronic or intermittent watery diarrhea. The colon is normal both on endoscopic investigations and on imaging, so that a conclusive diagnosis can only be reached through biopsy and histological examination^[90]. Lately, however, endoscopic findings have been described in patients with MC (as carefully reviewed by Koulaouzidis *et al*^[91]), especially CC, such as colonic mucosal defects (mucosal tears or fractures)^[92-94] and alterations of vascular patterns, e.g. crowding of vessels, with dilated, circling or winding blood capillaries^[95]. Endomicroscopy^[96] and indigo carmine chromoendoscopy^[97] for the endoscopic diagnosis of MC have also been tested.

The severity of symptoms is variable: up to 22% of patients have > 10 bowel movements per day and up to 27% having nocturnal diarrhea^[32].

Diarrhea may be accompanied by symptoms such as abdominal pain, weight loss, incontinence^[88,98-103]. MC has been associated with a significant impairment of the overall quality of life, comparable to if not worse than that of other severe gastroenterological conditions such as anal fissures, severe chronic constipation, faecal incontinence^[104].

MC may be associated with an increased risk of lymphoma^[105,106], and, in women, lung cancer, even though the overall risk of malignancy and mortality is not different from the general population^[107]. Cases of MC as paraneoplastic manifestation have been described, e.g. in colon cancer^[108].

The natural course of the disease is extremely variable. The rate of spontaneous symptomatic remission after many years was reported as varying between 60% and 93% in LC^[88,109] and as much as from 2% to 92% in CC^[32,110-112]. More informative data come from studies that have analysed the smaller timeframe of 6/8

wk, highlighting a general tendency to lower response rates to placebo for CC (12%-20%)^[109-111] than for LC (48%)^[112]. These findings suggest that LC might have an intrinsically higher tendency to spontaneous remission than CC.

TREATMENT

More evidence is available on the treatment of CC than LC, but there is general agreement that there is no reason why the approach to their treatment should differ^[113]; available studies also confirm that therapies that proved effective in one tend to be confirmed as effective for the other as well^[112]. Another accepted guideline for medical therapy, based on the finding that MC has a high rate of spontaneous remission and remission after therapy, is that intermittent therapy should be favoured and that long-term therapy should only be considered when relapses or refractory symptoms leave no other choice^[114]. Therapeutic algorithms that combine available therapies in a meaningful way were proposed in 2003^[10], 2008^[113] and 2011^[90].

Proved association of MC with a range of drugs and with coeliac sprue suggests that both these etiologies are excluded before any medical therapy is initiated. Since patient with coeliac sprue presenting as MC tend to be negative for the typical immune markers of coeliac sprue, a small intestine biopsy remains the most reliable way of ruling it out^[115,116]; however, the invasive nature of the test makes it advisable to use it only for patients with a clinical situation strongly suggesting coeliac disease or with a previously diagnosed MC that has already proved refractory to therapy^[90].

A number of medical treatments have been attempted in patients with MC. A general consensus persists as to the use of traditional antidiarrhoic agents (loperamide, diphenoxylate) as first-line medical therapy, especially for patients with mild symptoms. The main options to be used as second line therapy are represented by bismuth subsalicylate^[117-119] and mesalazine, with or without colestyramine^[120]. Despite the efficacy of both is proved by trials, bismuth subsalicylate presents the drawback that it is not suitable for long-term therapy because of its unfavourable side effects profile. The association mesalazine-colestyramine, on the other end, is made physiologically reasonable by previous evidence on the association between MC and bile acids malabsorption^[121,122], and probably deserved to be further investigated.

There is now overwhelming evidence proving the efficacy of short-term therapy with oral budesonide in MC, a corticosteroid that is quickly metabolized by the liver and has therefore limited side effects compared to other steroids^[109-112]. Still, the overall adverse effects profile and the high risk of steroid dependency impose that this option be kept for second line or third line after the above mentioned agents, or at the very least that it only be considered as first line in cases with severe symptoms. The efficacy of prednisone has also been assessed^[114,123],

but because of its intrinsically severe adverse effects profile it should only be used in patients refractory to budesonide, if at all.

Studies on immunosuppressive therapy for refractory MC have made their appearance since budesonide was first proposed as a treatment, mainly because of the similarity between MC and inflammatory bowel diseases. The first ones to be studied were azathioprine and 6-mercaptopurine^[124-126], so that were recognized as a valid choice for steroid refractory MC early on^[127]. Later on, methotrexate was also proved to be effective^[128].

In patients refractory to all medical therapy, the surgical option should be considered^[125]. A number of interventions, which substantially overlap with the surgical approaches to the inflammatory bowel disease, have shown to have a potential of leading to a complete resolution of the symptoms^[13,14,129].

Boswellia serrata extract and probiotics have been found ineffective by one radionuclide computed tomography each^[130,131] so that they have since not been proposed in any therapeutic algorithm or reconsidered for subsequent trials.

The most recent developments appear to be focused on two lines of research. The first is the use of low-dosage budesonide as maintenance therapy; this approach appears to be more clinically viable than expected^[132,133] and has been deemed as promising^[134]. The second is the use of a new category of immunosuppressive agents, anti-TNF- α agents, already used with great results for the treatment of inflammatory bowel diseases: good results in therapies with both infliximab and adalimumab have been recently reported in experiences of limited size^[135-137], providing a valid rationale for a future larger controlled study.

Based on the accepted body of evidence on MC in combination with the most recent developments, we suggest a systematic treatment algorithm for MC (Figure 1).

CONCLUSION

Microscopic colitis is a quite common cause of chronic watery diarrhea, whose epidemiological impact has grown in the last years. Colon biopsies are required for the definitive diagnosis, and for the histological characterisation of the two subtypes of the disease (CC and LC), whose clinical features and management do not present however differences.

Over the last years, a series of new pharmacological agents for the treatment of MC has been proposed. The role of steroidal therapy, especially regarding the use of oral budesonide, has gained relevance, as well as immunosuppressive agents as azathioprine and 6-mercaptopurine. Few and very recent evidences has introduced anti-TNF- α agents (infliximab and adalimumab) as the possible cutting-edge for the treatment of MC, but larger and adequately designed studies are required to confirm these data.

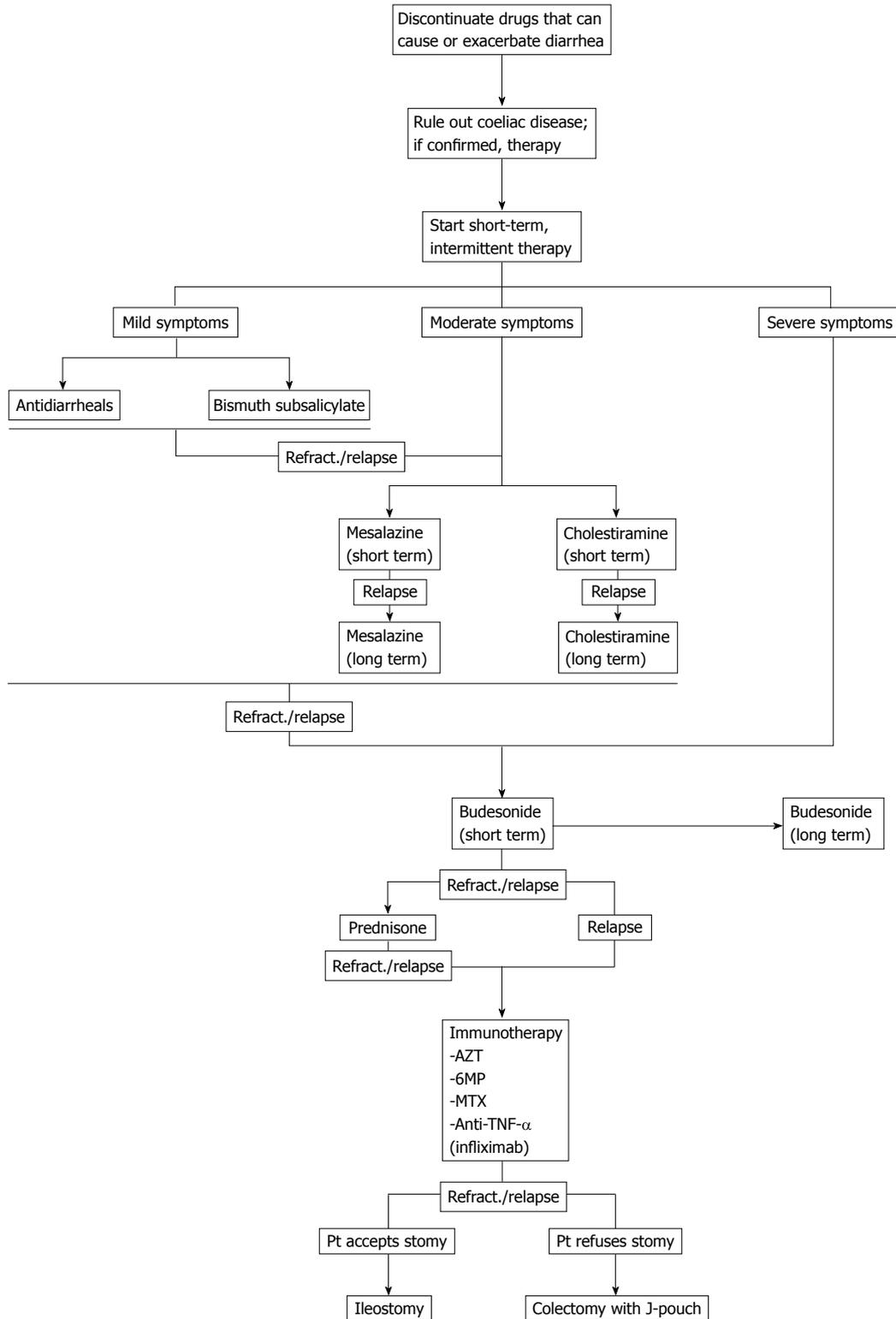


Figure 1 A proposed algorithm for the systemic treatment of microscopic colitis. TNF- α : Tumor necrosis factor- α ; Refract.: Refractory; AZT: Azathioprine; MP: Mercaptopurine; MTX: Methotrexate; Pt: Patient.

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Management strategies of Barrett's esophagus

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Abstract

Barrett's esophagus is a condition resulting from chronic gastro-esophageal reflux disease with a documented risk of esophageal adenocarcinoma. Current strategies for improved survival in patients with Barrett's adenocarcinoma focus on detection of dysplasia. This can be obtained by screening programs in high-risk cohorts of patients and/or endoscopic biopsy surveillance of patients with known Barrett's esophagus (BE). Several therapies have been developed in attempts to reverse BE and reduce cancer risk. Aggressive medical management of acid reflux, lifestyle modifications, antireflux surgery, and endoscopic treatments have been recommended for many patients with BE. Whether these interventions are cost-effective or reduce mortality from esophageal cancer remains controversial. Current treatment requires combinations of endoscopic mucosal resection techniques to eliminate visible lesions followed by ablation of residual metaplastic tissue. Esophagectomy is currently indicated in multifocal high-grade neoplasia or mucosal Barrett's carcinoma which cannot be managed by endoscopic approach.

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Key words: Barrett's esophagus; Diagnosis; Manage-

ment strategies; Esophagectomy; Esophageal adenocarcinoma

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INTRODUCTION

Barrett's esophagus (BE) is a condition resulting from chronic gastro-esophageal reflux disease (GERD). The clinical importance of the definition of BE is that it should identify a lesion documented to be at risk of esophageal adenocarcinoma.

Presently, the diagnosis of BE is based on a combination of endoscopic and histologic criteria^[1,2]. The diagnosis of BE is established when intestinal metaplasia (IM) is found in biopsy specimens obtained from salmon-colored mucosa in the distal esophagus proximal to the gastro-esophageal junction (Figure 1).

DIAGNOSIS

The diagnosis of BE requires systematic biopsy of the abnormal-appearing esophageal mucosa to document IM and to detect dysplasia^[1]. The "Seattle" protocol with random four-quadrant biopsies taken at 1-2-cm intervals along the endoscopically visible BE is the current recommended procedure in guidelines for the detection of dysplasia in patients with established BE^[3-6].

BE is currently graded with use of the Prague circumference and maximum criteria, which is a standardized, validated system based on the circumferential and

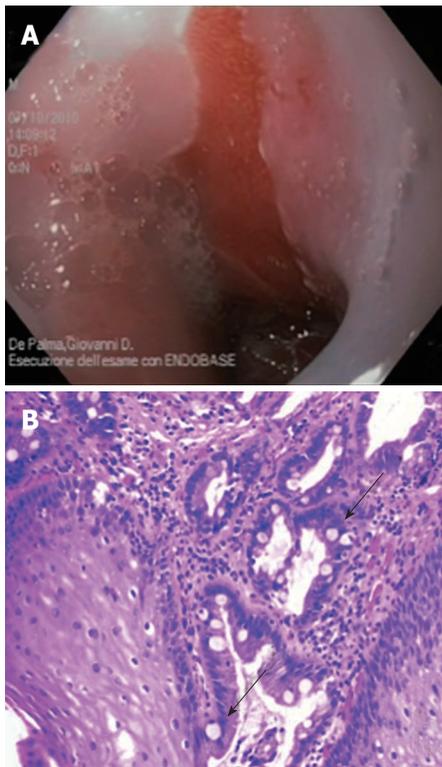


Figure 1 Endoscopic and histologic images of Barrett's esophagus. A: Endoscopic view of salmon-colored mucosa above the gastro-esophageal junction; B: Intestinal metaplasia with goblet cells (arrows) was found in biopsy specimens at histology.

maximal extent of the columnar-lined esophagus^[7-9].

THE PROBLEM: WHY, WHO, WHEN AND HOW TO TREAT FOR BE

BE develops in approximately 5% to 15% of patients with gastro-esophageal reflux undergoing endoscopic evaluation and in 1% to 2% of unselected population undergoing endoscopy^[10-14]. Evidence from one case series suggests that at least 60% of patients with BE develop the disease as a result of chronic reflux; other condition of mucosal inflammation of the lower esophagus, such as mucosal damage by chemotherapy, non-steroidal anti-inflammatory drugs, and viral infections are associated with the development of BE in about 1% of cases respectively^[15-19].

BE is associated with an increased risk of adenocarcinoma of the esophagus. Patients with BE are about 40 times more likely to have esophageal adenocarcinoma (EAC) than those without IM. The risk for an individual patient with BE has been estimated to range from 1 in 50 to 1 in 200 patient-years, or roughly 0.5% per year. Recent large cohort studies suggest the rate of progression is 0.1%-0.3% per year^[20-23].

Barrett's adenocarcinoma is considered a multistep process evolving from normal squamous mucosa to metaplasia to dysplasia to carcinoma. Why such a progression occurs, who is at risk, and what promotes these

changes remain unclear. Clinical and demographic factors, such as, age, male gender, longer duration and increased frequency of GERD symptoms, length of BE segment are associated with modestly increased odds of progression to EAC in some studies^[24-29]. Biomarkers^[30-33] such as aneuploidy and p53 loss have been recently associated with increased risk of progression to high-grade dysplasia (HGD) and/or EAC^[34-37].

At present, the strongest known predictor of cancer risk in the setting of BE is the degree of dysplasia. Subjects with no dysplasia have extremely low cancer rates for the five years following the index endoscopy. Conversely, subjects with HGD have rates reported as high as 10% per year^[38-40].

It is of paramount importance that the correct diagnosis is established. In many instances, especially in the presence of severe inflammation, there is an inter-observer disagreement on the diagnosis and grading of dysplasia. All biopsies with suspected dysplasia should be reviewed by a second "expert" pathologist^[41-43].

Several therapies have been developed in attempts to reverse BE and reduce cancer risk. Aggressive medical management of acid reflux, lifestyle modifications, anti-reflux surgery^[44-49], and endoscopic treatments^[50-52] have been recommended for many patients with BE. Whether these interventions are cost-effective or reduce mortality from esophageal cancer remains controversial.

MANAGEMENT STRATEGIES

Screening and surveillance for BE

Current strategies for improved survival in patients with Barrett's adenocarcinoma focus on detection of dysplasia. This can be obtained by screening programs in high-risk cohorts of patients and/or endoscopic biopsy surveillance of patients with known BE.

There is inadequate evidence of benefit to recommend endoscopic screening for BE in the general population of patients with GERD who do not have risk factors^[53-58]. Well-established risk factors for BE include age older than 50 years, male sex, white race, chronic GERD^[11-5], hiatal hernia^[59], elevated body mass index, and intra-abdominal distribution of body fat^[60,61]. The risk factors can be used to determine the threshold for endoscopy in patients with GERD to screen for the presence of BE^[2].

Endoscopic surveillance for patients with BE is recommended to identify curable neoplasia. Survey data indicate that although surveillance is widely practiced, there is marked variability in the technique and interval of surveillance because practice guidelines are not widely followed (Table 1)^[62].

Endoscopic imaging for the detection of dysplasia and early cancer: Endoscopy with multiple systematic biopsies (the "Seattle" protocol) is needed for the detection of dysplasia or adenocarcinoma for the surveillance of BE. This approach, requiring a large number of

Table 1 Guidelines for evaluation and management of Barrett's esophagus

	ACG	ASGE	AGA	BSG
No-dysplasia	Two esophageal examination with biopsy within 1 yr and follow up with endoscopy every 3 yr	Two consecutive esophageal examination with biopsy within 1 yr and follow up with endoscopy every 3 yr	Assess within 1 yr and if no dysplasia, defer for 5 yr or until cancer therapy is not possible of life expectancy is limited	Surveillance every 2 yr, if appropriate
Indefinite	-	Repeat biopsy after 8 wk of acid suppression, if evidence of acute inflammation due to gastro-esophageal acid reflux	-	Assess with extensive biopsies after course of proton pump inhibitors and return to routine surveillance, if no definite dysplasia at 6 mo
LGD	Treat based on highest grade of dysplasia seen on two esophageal examinations within 6 mo, with pathologist's confirmation, and follow up with endoscopy every year until dysplasia is absent on two subsequent examinations	Follow up after 6 mo with concentrated biopsies in area of dysplasia; follow up every 12 mo if dysplasia persists	Assess in 1 yr and re-examine every year if dysplasia is confirmed by two pathologists (if there is disagreement about the presence of dysplasia then re-examine in 2 yr)	Extensive biopsy after intensive acid suppression for 8-12 wk; surveillance every 6 mo if dysplasia persist; surveillance intervals of 2-3 yr if regression occurs on two sequential examinations
HGD	Document any mucosal irregularities, repeat esophageal examination with biopsy within 3 mo, with pathologist's confirmation, to eliminate the possibility of cancer; follow up with endoscopic mucosal resection in the case of any mucosal irregularity; then intensive endoscopic surveillance every 3 mo or an intervention, such as esophagectomy or ablation, in the case of flat mucosa	Diagnosis should be confirmed by a pathologist; surgical candidates can choose to have a surgery or endoscopic therapy; follow up patients who choose surveillance every 3 mo for 1 yr with several large biopsies every 1 cm along esophagus; after 1 yr without cancer detection, surveillance duration can be lengthened, provided dysplastic changes are absent on two subsequent examinations	Diagnosis should be confirmed by two pathologists; patients should be treated with surgical resection or endoscopic therapy; surveillance can be offered provided follow up with endoscopy is every 3 mo with a minimum of eight biopsies every 2 cm along esophagus	Esophagectomy recommended if changes persist after intensive acid suppression, if confirmed by two pathologists, and if patient considered fit for surgery; if unfit for surgery, use endoscopic ablation or mucosal resection

ACG: American College of Gastroenterology; ASGE: American Society for Gastrointestinal Endoscopy; AGA: American Gastroenterological Association; BSG: British Society of Gastroenterology; LGD: Low-grade dysplasia; HGD: High-grade dysplasia.

biopsies, is time consuming and has several limitations, including sampling error and inconsistent histological interpretation^[2-6].

Several endoscopic imaging techniques to improve the accuracy of endoscopic diagnosis, have been developed and tested recently, with variable results^[63-67]. Enhanced optical imaging techniques may improve the efficiency and accuracy of endoscopic surveillance^[68-72]. Enhanced techniques can generally be categorized as broad-field (red-flag) techniques, such as high-definition/high-resolution white-light endoscopy (HD-WLE) and narrow-band imaging (NBI)^[73-75], and focal techniques, such as confocal laser endomicroscopy (CLE)^[76-80]. The broad-field techniques are good for providing an overview of the entire BE segment, and point out an area of interest, whereas focal techniques can provide greater detail of the area of interest (Figures 2 and 3)^[81-85].

Recent reports demonstrated that, in BE patients undergoing surveillance endoscopy, CLE imaging with targeted biopsies significantly improved the yield of biopsies for dysplasia compared with standard endoscopy with random biopsies when CLE imaging is conducted on suspect areas evidenced with both HD-WLE and NBI. Similarly, CLE was useful as a tool to identify non-dysplastic BE, and hence potentially to reduce the number of biopsies needed^[86-89].

DRUG THERAPY

Acid suppressive therapy, specifically proton pump inhibitors (PPIs), has been shown to improve symptoms and to heal and prevent relapse of erosive esophagitis in patients with BE^[4,90,91]. Evidence to support use of PPIs, in patients with BE solely to reduce risk of progression to dysplasia or cancer is indirect and has not been proven in a long-term controlled trial^[92-96]. Epidemiologic data suggest a lower risk of progression in PPI users. There is also some evidence to suggest that long-term therapy may induce regression of IM and promote the development of squamous islands^[97-99].

There is epidemiologic and experimental evidence to suggest that chemoprevention using non-steroidal anti-inflammatory drugs, aspirin^[100-104], and selective cyclooxygenase-2 inhibitors^[105-107] may reduce the risk of cancer in BE patients. However, human trials to date has not proved that these treatments are associated with a lower risk for neoplastic progression^[108].

The A phase III, randomised study of aspirin and esoprazole chemoprevention in Barrett's metaplasia trial currently underway is seeking to determine the effects of high- and low-dose proton pump inhibitor therapy with and without low-dose aspirin as BE chemoprevention^[109,110].

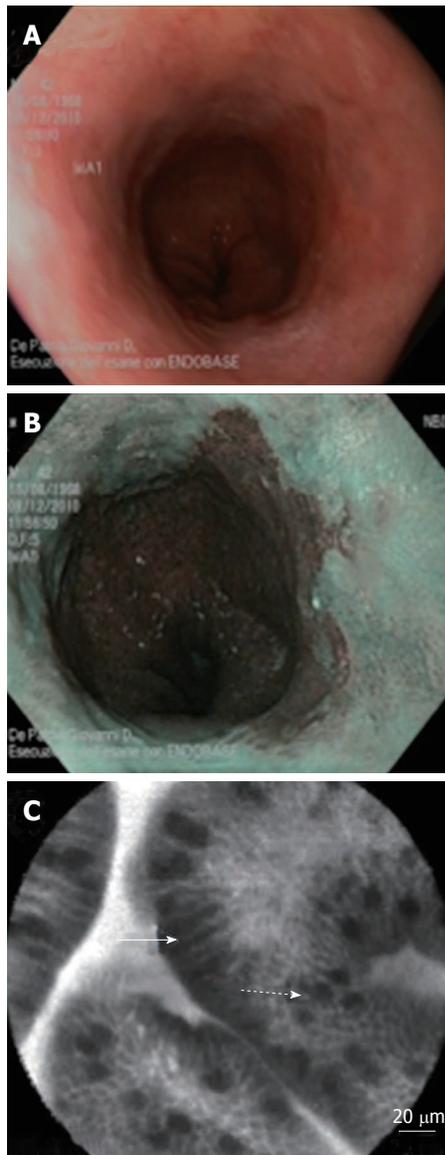


Figure 2 White-light and enhanced endoscopic images of non-dysplastic Barrett's esophagus. A: White-light of Barrett's esophagus; B: Narrow-band imaging endoscopic of Barrett's esophagus; C: Probe-based confocal laser endomicroscopy (pCLE) images of Barrett's esophagus. p-CLE image shows uniform villiform architecture, columnar cells (solid arrow) and dark goblet cells (dash arrow) predictive of non-dysplastic Barrett's esophagus.

ENDOSCOPIC THERAPY

Endoscopic treatment is focused on destruction of the existing metaplastic-dysplastic tissue using different modalities that eliminate the mucosa. The theory behind endoscopic treatment is that the injury of the metaplastic-dysplastic BE combined with vigorous acid suppression or with antireflux surgery would lead to reversion of the BE to squamous epithelium and reduce the risk of progression to cancer^[111-115].

Endoscopic treatment modalities include endoscopic resection techniques such as endoscopic mucosal resection and endoscopic submucosal dissection^[114] and endoscopic ablation therapy^[116,117], such as argon plasma coagulation (APC)^[118,119], laser ablation, photodynamic

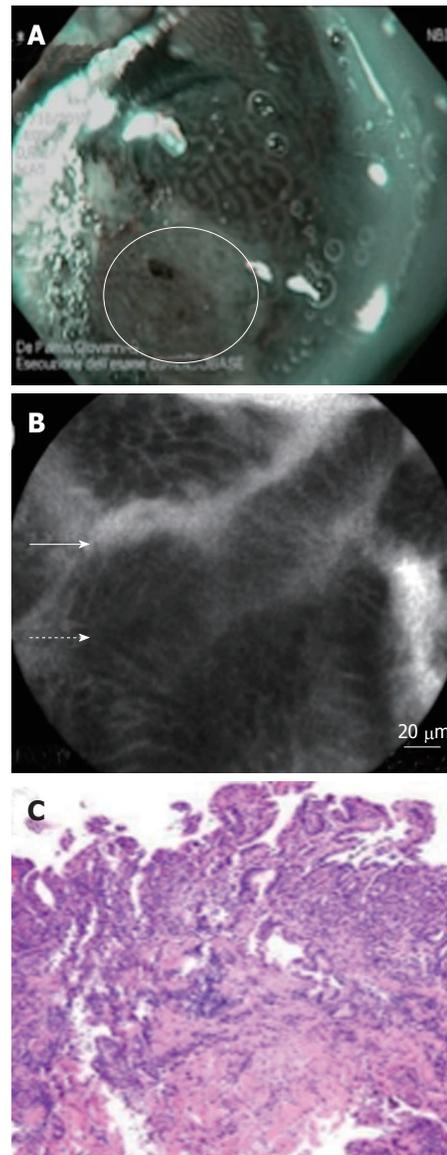


Figure 3 Enhanced narrow-band imaging and probe-based confocal laser endomicroscopy images of dysplastic Barrett's esophagus. A: Narrow-band imaging images shows distorted pits with irregular microvasculature (white circle); B: The corresponding probe-based confocal laser endomicroscopy image shows disorganized, distorted villiform structure and crypts, dark columnar cells (dash arrow) and dilated irregular vessels (solid arrow); C: High-grade dysplasia was found at histology in biopsy specimens performed at this level.

therapy (PDT)^[120], radiofrequency ablation (RFA)^[121,122], and cryotherapy^[123-127].

Current treatment requires combinations of mucosal resection techniques to eliminate visible lesions followed by ablation of residual metaplastic tissue. Endoscopic resection of focal lesions is currently the only method to accurately and reliably determine the depth of invasion of a superficial lesion since it is the only endoscopic technique that provides histology.

Several studies have reported on a variety of ablation methods and have demonstrated difficulty in achieving complete eradication of BE. Thermal ablative modalities, such as APC, and laser therapy suffer from several pitfalls including a not homogeneous ablation of the

mucosa and inconsistent depth of tissue penetration causing that some glands can persist under the neosquamous epithelium^[128,129].

At present, after the areas of mucosal abnormality are removed, ablation of the residual Barrett's mucosa is most commonly performed with PDT or RFA. Photodynamic therapy has been proved to be effective for dysplasia, with a success rate of > 90%. However, following this treatment, there is a high rate of complication and side effects, mainly characterized by strictures and photosensitivity^[2,120,130-132]. Radiofrequency ablation is associated with fewer complications since it has a limited depth of injury, although stricture formation is approximately 6% in some prospective series^[133-137]. After RFA, complete eradication of dysplasia was reported in > 90% of patients with LGD and > 80% of patients with HGD, 1 year after the initial treatment. After 3 years, complete eradication of dysplasia and complete eradication of IM was reported in 98% and 91% of patients, respectively. At 5 years follow up, complete eradication of IM was demonstrated in 92% of the patients^[138-142].

Buried metaplasia is reported less frequently after RFA (< 1%) than after other different ablative endoscopic therapies, including PDT. However RFA is a relatively new procedure and, therefore, available studies on RFA describe only brief follow-up intervals^[143,144].

Because of the esophagus remains after endoscopic therapy, surveillance endoscopy at regular intervals, is necessary, even after complete ablation of BE has been accomplished.

SURGICAL THERAPY

As development of BE is based on gastro-esophageal reflux, a potential concept would be to stop reflux by anti-reflux surgery and thereby interrupt the mechanisms of malignant degeneration. Patients who are appropriate surgical candidates may elect anti-reflux surgery^[145-148]. Fundoplication effectively controls reflux symptoms in most patients^[149,150]. Surgical control of reflux disease, however, has not been found to be associated with a decrease in the incidence of esophageal cancer^[151-154].

Before the advent of endoscopic therapies, esophagectomy was the primary treatment option for patients with HGD.

Esophagectomy offers the most definite treatment in patients with BE with HGD (in particular in patients with multifocal HGD) since it eliminates all of the Barrett's epithelium preventing the risk of progression. In patients with HGD, a benefit of esophagectomy includes the treatment of an occult carcinoma (surgical series summarizing the incidence of occult adenocarcinoma, in patients with the preoperative diagnosis of HGD in resected series show an incidence ranging from 0% to 73%)^[155-159].

The standard surgical resection in most patients includes a total esophagectomy with a transhiatal or trans-thoracic approach, and reconstruction with gastric pull-

up or tubularized gastric conduit and the anastomosis performed in the neck or the high chest. In some cases esophageal resection could be performed minimally invasively. Limited vagal-sparing surgery like esophageal stripping or Merendino's operation is currently indicated in multifocal high-grade neoplasia or mucosal Barrett's carcinoma which cannot be managed by endoscopic approach. Strong consideration should be given for the performance of surgery in a high-volume hospital, by a specialty-trained surgeon with a large-volume esophageal practice^[160-162].

CONCLUSION

BE is a premalignant condition, with dysplasia usually preceding the development of adenocarcinoma. Patients with chronic reflux, especially white males, have the highest risk. Reducing reflux either medically or surgically may diminish the occurrence and/or progression of disease. Management of BE may vary from essentially a surveillance strategy to highly invasive esophagectomy.

Several therapies have been developed in attempts to reverse BE and reduce cancer risk, such as medical management of acid reflux, antireflux surgery, and endoscopic treatments. Whether these interventions are cost-effective or reduce mortality from esophageal cancer remains controversial. Endoscopic mucosal ablation techniques show promise as emerging therapeutic options. Current treatment requires combinations of endoscopic mucosal resection techniques to eliminate visible lesions followed by ablation of residual metaplastic tissue.

Esophagectomy is currently indicated in multifocal high-grade neoplasia or mucosal Barrett's carcinoma which cannot be managed by endoscopic approach.

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Insights into erlotinib action in pancreatic cancer cells using a combined experimental and mathematical approach

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Abstract

AIM: To gain insights into the molecular action of erlotinib in pancreatic cancer (PC) cells.

METHODS: Two PC cell lines, BxPC-3 and Capan-1, were treated with various concentrations of erlotinib, the specific mitogen-activated protein kinase kinase (MEK) inhibitor U0126, and protein kinase B (AKT) inhibitor XIV. DNA synthesis was measured by 5-bromo-2'-deoxyuridine (BrdU) assays. Expression and phosphorylation of the epidermal growth factor receptor (EGFR) and downstream signaling molecules were

quantified by Western blot analysis. The data were processed to calibrate a mathematical model, based on ordinary differential equations, describing the EGFR-mediated signal transduction.

RESULTS: Erlotinib significantly inhibited BrdU incorporation in BxPC-3 cells at a concentration of 1 $\mu\text{mol/L}$, whereas Capan-1 cells were much more resistant. In both cell lines, MEK inhibitor U0126 and erlotinib attenuated DNA synthesis in a cumulative manner, whereas the AKT pathway-specific inhibitor did not enhance the effects of erlotinib. While basal phosphorylation of EGFR and extracellular signal-regulated kinase (ERK) did not differ much between the two cell lines, BxPC-3 cells displayed a more than five-times higher basal phospho-AKT level than Capan-1 cells. Epidermal growth factor (EGF) at 10 ng/mL induced the phosphorylation of EGFR, AKT and ERK in both cell lines with similar kinetics. In BxPC-3 cells, higher levels of phospho-AKT and phospho-ERK (normalized to the total protein levels) were observed. Independent of the cell line, erlotinib efficiently inhibited phosphorylation of EGFR, AKT and ERK. The mathematical model successfully simulated the experimental findings and provided predictions regarding phosphoprotein levels that could be verified experimentally.

CONCLUSION: Our data suggest basal AKT phosphorylation and the degree of EGF-induced activation of AKT and ERK as molecular determinants of erlotinib efficiency in PC cells.

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Key words: Erlotinib; Pancreatic cancer; Epidermal growth factor receptor; Signal transduction; Mathematical modeling

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INTRODUCTION

Pancreatic cancer (PC) is the fourth leading cause of cancer death in the western hemisphere, with an overall five-year survival rate less than 6%^[1]. The most common kind of PC is pancreatic ductal adenocarcinoma, which accounts for more than 90% of all cases. **Major reasons** for this poor outcome are a late diagnosis and the lack of appropriate therapy approaches. Aside from genetic alterations in oncogenes like *KRAS*, or tumor suppressor genes such as *TP53*, *p16/CDNK2A*, and *SMAD4/DPC4*^[2], an increased expression of protein kinase B 2 (AKT2) and epidermal growth factor receptor (EGFR) can be found in a broad range of patient samples^[3-8]. Overexpression of EGFR was accompanied by a worse overall survival^[9]. Most carcinomas are diagnosed in an advanced non-resectable state, with palliative care remaining as the only treatment option.

Erlotinib, a small molecule inhibitor of the EGFR, is approved for the treatment of advanced PC. Combination treatment with gemcitabine has a moderate, but significant, survival benefit over standard treatment with gemcitabine alone^[10]. Rash is a prominent side effect of EGFR-targeted therapies with monoclonal antibodies and small molecule inhibitors. In various studies, a correlation of the efficacy of a targeted therapy with erlotinib and rash was observed^[10,11].

In non-small-cell lung cancer (NSCLC), activating EGFR mutations were identified as an indicator of a good response to small molecule inhibitors targeting this receptor^[12]. However, EGFR activating mutations are uncommon in PC^[13-15]. **Unlike in NSCLC, no predictive marker** (besides rash) for a response to erlotinib has been established to date in PC. It is believed that the identification of such markers holds promise for the classification of patient subgroups that would benefit most from targeted therapy in PC.

Erlotinib binds to the adenosine-5'-triphosphate (ATP) binding site of the EGFR and prevents ligand-induced receptor activation. Hence, no transphosphorylation of receptor complexes takes place, and executive downstream signaling pathways, like Ras-Raf-mitogen-activated protein kinase kinase (MEK)-extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)-AKT, are not activated. To this day, erlotinib-attenuated signal transduction in PC is poorly understood.

Computational approaches for analyzing biochemical reactions are increasingly recognized as useful tools for the study of signaling networks and gaining deeper insights into dynamic processes^[16,17]. We, along with others, have previously shown that a combination of experimental and mathematical approaches can also be successfully applied to the analysis of pathophysiological mechanisms in PC and pancreatic fibrosis^[18-20].

In this study, we have addressed the question of how erlotinib modulates signal transduction *via* the EGFR, in order to determine molecular predictors of erlotinib sensitivity and resistance. To this end, two commonly-used and well-characterized human PC cell lines that differ in their biological sensitivity to erlotinib were chosen for a comparison of the molecular effects of the small molecule inhibitor. Experimental findings were used to establish a mathematical model that simulated major signaling pathways downstream of the EGFR. Together, our data suggest basal AKT phosphorylation and the degree of EGF-induced activation of downstream signaling pathways as molecular determinants of erlotinib efficiency.

MATERIALS AND METHODS

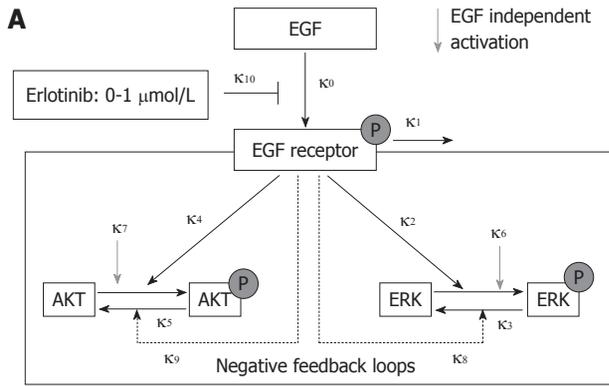
Materials

Iscove's modified Dulbecco's medium (IMDM) was from Biochrom (Berlin, Germany), and RPMI 1640 and fetal calf serum (FCS) was from PAA Laboratories (Pasching, Austria). Erlotinib was supplied by Biaffin (Kassel, Germany), and AKT inhibitor XIV and U0126 by Merck (Darmstadt, Germany). Recombinant human EGF and bovine serum albumin (BSA) were delivered by Sigma-Aldrich (St Louis, MO, United States).

Phospho-EGFR (pEGFR) (Tyr1068) rabbit mAb, phospho-AKT (pAKT) (Ser473) rabbit mAb, phospho-p44/42 mitogen-activated protein kinase (phospho-extracellular signal-regulated kinase 1/2, pERK1/2) (Thr202/Tyr204) rabbit pAb, AKT rabbit mAb, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) rabbit mAb, were purchased from New England Biolabs (Frankfurt, Germany). EGFR rabbit mAb was from Epitomics (Burlingame, CA, United States) and MAP Kinase 1/2 (ERK1/2) rabbit pAb from Millipore (Billerica, MA, United States). **Fluorescently-labeled secondary antibodies** for immunoblot analysis were delivered by LI-COR (Lincoln, NE, United States). **Polyvinylidene fluoride (PVDF)** membrane was obtained from Millipore. Standard laboratory chemicals were from Sigma-Aldrich.

Cell culture

The human PC cell lines BxPC-3 and Capan-1 were obtained from the American Type Culture Collection. BxPC-3 was cultured in RPMI 1640 medium supplemented with 10% FCS, 10⁵ U/L penicillin, and 100 mg/L streptomycin. Capan-1 was cultured in IMDM medium supplemented with 17% FCS, 10 mL/L non-essential amino acids (dilution of a 100 × stock solution), 10⁵ U/L penicillin, and 100 mg/L streptomycin. **The cells were grown** at 37 °C in a 5% CO₂ humidified atmosphere.



B ODE network

$$\frac{d}{dt} pEGFR = \frac{\kappa_0 \cdot (EGFR_{tot} - pEGFR)}{1 + \kappa_{10} \cdot erlotinib} - \kappa_1 \cdot pEGFR$$

$$\frac{d}{dt} pERK = (\kappa_6 + \kappa_2 \cdot pEGFR) \cdot (ERK_{tot} - pERK) - (\kappa_3 + \kappa_9 \cdot \int_0^{\infty} \Gamma_{q_1}^4(\tau) \cdot pEGFR(t - \tau) d\tau) \cdot pERK$$

$$\frac{d}{dt} pAKT = (\kappa_7 + \kappa_4 \cdot pEGFR) \cdot (AKT_{tot} - pAKT) - (\kappa_5 + \kappa_9 \cdot \int_0^{\infty} \Gamma_{q_2}^4(\tau) \cdot pEGFR(t - \tau) d\tau) \cdot pAKT$$

The parameters κ_i are reaction constants.
 $\Gamma_{q_i}^p(\tau) = \frac{q_i^p}{(p-1)!} \tau^{p-1} \cdot e^{-q_i \tau}$ Kernel of the gamma function
 The shape is determined by the parameters p and the mean delay time $\bar{\tau}$.

C Scaling of phosphoprotein levels for Capan-1 with respect to BxPC-3 cells

Erlotinib ($\mu\text{mol/L}$)	$\frac{pAKT(0)^c}{pAKT(0)^b} = r_{pA}$	$\frac{pERK(0)^c}{pERK(0)^b} = r_{pE}$
0.00	0.18 \pm 0.01	0.89 \pm 0.04
0.11	0.22 \pm 0.03	1.56 \pm 0.15
0.33	0.31 \pm 0.06	1.14 \pm 0.12
1.00	0.38 \pm 0.06	1.49 \pm 0.36

$$\kappa_3^c = \frac{\kappa_3^b \cdot \kappa_6^c + [1 - r_{pE}(erl)] \cdot \kappa_6^b \cdot \kappa_6^c}{r_{pE}(erl) \cdot \kappa_6^b}$$

$$\kappa_5^c = \frac{\kappa_5^b \cdot \kappa_7^c + [1 - r_{pA}(erl)] \cdot \kappa_7^b \cdot \kappa_7^c}{r_{pA}(erl) \cdot \kappa_7^b}$$

$$pERK(0) = \frac{\kappa_6}{\kappa_3 + \kappa_6} \quad pAKT(0) = \frac{\kappa_7}{\kappa_5 + \kappa_7}$$

D Relations between observables and model variables

$$pEGFR_{exp} = pEGFR \cdot WB_{pEGFR}$$

$$pAKT_{exp} = pAKT \cdot WB_{pAKT}$$

$$pERK_{exp} = pERK \cdot WB_{pERK}$$

WB_x are Western blot scaling factors

Figure 1 Flow chart of epidermal growth factor signal transduction and the ordinary differential equation network. A: Simplified reaction network. Solid black arrows show epidermal growth factor-dependent processes, whereas grey arrows represent basal phosphorylation. Two epidermal growth factor receptor (EGFR)-dependent negative feedback loops are shown by black dotted lines; B: Translation of the reaction network into an ordinary differential equation (ODE) model describing EGFR-mediated signal transduction; C: Ratio of basal levels of phosphorylated protein kinase B (AKT) (r_{pA}) and extracellular signal-regulated kinase (ERK) (r_{pE}) in BxPC-3 (superscript B) and Capan-1 (superscript C) cells. These calculations are implemented in the mathematical model; D: Equations describe relations between observables (fitted to experimental data) and model variables. For further description see “mathematical model” in the “materials and methods” section. EGF: Epidermal growth factor; pAKT: Phospho-AKT; ERK: Extracellular signal-regulated kinase; pERK: Phospho-ERK; WB: Western blot.

Cell proliferation assay

To analyze the inhibitory effects of erlotinib, AKT inhibitor XIV, U0126, and combinations thereof on cell proliferation, DNA synthesis was measured using a 5-bromo-2'-deoxy-uridine (BrdU) incorporation assay (Roche Applied Science, Mannheim, Germany). Therefore, the cells were seeded in 96 half-area plates. The following day, the cells were serum-starved and the inhibitors alone or in combination were applied as indicated. After 24 h, BrdU labeling solution was added for an additional 8 h and DNA synthesis was measured following the instructions of the manufacturer.

Immunoblotting analysis

Serum-starved BxPC-3 and Capan-1 cells were preincubated with different doses of erlotinib, AKT inhibitor XIV, or U0126, for 4 h before they were stimulated with 10 ng/mL human recombinant EGF. The cells were harvested by medium aspiration and boiled in lysis buffer [2% sodium dodecyl sulphate (SDS), 10% glycerol, 5 mmol/L ethylenediaminetetraacetic acid (pH 8.0), 62.5 mmol/L Tris-HCl (pH 6.8), 0.01% 3,3',5,5'-tetrabromophenol-sulfonphthalein, 5% β -mercaptoethanol]. The cellular proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto PVDF membrane.

Afterwards, the blots were blocked with 1% BSA dissolved in tris-buffered saline [TBS; 20 mmol/L Tris-HCl (pH 7.6), 140 mmol/L NaCl] for 1 h, followed by incubation with primary antibody in TBST (TBS + 0.1% Tween 20) overnight at 4 °C. After washing with TBST, the blots were incubated in secondary antibody solution for 30 min. Immunofluorescence was detected using an Odyssey Infrared Imaging system. Signals for pERK, pAKT, pEGFR, the corresponding total proteins, and GAPDH were quantified using Odyssey® Application Software 3.16.

Phosphoprotein and total protein fluorescence intensities were adjusted to GAPDH and the potential effects of gel inhomogeneities were minimized by normalizing the individually-adjusted signal intensities to the mean of all samples of the gel. At least six independent experiments were performed to calculate mean and SE.

Potential inhibitory effects of erlotinib on the basal phosphorylation of the proteins (prior to the application of EGF) were considered as follows: the time curves of EGF stimulation were adjusted with experimentally-measured basal pERK/ERK and pAKT/AKT ratios for different erlotinib concentrations in both cell lines. Results for Capan-1 cells were related to the corresponding data for BxPC-3 cells (Figure 1). However, no meaning-

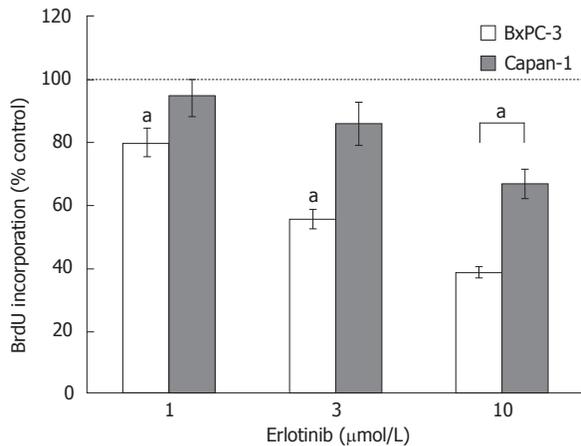


Figure 2 Effects of erlotinib on the proliferation of pancreatic cancer cell lines. BxPC-3 and Capan-1 cells growing in 96 half-area well plates were starved from serum and treated with different doses of erlotinib for a total of 32 h. DNA synthesis was measured by 5-bromo-2'-deoxyuridine (BrdU) incorporation over the last 8 h. Data represent mean \pm SE ($n = 6$). ^a $P < 0.05$ between control cultures without erlotinib.

ful scaling of pEGFR for different erlotinib concentrations without EGF stimulation could be performed, as basal phosphorylation of the EGFR was too weak.

Mathematical model

To examine the signal transduction dynamics downstream of the EGFR, we combined experimental data and mathematical modeling in a systems biological approach. A simplified network of EGFR signaling (Figure 1A) was chosen to describe the different steps in Figure 1A with the help of ordinary differential equations (ODE), whose terms are interpreted using mass action kinetics (Figure 1B). In the ODE model, EGF binds to the EGFR and triggers the phosphorylation of the receptor. Experimentally, only a low level of phosphorylated receptor was found in the absence of EGF. Therefore, no EGF-independent receptor phosphorylation was assumed. The receptor activation can be attenuated directly by erlotinib in a dose-dependent manner and by dephosphorylation. In turn, the phosphorylated receptor triggers the activation of downstream signaling pathways, where AKT and ERK were chosen as representative components. For simplicity, only two individual EGFR-induced feedback loops enhancing the dephosphorylation of AKT and ERK were assumed, although both kinases are targets of multiple inhibitory pathways^[21,22].

It was shown that PC cells may secrete EGF in an autocrine loop^[23]. Taking this into account, and to simulate an oncogenic *KRAS*-driven activation of the Ras-Raf-MEK-ERK pathway in Capan-1 cells, a phosphorylation of AKT and ERK independent of exocrine EGF was considered in the model.

We also included the experimentally measured ratios of basal phosphorylated AKT and ERK in Capan-1 versus BxPC-3 cells for all erlotinib concentrations. This information led to algebraic relations between the model

parameters, and between the initial conditions and the model parameters (Figure 1C).

The relationship between the observables which are fitted to the experimental time series and the variables of the mathematical model include scaling parameters, since the levels of the phosphorylated proteins could not be quantified in an absolute manner (Figure 1D).

To optimize the parameter values, the mathematical model was trained against quantitative immunoblot data. The optimization was done with a hybrid algorithm combining a global and a local search implemented in pWFitBoost of the MATLAB Toolbox Potter's Wheel^[24].

Statistical analysis

All experimental results represent mean \pm SE for the indicated number of experiments. The Wilcoxon rank-sum test was used to test differences for statistical significance. $P < 0.05$ was considered statistically significant.

RESULTS

Erlotinib and pathway-specific inhibitors reduce DNA synthesis of PC cells

In initial experiments, two PC cell lines with different *KRAS* status, BxPC-3 (wild-type) and Capan-1 (harboring mutant *KRAS*), were tested for their sensitivity to erlotinib. Therefore, the effects of clinically achievable concentrations of erlotinib^[25,26] on DNA synthesis were measured using a BrdU assay (Figure 2). Erlotinib significantly inhibited the incorporation of BrdU into newly synthesized DNA in BxPC-3 cells in a dose-dependent manner. Capan-1 cells were much more resistant to erlotinib treatment, and only the highest concentration of 10 $\mu\text{mol/L}$ significantly reduced the DNA synthesis of the cells. Neither of the two cell lines carried genetic alterations in exons 19 and 21 of EGFR, the sites of hotspot mutations sensitizing the receptor to erlotinib in NSCLC^[27] (data not shown).

Next, the question was addressed if one of the two major pathways downstream of the EGFR, Ras-Raf-MEK-ERK and PI3K-AKT, is more sensitive against a perturbation at the EGFR level than the other. Therefore, two pathway-specific inhibitors were used. As shown in Figure 3, AKT inhibitor XIV at a concentration of 10 $\mu\text{mol/L}$ diminished DNA synthesis in both cell lines. When AKT inhibitor XIV and erlotinib treatment were combined, no additional growth reduction over erlotinib alone was observed.

At a concentration of 10 $\mu\text{mol/L}$, the MEK inhibitor U0126 inhibited the DNA synthesis of both cell types. Unlike in the case of AKT inhibitor XIV, an additional treatment with erlotinib further increased the inhibition of DNA synthesis of the cells. Comparing both cell lines, BxPC-3 cells were much more sensitive to U0126 than Capan-1.

Erlotinib inhibits EGFR, ERK and AKT phosphorylation

The differences in the biological response of BxPC-3

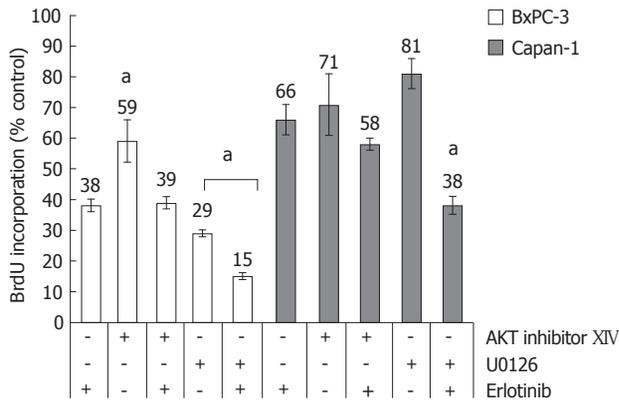


Figure 3 Effects of U0126, protein kinase B inhibitor XIV and erlotinib on proliferation of BxPC-3 and Capan-1 cells. The cells were seeded in 96 half-area well plates, starved from serum and treated with protein kinase B (AKT) inhibitor XIV or mitogen-activated protein kinase kinase inhibitor U0126 in the presence or absence of erlotinib for a total of 32 h. DNA synthesis was measured by 5-bromo-2'-deoxyuridine (BrdU) incorporation over the last 8 h. Data represent mean \pm SE ($n = 6$). ^a $P < 0.05$ vs cultures with erlotinib only. $P < 0.05$ between all samples vs untreated cells (not indicated in the figure).

and Capan-1 cells to erlotinib and the two pathway-specific inhibitors raised the question of the underlying molecular mechanism. In our approach, we focused on the EGFR and the major downstream signaling cascades, where AKT and ERK were chosen as representative components.

The basal (EGF-independent) phosphorylation level of the EGFR and ERK did not differ much between both cell lines; while pEGFR was barely detectable, pERK1/2 was present at a low level. In contrast, BxPC-3 cells displayed a more than five-times higher basal pAKT/AKT ratio than Capan-1 cells (Figures 4 and 5).

To activate the EGFR and its related pathways, cells were stimulated with 10 ng/mL human EGF. As shown in Figure 4, in response to EGF, a phosphorylation of EGFR, AKT and ERK was observed in both cell lines. The pEGFR level increased over the first 60 min of stimulation (Figure 4) and only slightly attenuated afterwards (data not shown). AKT had its maximum phosphorylation at 5 min and ERK at 10-15 min, respectively. Subsequently, pAKT decreased to the initial phosphoprotein level, while ERK phosphorylation remained above the basal level until the end of treatment (Figures 4 and 5). As shown in Figure 5, BxPC-3 cells displayed, at all time points, higher levels of pAKT and pERK than Capan-1 cells.

The phosphorylation of all three signaling components was efficiently inhibited by preincubation of the cells with 1 μ mol/L erlotinib (Figures 4 and 5).

The mathematical model describes the experimental data and predicts phosphoprotein levels of AKT and ERK

To further characterize the signal transduction dynamics downstream of the EGFR, a mathematical model was established. Towards this goal, the experimental data set presented in Figure 4 was extended with phosphopro-

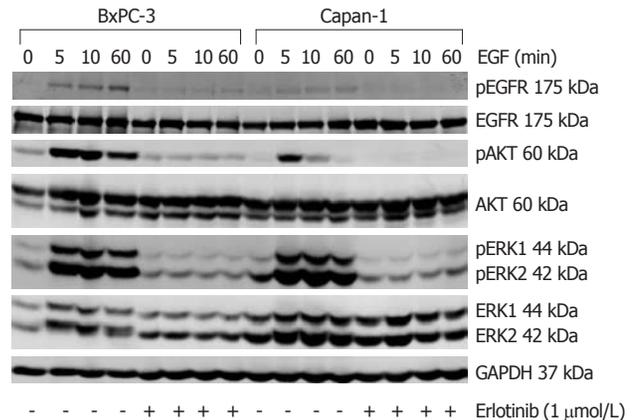


Figure 4 Effects of erlotinib on epidermal growth factor receptor signal transduction in BxPC-3 and Capan-1 cells. Serum-starved pancreatic cancer cells were preincubated with erlotinib at 1 μ mol/L for 4 h, as indicated, before they were stimulated with 10 ng/mL epidermal growth factor (EGF) for the indicated times. Protein extracts from equal amounts of cells were subjected to Western blot analysis. Phospho-epidermal growth factor receptor (pEGFR), phospho-protein kinase B (pAKT), phospho-extracellular signal-regulated kinase 1/2 (pERK1/2), their respective total proteins and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were detected using fluorescein (IRDye[®])-labeled secondary antibodies. One representative Western blot is shown. For mean values of independent experiments, please refer to Figure 5. AKT: Protein kinase B; ERK: Extracellular signal-regulated kinase; EGFR: Epidermal growth factor receptor.

tein data obtained by using additional erlotinib concentrations and time points of EGF stimulation. Figure 5 shows for pAKT and pERK the comparison of the experimental data and model simulations, using optimized parameter values for all erlotinib concentrations and both cell lines. Parameter values and initial conditions of fixed parameters are summarized in Table 1.

As shown by experimental data and model simulation (Figure 5), erlotinib attenuated the activation of AKT and ERK in a dose-dependent manner in both cell types. Phosphoprotein levels in the two cell lines were diminished to a similar degree, except for ERK phosphorylation being more sensitive to erlotinib treatment in BxPC-3 than in Capan-1 cells.

To perform a validation of our mathematical model, we experimentally verified the peaks of the phosphoprotein levels of AKT and ERK for different doses of erlotinib that were previously predicted by computational simulation. Therefore, pAKT and pERK levels were quantified for the indicated times and compared with the model calculations (Figure 6). As shown, the model was able to provide suitable predictions of phosphoprotein peaks of both signaling components in BxPC-3 and Capan-1 cells.

DISCUSSION

In this study, a combined experimental and mathematical approach was chosen to gain deeper insights into the mechanisms of EGFR signaling and erlotinib action in PC cells. In agreement with previous studies, we observed a high biological erlotinib sensitivity of BxPC-3

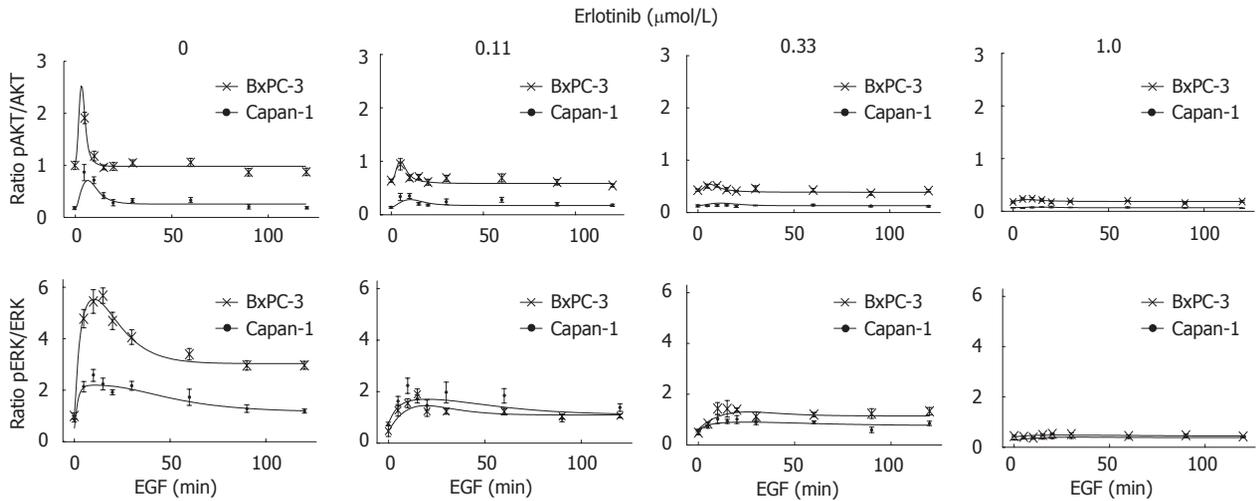


Figure 5 Simulations of the mathematical model reflect the experimental time series. Serum-starved BxPC-3 and Capan-1 cells were preincubated with different doses of erlotinib for 4 h and stimulated with 10 ng/mL epidermal growth factor (EGF) for the indicated times. Cellular lysates were analyzed by quantitative immunoblotting, and phospho-protein kinase B (pAKT), protein kinase B (AKT), phospho-extracellular signal-regulated kinase (pERK), extracellular signal-regulated kinase (ERK) and glyceraldehyde-3-phosphate dehydrogenase levels were determined. Plotted are experimental results (BxPC-3 and Capan-1); mean \pm SE of at least six independent experiments and model simulation (lines) for BxPC-3 and Capan-1 cells for different times of EGF stimulation. All data were scaled to the untreated BxPC-3 cells (absence of EGF and erlotinib), where the ratio was set as 1.

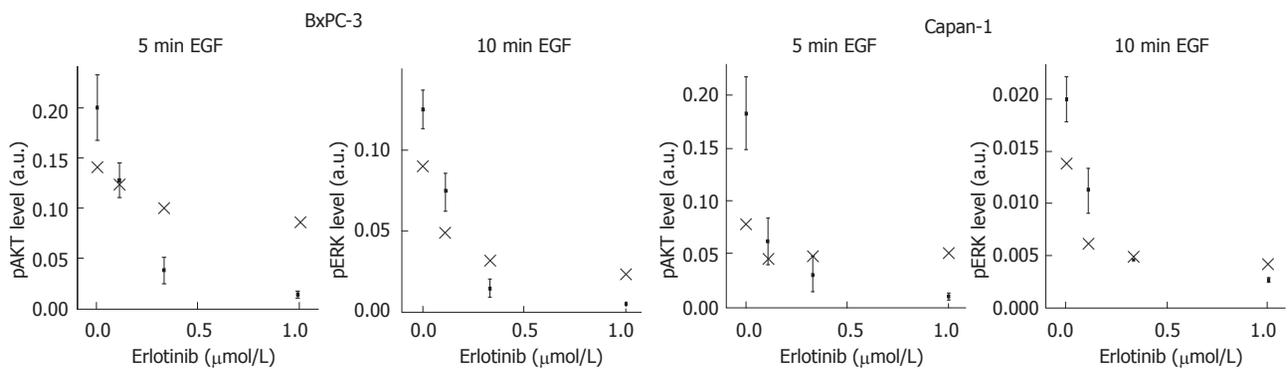


Figure 6 Mathematical prediction of phospho-extracellular signal-regulated kinase and phospho-protein kinase B levels after erlotinib treatment. Serum-starved BxPC-3 and Capan-1 cells were preincubated with different doses of erlotinib for 4 h and stimulated with 10 ng/mL human epidermal growth factor for the indicated times afterwards. Protein kinase B (AKT) and extracellular regulated protein kinases (ERK) phosphorylation were detected by immunoblot analysis. Experimental data (black dots) are expressed as arbitrary units (a.u.) of four independent experiments (mean \pm SE). Model predictions are indicated by cross symbols. EGF: Epidermal growth factor; pAKT: Phospho-AKT; pERK: Phosphomito-gen-extracellular signal-regulated kinase.

cells and a lesser sensitivity for Capan-1 cells^[28-31].

In the two cell lines, EGF induced the phosphorylation of EGFR, AKT, and ERK with similar kinetics, but different amplitudes (higher ratios of pAKT/AKT and pERK/ERK in BxPC-3 than in Capan-1 cells). Furthermore, BxPC-3 cells displayed a more than five-times higher basal pAKT level than Capan-1 cells. Factors that may contribute to the increased AKT phosphorylation are the amplification of *AKT2*, and the existence of an autocrine EGF loop in BxPC-3 but not Capan-1 cells^[32,33]. Despite the presence of an oncogenic *KRAS* allele in Capan-1 cells, basal pERK levels were similarly low in both cell lines. This seemingly surprising finding is in agreement with previous studies^[34], where low levels of pERK in *KRAS* mutant PC cells were linked to the activity of MKP-2, a member of the dual-specificity phosphatase family that acts in a negative feedback loop^[35].

In both cell lines, erlotinib efficiently inhibited phosphorylation of EGFR, ERK and AKT.

Next, we analyzed if both the PI3K-AKT and the Ras-Raf-MEK-ERK pathway were involved in mediating the anti-proliferative effects of erlotinib. We therefore challenged the cells with erlotinib or additional MEK and AKT-specific inhibitors to compare the effects on cell proliferation. In both cell types, erlotinib enhanced inhibition of cell proliferation by the pathway-specific inhibitors. U0126, but not AKT inhibitor XIV, was able to increase the growth-inhibitory effect of erlotinib. Together, these data are compatible with the hypothesis that both the AKT and ERK pathway are involved in the mediation of the antiproliferative effects of erlotinib in BxPC-3 and Capan-1 cells. The additional growth inhibitory effect of U0126 plus erlotinib versus erlotinib alone might possibly be explained by off-target effects of the MEK inhibitor, which have previously been described^[36].

Table 1 Optimized parameter values for the BxPC-3 and Capan-1 model

Model parameter	Value	
	BxPC-3	Capan-1
Global parameter		
κ_0 = EGFR phosphorylation (min^{-1})	0.293	0.438
κ_1 = pEGFR attenuation (min^{-1})	0.067	0.155
κ_2 = EGF-dependent ERK phosphorylation ($\text{a.u.}^{-1}\text{min}^{-1}$)	0.388	0.482
κ_3 = basal pERK dephosphorylation (min^{-1})	3.688	a
κ_4 = EGF-dependent AKT phosphorylation ($\text{a.u.}^{-1}\text{min}^{-1}$)	0.225	0.044
κ_5 = basal pAKT dephosphorylation (min^{-1})	0.149	a
κ_6 = basal ERK phosphorylation (min^{-1})	0.067	0.104
κ_7 = basal AKT phosphorylation (min^{-1})	0.013	0.007
κ_8 = EGF-dependent pERK dephosphorylation ($\text{a.u.}^{-1}\text{min}^{-1}$)	4.512	8.520
κ_9 = EGF-dependent pAKT dephosphorylation ($\text{a.u.}^{-1}\text{min}^{-1}$)	2.914	1.771
κ_{10} = pEGFR inhibition by erlotinib ($\mu\text{mol/L}^{-1}$)	39.066	62.143
τ_{u1} = delay for pERK (min)	29.766	66.809
τ_{u2} = delay for pAKT (min)	3.109	13.803
Scaling parameter		
erlotinib = 0 $\mu\text{mol/L}$		
scale_pEGFR	0.858	0.540
scale_pERK	61.339	33.035
scale_pAKT	13.483	12.039
erlotinib = 0.11 $\mu\text{mol/L}$		
scale_pEGFR	1.594	1.191
scale_pERK	27.224	29.561
scale_pAKT	8.231	8.254
erlotinib = 0.33 $\mu\text{mol/L}$		
scale_pEGFR	2.522	1.955
scale_pERK	35.326	29.479
scale_pAKT	5.528	5.237
erlotinib = 1.0 $\mu\text{mol/L}$		
scale_pEGFR	6.242	9.296
scale_pERK	15.631	15.464
scale_pAKT	2.569	2.340
Fixed parameter and initial condition		
pEGFR (a.u.)	0	0
EGFR _{tot} = total EGFR (a.u.)	1	1
AKT _{tot} = total AKT protein (a.u.)	1	1
ERK _{tot} = total ERK protein (a.u.)	1	1

a: Calculated according to Figure 1C; EGFR: Epidermal growth factor receptor; AKT: Protein kinase B; ERK: Extracellular signal-regulated kinase; EGF: Epidermal growth factor; pAKT: Phospho-AKT; pERK: Phosphomito-gen-ERK; a.u.: Arbitrary units.

Our observations of BxPC-3 cells are in agreement with a previous study by Diep *et al.*³⁷, who showed that in *KRAS* wild-type PC cells, erlotinib-attenuated cell proliferation could be further diminished with MEK inhibitors. The results of the two studies, however, differ in that we observed a similar effect of the drug combination in *KRAS* mutant cells, while Diep *et al.*³⁷ did not. These contradictory findings are possibly due to the fact that different *KRAS* mutant cell lines and non-identical MEK inhibitors were used.

The antiproliferative effect of erlotinib in PC cells has previously already been linked to the expression of HER3 (ErbB3)^{28,38,39}. Interestingly, HER3 has also been shown to act upstream of AKT in a pathway that is activated by EGF-induced formation of EGFR/HER3 heterodimers³⁹. Thus, by blocking EGFR-mediated transphosphorylation of HER3, erlotinib may effectively

interfere with AKT activation in EGF-treated PC cells. Furthermore, using a systems biology approach, Schoeberl *et al.*⁴⁰ found that, in ovarian cancer cells, HER3 and AKT are particularly sensitive components of the HER receptor signaling network.

In conclusion, our data are in agreement with recent publications suggesting inhibition of EGF-induced ERK and AKT signaling as key components of erlotinib action in PC cells. A new finding of this study is that cells with high and low erlotinib sensitivity differed in their basal pAKT level. Furthermore, erlotinib displayed a stronger growth-inhibitory effect in the cells with a more pronounced activation of AKT and ERK in response to EGF (BxPC-3). Although we found a correlation between *KRAS* status and the growth-inhibitory effect of erlotinib, our data did not reveal a causal relationship, since the drug blocked ERK phosphorylation in *KRAS* wild-type and mutant cells with equal efficiency.

The ODE model of EGFR signaling in PC cells, established in the course of this study, accurately reflected the experimental findings. This observation suggested that the model, despite its simplifications, still contained all the components crucial to reproducing EGFR-triggered activation of AKT and ERK *in silico*. In support of this conclusion, the model also provided predictions regarding erlotinib-dependent changes of the phosphoprotein levels that could be verified experimentally. We consider the introduction of a mathematical model of EGFR signaling in PC cells as a first step on a path towards the identification of promising drug targets by means of computational modeling. Potential applications also include the *in silico*-testing of novel therapeutics targeting the EGFR pathway, and the further analysis of mechanisms of drug sensitivity and resistance.

Taken together, the results of this study may facilitate the search for molecular markers of erlotinib efficiency in PC patients. Mathematical models, like the one established here, are helping to gain further insights into signaling processes in cancer cells. In the long run, they may also become useful for predicting drug efficiencies in a clinical setting. In this regard, the particular advantages of mathematical models are that they can be based on a relatively small number of measurable parameters, and provide information about dynamic processes.

ACKNOWLEDGEMENTS

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COMMENTS

Background

Of all common malignancies, pancreatic cancer (PC) has the lowest survival rate. The epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor erlotinib is the only new drug for the treatment of locally advanced, unresectable, or metastatic PC that has been successfully introduced into the clinics in recent years, but its positive effect on survival time is small.

Research frontiers

To date, only rash, a common side effect of EGFR targeted therapies, is an indicator of a possible response to an erlotinib treatment. Identification of molecular markers holds promise for classifying subgroups of patients who would benefit most from erlotinib therapy.

Innovations and breakthroughs

This is the first study combining experimental data and mathematical modeling to elucidate epidermal growth factor and erlotinib action in PC cells. The authors observed that PC cells with a high biological sensitivity to erlotinib displayed a higher basal phospho-protein kinase B level and increased activation of EGFR-induced downstream signaling pathways than less sensitive cells. The mathematical model not only reflected the experimental findings, but also provided predictions regarding phosphoprotein levels that could be verified experimentally.

Applications

The results of this study may facilitate the search for molecular markers of erlotinib efficiency in PC patients. Mathematical models, like the one established here, are currently applicable in order to gain molecular insights into signaling processes in cancer cells. In the long run, they may also become useful for predicting drug efficiencies in a clinical setting.

Peer review

The authors present a manuscript with important data which may be helpful for patient stratification for the treatment of pancreatic carcinoma using erlotinib. Of course, the model as established in this study should be confirmed through further *in vivo* tests, including clinical trials, and a group of biomarkers may be developed in the future and applied for a selection of potentially sensitive patients.

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Distribution of bleeding gastrointestinal angioectasias in a Western population

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Abstract

AIM: To define which segments of the gastrointestinal tract are most likely to yield angioectasias for ablative therapy.

METHODS: A retrospective chart review was performed for patients treated in the Louisiana State University Health Sciences Center Gastroenterology clinics between the dates of July 1, 2007 and October 1, 2010. The selection of cases for review was initiated by use of our electronic medical record to identify all patients with a diagnosis of angioectasia, angiodysplasia, or arteriovenous malformation. Of these cases, chart reviews identified patients who had a complete evaluation of their gastrointestinal tract as defined by at least one upper endoscopy, colonoscopy and small bowel capsule endoscopy within the past three years. Patients without evidence of overt gastrointestinal bleeding or iron deficiency anemia associated with intestinal angioectasias were classified as asymptomatic and excluded from this analysis. Thirty-five patients with confirmed, bleeding intestinal angioectasias who had undergone complete endoscopic evaluation of the gas-

trointestinal tract were included in the final analysis.

RESULTS: A total of 127 cases were reviewed. Sixty-six were excluded during subsequent screening due to lack of complete small bowel evaluation and/or lack of documentation of overt bleeding or iron deficiency anemia. The 61 remaining cases were carefully examined with independent review of endoscopic images as well as complete capsule endoscopy videos. This analysis excluded 26 additional cases due to insufficient records/images for review, incomplete capsule examination, poor capsule visualization or lack of confirmation of typical angioectasias by the principal investigator on independent review. Thirty-five cases met criteria for final analysis. All study patients were age 50 years or older and 13 patients (37.1%) had chronic kidney disease stage 3 or higher. Twenty of 35 patients were taking aspirin (81 mg or 325 mg), clopidogrel, and/or warfarin, with 8/20 on combination therapy. The number and location of angioectasia was documented for each case. Lesions were then classified into the following segments of the gastrointestinal tract: esophagus, stomach, duodenum, jejunum, ileum, right colon and left colon. The location of lesions within the small bowel observed by capsule endoscopy was generally defined by percentage of total small bowel transit time with times of 0%-9%, 10%-39%, and 40%-100% corresponding to the duodenum, jejunum and ileum, respectively. Independent review of complete capsule studies allowed for deviation from this guideline if capsule passage was delayed in one or more segments. In addition, the location and number of angioectasias observed in the small bowel was further modified or confirmed by subsequent device-assisted enteroscopy (DAE) performed in the 83% of cases. In our study population, angioectasias were most commonly found in the jejunum (80%) followed by the duodenum (51%), stomach (22.8%), and right colon (11.4%). Only two patients were found to have angioectasias in the ileum (5.7%). Twenty-one patients (60%) had angioectasias in more than one location.

CONCLUSION: Patients being considered for endoscopic ablation of symptomatic angioectasias should undergo push enteroscopy or antegrade DAE and re-inspection of the right colon.

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Key words: Intestinal angioectasias; Intestinal angiodysplasias; Intestinal arteriovenous malformations; Obscure gastrointestinal bleeding

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INTRODUCTION

Intestinal angioectasias are culprit lesions in up to 5%-6% of gastrointestinal bleeding cases and are the most common source of bleeding from the small intestine in patients older than 50-60 years^[1]. In patients who experience symptomatic blood loss due to these lesions, approximately 40%-50% will experience recurrent bleeding^[2]. Endoscopic ablation of these lesions using bipolar cautery or argon plasma coagulation is a standard therapy to prevent bleeding recurrence^[1,3]. However, localization of these lesions can be challenging as they are commonly small, evanescent and/or located deep in the small intestine. These lesions are also multiple in many cases^[4]. Understanding the natural distribution of these lesions in the gastrointestinal tract is useful to guide endoscopic evaluation and therapy.

Endoscopic visualization of the entire gastrointestinal tract has not been readily available until the advent of wireless capsule endoscopy and double balloon enteroscopy in 2001^[5,6]. Prior to the introduction of these technologies, complete examinations of the intestinal lumen were limited to cases in which intraoperative enteroscopy was performed. Recent studies of the distribution of angioectasias using capsule endoscopy and balloon enteroscopy demonstrate that these lesions are common causes of bleeding from the small intestine. However, the most frequent location of lesions within the small intestine has varied between populations. In Eastern populations, angioectasias have been found to be distributed almost equally between the proximal and distal small intestine (44%-69% and 31%-56%, respectively)^[7-9]. These findings are in contrast to a recent study in the United States which found that the vast majority

of lesions occur in the jejunum (93%) rather than the ileum (7%)^[10]. Anecdotal reports from our center or other centers performing balloon enteroscopy in the United States are similar to the findings of Gerson *et al*^[10].

In order to better define the distribution of angioectasias in the gastrointestinal tract, we studied the location of specific lesions in patients with overt or occult gastrointestinal blood loss attributed to symptomatic angioectasias.

MATERIALS AND METHODS

This study was designed as a single-center retrospective chart review. Institutional review board approval was obtained during final protocol development. Prior to study initiation, the terminology regarding intestinal vascular lesions was delineated. Angioectasias were defined pathologically as dilated submucosal veins with overlying ectasia of mucosal venules and capillaries. The term angiodysplasia was used interchangeably with angioectasia although the equivalence of these terms has been debated. Due to the presence of arteriovenous communications in some angioectasias, lesions identified as arteriovenous malformations were also included in this study provided that the observed lesion was not protruding or obviously deep to the submucosa. Using these pathologic definitions as a reference, all superficial lesions 1mm or greater in size which are cherry-red in color with a fern-like pattern were included for analysis (Figure 1). Lesions characterized by pulsatile bleeding but no mucosal defect were generally classified as Dieulafoy lesions and were excluded^[1,11].

Selection of cases for review was initiated by use of our electronic medical record to identify cases in which a diagnosis of angioectasia, angiodysplasia, or arteriovenous malformation was made between the dates of July 1, 2007 and October 1, 2010. Of these cases, chart reviews identified patients who had a complete evaluation of their gastrointestinal tract as defined by at least one upper endoscopy, colonoscopy and small bowel capsule endoscopy within the past three years. Patients without evidence of overt gastrointestinal bleeding or iron deficiency anemia associated with intestinal angioectasias were classified as asymptomatic and excluded from this analysis. Patients with a diagnosis of hereditary hemorrhagic telangiectasia or gastric antral vascular ectasia were also excluded.

RESULTS

A total 127 cases were initially reviewed. A large number (66) of these were excluded during subsequent screening due to lack of complete small bowel evaluation (primarily lack of capsule endoscopy) and/or lack of documentation of overt bleeding or iron deficiency anemia. The 61 remaining cases were carefully examined with independent review of endoscopic images as well as complete capsule endoscopy videos. This analysis excluded 26



Figure 1 A typical angioectasia (arrow) observed in the small intestine by capsule endoscopy.

additional cases due to insufficient records/images for review, incomplete capsule examination, poor capsule visualization or lack of confirmation of typical angioectasias by the principal investigator on independent review. A total of 35 cases met criteria for final analysis. The number and location of angioectasias were documented for each case. Lesions were then classified into the following segments of the gastrointestinal tract: esophagus, stomach, duodenum, jejunum, ileum, right colon and left colon. The location of lesions within the small bowel observed by capsule endoscopy was generally defined by percentage of total small bowel transit time with times of 0%-9%, 10%-39%, and 40%-100% corresponding to the duodenum, jejunum and ileum, respectively. Independent review of complete capsule studies allowed for deviation from this guideline in cases in which capsule passage was delayed in one or more segments. In addition, the location and number of angioectasias observed in the small bowel was further modified or confirmed by subsequent device-assisted enteroscopy (DAE) performed in the 83% of cases.

In the final analysis, all study patients were age 50 years or older (age range 50-91 years) and 13 patients (37.1%) had chronic kidney disease stage 3 or higher (defined by Kidney disease outcomes quality initiative staging: stage 3 = glomerular filtration rate (GFR) 30-59, stage 4 = GFR 15-29, stage 5 = GFR < 15 or on dialysis). Twenty of 35 patients were taking aspirin (81 mg or 325 mg), clopidogrel, and/or warfarin. Of these 20 patients, 8 were on a combination of these medical therapies.

In our study population, the jejunum was the most common location of symptomatic angioectasias in the gastrointestinal tract. Out of 150 total angioectasias observed in 35 patients, 78 were localized to the jejunum (52%), 34 to the duodenum (23%), 22 to the stomach (15%), 9 to the ileum (6%), and 7 to the right colon (4.7%). Twenty-eight patients had lesions in the jejunum (80%), 18 had lesions in the duodenum (51%), 8 had lesions in the stomach (22.8%), 4 had lesions in the right colon (11.4%) and 2 had lesions in the ileum (5.7%). There were no angioectasias localized to the esophagus or left colon. Twenty-five patients had more than one

lesion (71.4%). Twenty-one patients had lesions in more than one location (60%).

Spatial clustering of lesions was also observed. In patients with lesions in multiple segments, the most common sites were the duodenum and jejunum (14 patients or 40%). Other clustering occurred in the stomach and jejunum (6 patients or 17%), stomach and duodenum (5 patients or 14.3%), jejunum and right colon (5 patients or 14.3%), duodenum and right colon (3 patients or 8.6%), stomach and right colon (1 patient or 2.9%), and duodenum and ileum (1 patient or 2.9%).

Eight patients had angioectasias isolated to the jejunum (22.8%), while two patients had angioectasias isolated to the duodenum (5.7%). Other angioectasias isolated to a single segment were found in the stomach and ileum in one patient each (2.9%). None of our patients had angioectasias located in the esophagus or left colon and no patients had isolated angioectasias in the right colon.

DISCUSSION

Recent studies of obscure bleeding in Eastern populations indicate that angioectasias are not the most common cause of small bowel hemorrhage and that these lesions are distributed fairly equally throughout the small intestine^[7-9,12-14]. However, studies of Western populations report that angioectasias account for 60%-70% of bleeding sources in the small bowel and are generally found in the proximal small bowel^[10,15,16]. One study by May *et al*^[17] described a high incidence of symptomatic small bowel angioectasias in a United States population, with lesions seen in 17 of 52 push enteroscopies, 21 of 52 oral push-and-pull enteroscopies, and 2 of 52 anal push-and-pull enteroscopies. In our study population, angioectasias were most commonly found in the proximal small bowel, namely the jejunum and duodenum. Spatial clustering of these lesions also occurred most frequently in these segments.

Treatment of bleeding angioectasias is difficult. Data regarding the use of hormones, thalidomide or octreotide is limited with pharmacologic therapy reported as effective in some studies but not others. Treatment with estrogen and progesterone is the most common pharmacologic therapy, although recent studies have shown failed to confirm the efficacy observed in smaller, earlier trials^[1,18-20]. Somatostatin and octreotide have reportedly shown some reduction of blood loss from angioectasias, although only in case reports and one small study. It is theorized that somatostatin may prevent recurrent bleeding from angioectasias by reduction in mesenteric blood flow and inhibition of vasodilator peptides^[19]. Although this rationale seems plausible, there are currently no randomized, double-blinded studies supporting these hypotheses^[1]. Endoscopic ablation is a commonly utilized therapy in patients with overt bleeding or iron deficiency anemia attributed to intestinal angioectasias. Although the effectiveness of this therapy has not been proven in a randomized, controlled trial, the practice of endoscop-

ic ablation is supported by the lack of compelling data for medical therapy. Small studies of rebleeding rates after ablative therapy support this practice. In one study conducted in the United Kingdom, heater probe ablation of angioectasias was performed in 23 patients during push enteroscopy. The authors found this therapy to be effective although repeated ablation was required in 30% of cases^[21]. Another early study of endoscopic therapy in London found that argon lasers, although seemingly promising, did not completely ablate the angioectasia. The first of their 18 studied cases ultimately required an emergency gastrectomy, so the investigators observed the argon laser's effects on the resected specimen. Despite the laser's small ulcer created during endoscopy, the arteriovenous malformation remained intact under normal mucosa, increasing the likelihood of rebleeding. In a separate case, investigators had better results with the neodymium Yag (Nd YAG) laser, which created fibrotic zones three to four times thicker than the argon laser^[22]. This finding was validated by two separate studies which showed a reduction in blood transfusion requirements after treating patients with angioectasias with endoscopic Nd YAG laser. However, the investigators of these studies performed only upper gastrointestinal endoscopy, and admitted that the Nd YAG laser would need to be used with caution in the thinner bowel walls of the lower gastrointestinal tract^[23,24]. In Japan, Ohmiya *et al.*^[25] used a combination of double balloon endoscopy with enteroscopic electrocoagulation to locate and treat 19 patients with small bowel angioectasias. Of the 19 patients, 7 rebled after treatment. Nakase *et al.*^[26] also used DBE to localize bleeding angioectasias in the small bowel of 8 patients, and reported successful hemostasis in all cases using combination therapy of local injection of hypertonic saline epinephrine and heat coagulation. However, since bleeding from angioectasias often occurs intermittently, it is difficult to evaluate the effects of any therapy on rebleeding rates. For this reason, both endoscopic therapy and medical therapy are still used in clinical practice with medical therapy often reserved for patients with comorbidities preventing endoscopic ablation, patients with multiple lesions or in cases in which bleeding recurs despite ablation^[1].

Weaknesses of our study include retrospective design and relatively small number of patients included in the final analysis. Also, patients with angioectasias located within reach of standard endoscopy may not have been included if additional evaluation by capsule endoscopy was not pursued thus leading to overstatement of the percentage of patients with small bowel lesions. The lack of angioectasias found in the ileum is still a compelling finding despite these considerations. When applying this finding towards clinical practice, examination of the ileum by retrograde DAE or attempted total enteroscopy by antrograde DAE is unlikely to yield angioectasias candidate for ablation unless lesions are observed in this segment by capsule study. Close inspection of the duodenum and jejunum using push enteroscopy or

anterograde DAE combined with re-inspection of the right colon and terminal ileum may be the most effective approach in these patients.

COMMENTS

Background

Intestinal angioectasias are the most common source of bleeding from the small intestine in patients older than 50-60 years. Nearly half of patients who experience symptomatic blood loss due to these lesions will experience recurrent bleeding. Additionally, many patients have multiple angioectasias that may bleed. Localization and endoscopic treatment of these lesions can be challenging as they are commonly small, evanescent and/or located deep in the small intestine. In order to better define the distribution of angioectasias in the gastrointestinal tract, we studied the location of specific lesions in patients with overt or occult gastrointestinal blood loss attributed to symptomatic angioectasias.

Research frontiers

Endoscopic visualization of the entire gastrointestinal tract has not been readily available until the advent of wireless capsule endoscopy and double balloon enteroscopy in 2001. Since that time, localization and endoscopic treatment of angioectasias have become areas of interest for clinicians and researchers alike.

Innovations and breakthroughs

Recent studies of the distribution of angioectasias using capsule endoscopy and balloon enteroscopy demonstrate that these lesions are common causes of bleeding from the small intestine. However, the most frequent location of lesions within the small intestine has varied between populations. In Eastern populations, angioectasias are almost equally distributed between the proximal and distal small intestine. In Western populations, however, the vast majority of lesions occur in the proximal small bowel.

Applications

The majority of small bowel angioectasias in the study were located in the proximal small bowel. When applying this finding towards clinical practice, examination of the ileum by retrograde device-assisted enteroscopy (DAE) or attempted total enteroscopy by antrograde DAE is unlikely to yield angioectasias candidate for ablation unless lesions are observed in this segment by capsule study. Close inspection of the duodenum and jejunum using push enteroscopy or antrograde DAE combined with re-inspection of the right colon and terminal ileum may be the most effective approach in these patients.

Peer review

It is a retrospective study of 35 patients with bleeding intestinal angioectasias who had undergone complete endoscopic evaluation of the gastrointestinal tract. They showed that angioectasias were most commonly found in the jejunum (80%) followed by the duodenum (51%), stomach (22.8%), and right colon (11.4%). It has an interesting report but the main limitation its small sample size.

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Understanding the health and social care needs of people living with IBD: A meta-synthesis of the evidence

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Abstract

AIM: To undertake a metasynthesis of qualitative studies to understand the health and social needs of people living with inflammatory bowel disease (IBD).

METHODS: A systematic search strategy identified qualitative studies exploring the phenomenon of living with inflammatory bowel disease. Databases included MEDLINE, PsychInfo, EMBASE, CINAHL and the British Nursing Index *via* the OVID platform. Qualitative search filters were adapted from Hedges database (http://www.urmc.rochester.edu/hslt/miner/digital_library/tip_sheets/Cinahl_eb_filters.pdf). Qualitative empirical studies exploring the health and social needs of people living with inflammatory bowel disease were selected. Study eligibility and data extraction were independently completed using the Critical

Appraisal Skills Programme for qualitative studies. The studies were analysed and synthesised using meta-synthesis methodology. The themes from the studies allowed for common translations into a new interpretation of the impact of living with inflammatory bowel disease.

RESULTS: Of 1395 studies, six published studies and one unpublished thesis fulfilled the inclusion criteria. First iteration of synthesis identified 16 themes, 2nd iteration synthesised these into three main 2nd order constructs: "detained by the disease"; "living in a world of disease" and "wrestling with life". "Detained by the disease" is the fear of incontinence, the behaviour the patients display due to the fear, and the impact this has on the individual, such as social isolation and missing out on life events. All of these serve to "pull" the patient back from normal living. "Living in a world of disease" is the long term effects of living with a long term condition and the fear of these effects. "Wrestling with life" is the continued fight to thrive, the "push" to continue normal living.

CONCLUSION: The metasynthesis provides a comprehensive representation of living with IBD. The unmistakable burden of incontinence is exposed and its ongoing effects are demonstrated. The combined overall impact of living with IBD is the tension these patients live with: "Pushed and pulled: a compromised life", people living with IBD experience a constant conflict throughout their lives, they push to be normal but IBD pulls them back. The impact of the fear of incontinence and behaviour of the individual as a result, requires further qualitative enquiry.

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Key words: Inflammatory bowel disease; Metasynthesis; Qualitative; Incontinence

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the gastrointestinal tract that is divided into two subgroups: Crohn's disease (CD) and ulcerative colitis (UC). Characterised by periods of remission and relapse, bowel movements may be up to 20 times per day with associated faecal urgency and incontinence. IBD is also associated with extra intestinal manifestations, affecting joints, eyes, skin, bones and organs as a consequence of the disease process^[1].

The disease often has a negative effect on the patient's emotional and social life, which are not always visually apparent^[2]. Loss of bowel control, feeling dirty and smelly, producing offensive body odours, unfulfilled potential in the workforce and issues with sexual relationships, were concerns ranked highly in a patient survey of people with IBD^[5]. One of the most prevalent concerns is fatigue^[3,4]. Fatigue in people with IBD was found to be comparable to those suffering from cancer^[5]. Fatigue affects the ability to work and socialise, confirming the disability associated with IBD^[6]. Unemployment and sick leave is more common in IBD patients compared to the general population^[7,8], with ability to work regarded as a global marker of the total impact of IBD^[5].

People with IBD have a poorer quality of life than the general population^[9-13] and are more likely to report increased levels of anxiety and depression with increased disease activity^[14]. Evidence reveals that the disease continues to impact on the individuals psychological status even when in remission^[9,15]. Overall, evidence suggests that the subjective experience of ill health associated with IBD does not always correlate with clinical disease activity.

The health-related quality of life (HRQoL) of people with IBD has been extensively evaluated with the development of two key disease specific tools: the Inflammatory Bowel Disease Questionnaire (IBDQ)^[16] and the Rating Form for Inflammatory Bowel Disease Patient Concerns (RFIPC)^[4]. The IBDQ was developed using survey methodology and measures subjective emotional and social functioning. The RFIPC was developed to measure neglected but important IBD concerns including disease related, body related, and inter/intrapersonal and sex related.

Whilst useful measures, the IBDQ and RFIPC fail to capture the essence of living with IBD from the patient's

perspective^[4,16]. For example, the RFIPC includes loss of bowel control as a concern but fails to encapsulate the real impact this has on the individual^[17]. A study exploring concerns and worries of patients with CD identified other concerns and worries that were not captured within the RFIPC^[18]. Objective indices within the tools do not fully summarize the patient's clinical symptoms, nor reflect the individual's experience of IBD^[19,21]. Failure to capture the lived experience of IBD has been confirmed by the European Federation of Crohn's and Colitis Associations (EFCCA) patient survey^[22] which reported that quality of life (QoL) and patient concerns were not taken into account when caring for patients with IBD, despite the plethora of studies highlighting this fundamental principle^[23,25]. The EFCCA study identified that half of the patients surveyed were not questioned by their doctor about the impact of their symptoms on their QoL.

In contrast to quantitative measures, qualitative methods are more able to capture the essence of living with IBD from the patients perspective^[26,27]. They can provide insight into the meanings, behaviours, experiences and beliefs of the participants with the aim of "drawing out understandings and perceptions and understand the linkages between process and outcomes"^[28].

In order to understand IBD, tailor treatment and provide personalised care, capturing the patient experience is imperative. There are a number of small scale qualitative studies exploring the experience of living with IBD from the patient's perspective but there is a need to synthesis this evidence to further understand this before undertaking larger in-depth qualitative studies. The studies relating to IBD are small and often are not published in journals normally accessed by healthcare professionals responsible for managing these patients. Meta synthesis meets this need by the systematic selection, comparison and analysis of these qualitative combined studies and translating them to create new interpretations^[28].

The qualitative meta synthesis is a set of techniques for the interpretive integration of qualitative research findings^[29], it overcomes the limitations of small studies^[30] and has the ability to promote a greater understanding in a particular area^[31]. In this study, the purpose was to integrate and interpret the qualitative studies of the experience of living with IBD. Systematic reviews are accepted as the cornerstone of evidence based practice^[32] and are based on reviews of effectiveness and of "what works". However there is now a move toward addressing the wider questions, such as why there is a problem in the first place and how it has come about. These questions need to be answered in order to develop patient centred interventions^[33,34], implement studies of effectiveness and provide answers for the policy makers^[31,33].

MATERIALS AND METHODS

Inclusion criteria

Qualitative studies which explored the phenomena of

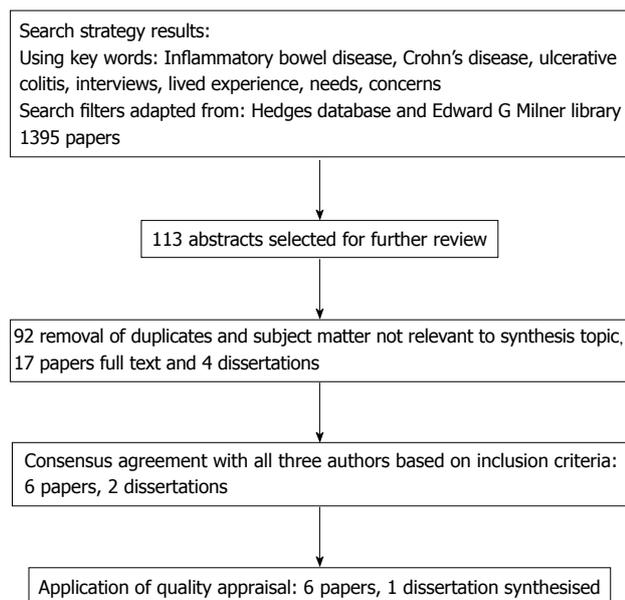


Figure 1 Flow chart summarising search strategy.

living with IBD from the patient's perspective were included in the synthesis. Additional inclusion criteria were studies restricted to English language only, published and unpublished studies and sample population adults > 16 years.

Exclusion criteria

The study focused on only one aspect e.g. living with a colostomy or diet, and mixed studies of irritable bowel syndrome and IBD.

Systematic search

Electronic literature searches were conducted in MEDLINE (1966-2010), PsychInfo (1967-2010), EMBASE (1980-2010) and CINAHL databases (1982-2010) and the British Nursing Index (1994-2010) *via* the OVID platform. Search filters developed by the Hedges database from McMaster University Health Information Research Unit and Kathryn Nesbit, Edward G Milner Library, University of Rochester Medical Centre, were adapted to aid the search (http://www.urmc.rochester.edu/hslt/miner/digital_library/tip_sheets/Cinahl_eb_filters.pdf). The search was conducted from the inception of the databases to August 2010. Web of Knowledge and CINAHL were used for citation searches, foot note chasing and journal runs. Author searches were also incorporated into the search of the literature from journals including Qualitative Health Research, Gastroenterology Nurse, and Inflammatory Bowel Diseases^[35].

Of the 1395 papers generated by the preliminary search of all the databases combined, 1282 were excluded as they were irrelevant to the study question. 113 abstracts were selected for further review, of which 92 were excluded based on duplication, quantitative methodology, and wrong subject matter. Four unpublished dissertations were identified within this and obtained,

two excluded due to the quantitative methodological approach used^[36,37], the remaining two were included for initial screening^[38,39]. Full text papers were obtained for the remaining 17 papers. These 17 papers and two dissertations were then screened for initial inclusion using three screening questions: Does this paper report on findings taken from qualitative work? Did the work involve both qualitative methods of data collection and analysis? Is this research relevant to the topic area?^[40].

Of the 17 papers and two dissertations screened, 11 papers and one dissertation were excluded from the synthesis. The dissertation was excluded as no qualitative methodological analysis was undertaken^[39]. The remaining papers were excluded based on: methodological approach used an online survey method^[19]; paediatric age group^[41]; subject matter focused on living with an ostomy^[42]; narrative journey with no qualitative methodology^[43]; participant responses used to validate commonly used indices^[21] and six papers excluded as the subject group was a mixture of patients with IBD and irritable bowel disease and unable to distinguish between responses from each group^[44-49].

The final selection of six papers^[17,50-54] and one unpublished dissertation^[38] were reviewed by all three authors. Data extraction forms were developed and data extraction, including study eligibility, study demographics, study characteristics, and themes, and data extraction were independently completed by three reviewers (Kemp K, Griffiths J, Lovell K).

The CASP^[55] tool was used to quality appraise the papers and also to aid the interpretation and exploration process of the synthesis^[56]. Further synthesis of the themes from the studies was agreed collectively at synthesis meetings to develop the new translations. The search summary is found in Figure 1 and full details of the search strategy are available from the authors.

RESULTS

Seven studies met the inclusion criteria. Summaries of the included studies are given in Table 1 and their corresponding demographics in Table 2. A list of excluded studies is available from the authors.

Characteristics of included studies

The seven selected studies were published from 1996-2010. Two were conducted in the United Kingdom^[50,54], one in Sweden^[52], one in Canada^[51], one in New Zealand^[53], and two in America^[17,38]. All of the studies used in depth interviews^[17,38,51-54] and one study combined interviews with focus groups^[50].

A total of 86 patients with an age range was 16-83 years were included and only one reported one patient from an ethnic background^[52]. Two studies focused on CD only^[52,53], and one study UC patients only^[17]. The remaining studies included people with both UC and CD. Patients were recruited from relevant national IBD charities^[38,53], directly from outpatients clinics^[17,52,54], media advertisements^[51], and from a previous community

Table 1 Characteristics of synthesised studies

Ref.	Theoretical perspective	Sampling strategy	Recruitment setting	Data collection method	Analytical approach
Dudley-Brown ^[17]	Phenomenological	Convenience sample (n = 3)	Patients sampled from IBD outpatient clinic when attending for their scheduled appointment	In depth semi structured interviews	Coding and memo system used, grouped and transformed into an interpretive understanding of the phenomenology of living with UC, with the extraction of emergent themes
Daniel <i>et al</i> ^[51]	Phenomenological	Purposive sample (n = 5)	Patients recruited by an advertisement in national newspaper	In depth semi structured interviews	Kings Goal Attainment Framework used as theoretical framework; thematic content analysis of interviews to develop themes in line with this framework
Hall <i>et al</i> ^[50]	Grounded theory	Purposive sample (n = 31)	Recruited from a previous unconnected study, sampled by lowest quintile of UK-IBDQ, established low quality of life	In depth interviews and focus groups	Concurrent data collection and analysis to identify emerging themes; selective coding was used to enabled theoretical framework
Burger <i>et al</i> ^[38]	Interpretive phenomenological design	Convenience sample (n = 8)	Participants from mailing list of Indiana Chapter of Crohn's and Colitis Foundation of America, answered advert and recruited according to inclusion/exclusion criteria	In depth interviews, each participant interviewed 3 times	Thematic analysis, identification analysis and identification of paradigm cases used
Lynch <i>et al</i> ^[53]	Phenomenological	Purposive sample (n = 4)	Participants recruited from Crohn's and Colitis New Zealand	Semi structured in depth interviews	Thematic analysis from transcribed data, ongoing process of interpretation used to refine themes to describe nature of the experience
Pihl-Lesnovska <i>et al</i> ^[52]	Grounded theory	Theoretical sample (n = 11)	Patients recruited from the gastroenterology outpatient clinic	Unstructured in depth interviews	Constant comparative analysis used, saturation determined sample size; core category and related categories identified; two authors analysed all interview transcripts
Cooper <i>et al</i> ^[54]	Framework	Purposive sampling (n = 24)	Patients sampled from IBD outpatient clinic when attending for their scheduled appointment	Semi structured in depth interviews	Thematic content analysis using framework

IBD: Inflammatory bowel disease; UK-IBDQ: United Kingdom version of the McMaster Inflammatory Bowel Disease Questionnaire; UC: Ulcerative colitis.

Table 2 Demographics of synthesised studies

Ref.	Date	Country	Age range	Gender	Disease	Disease duration	Sample size
Dudley-Brown ^[17]	1996	United States	30-50 yr	1 female; 2 male	3 ulcerative colitis	1-10 yr	3
Daniel <i>et al</i> ^[51]	2001	Canada	18-24 yr	2 female; 3 male	IBD not specified	< 2 yr	5
Hall <i>et al</i> ^[50]	2005	United Kingdom	Not specified but all > 16 yr	19 female; 12 male	14 Crohn's disease; 17 ulcerative colitis	Not specified but all > 2 yr	31
Burger <i>et al</i> ^[38]	2005	United States	30-65 yr	6 female; 2 male	6 Crohn's disease; 2 ulcerative colitis	2-40 yr	8
Lynch <i>et al</i> ^[53]	2007	New Zealand	16-21 yr	3 female; 1 male	All Crohn's disease	< 18 mo	4
Pihl-Lesnovska <i>et al</i> ^[52]	2010	Sweden	29-83 yr	5 female; 6 male	All Crohn's disease	2-33 yr	11
Cooper <i>et al</i> ^[54]	2010	United Kingdom	30-40 yr	11 female; 13 male	12 Crohn's disease; 12 ulcerative colitis	1- > 10 yr	24

IBD: Inflammatory bowel disease.

based study^[50]. The theoretical perspectives were mainly phenomenology^[17,38,51,53] and grounded theory^[50,52] with one study using framework^[54].

Synthesis of the evidence

The three authors independently reviewed all of the studies. The emergent themes were subject to constant examination until an argument to explain the data of the combined studies was developed. The themes and

findings of each study were compared with one another repeatedly to identify the 1st order constructs. This revealed the similarities and differences in the data, which led to 2nd order constructs and the interpretation of all of the synthesised studies. For example, study 1 may have had findings AB and C, study 2 may have findings AC and D, a new finding. The synthesis from studies 1 and 2 was compared to study 3 and so forth, until all of the papers were synthesised^[29,57]. Early on in the synthe-

1st order constructs	Ref.	2nd order constructs	Line of argument synthesis
Limitations/ missing out on life events	[17,38,50-54]	Detained by disease ("pull") Fear of incontinence - unpredictability, humiliation Behaviour due to fear of incontinence - avoidance Impact of behaviour - socially isolated, missing out on life events, limited life, relationship burden, feeling damaged Fatigue	"Pushed and pulled: a compromised life". Constant conflict between IBD and normal life results in a compromised life. Pushes to be normal but IBD pulls individual back.
Humiliation of incontinence	[17,50,51,53,54]		
Social isolation	[17,38,50,51,53,54]		
Unpredictability	[38,50-53]		
Powerlessness	[17,38,53,54]	Living in a world of disease	
Feeling damaged	[38,52-54]		
Impact on relationships	[17,38,50-54]		
Negative emotions	[17,50-54]		
Stress	[38,51-54]	Wrestling with life ("push") Striving to thrive	
Fatigue	[38,50-53]		
A disease for life	[38, 51-53]		
Fear of long term effects	[38, 51-53]		
Invisible disease	[38,50,53,52]		
Acceptance yet fight	[38,53,54]		
Knowing my body	[38,53,54]		
Control	[38,51-54]		
Maintaining normality	[38,50,52,53]		

IBD: Inflammatory bowel disease.

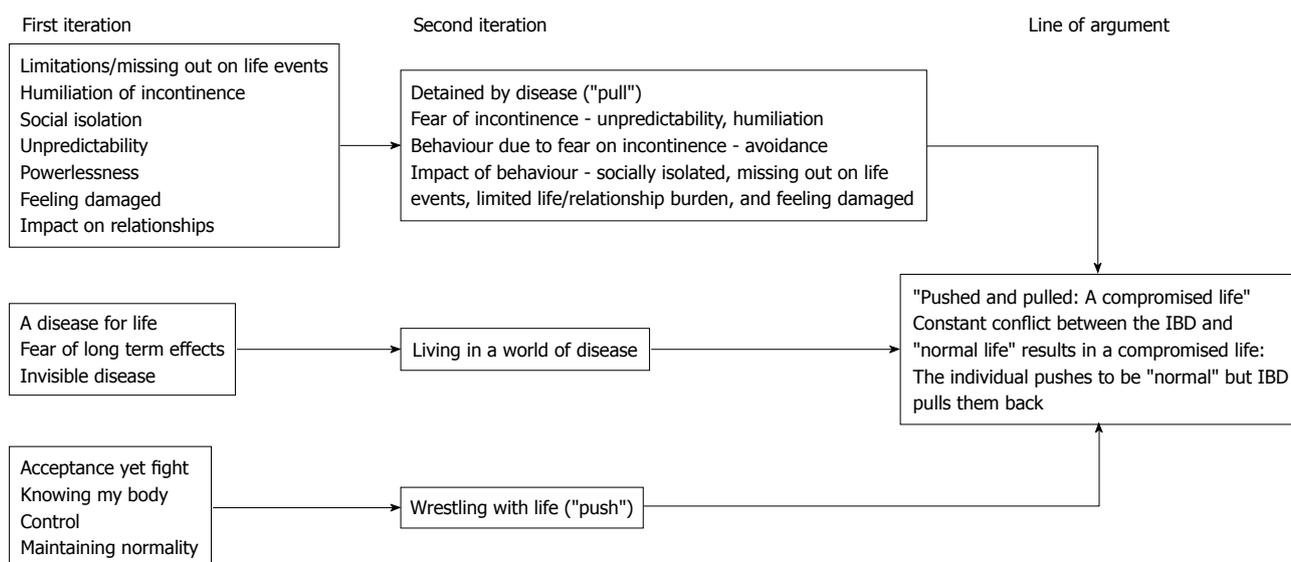


Figure 2 Relationship between synthesised studies. IBD: Inflammatory bowel disease.

sis it was clear that the relationship between the studies was mutual, all sharing common themes^[58]. As the studies had a “reciprocal” arrangement, a new argument was developed. This process was followed systematically, starting with the oldest study first^[17] in keeping with the model of “line of argument” synthesis^[58]. The themes and concepts are illustrated in Table 3 and the relationship between them identified in Figure 2.

Results - synthesis of the evidence

The synthesis of the seven studies identified that people with IBD endure many daily challenges, stress, pain, fatigue, and fighting for control. The combined impact of living with IBD is the tension they live with. The meta-synthesis has provided an in-depth exploration of living

with IBD: “pushed and pulled: a compromised life”, people living with IBD experience a constant conflict throughout their lives, they push to be normal but IBD pulls them back.

Living in a world of disease

A disease for life: Participants were acutely aware that they had been diagnosed with a long term condition with no cure. Facing and accepting the incurable illness was met with a variety of responses yet the need to get back to normal, but inability to do so, was a theme running through all of the studies.

Fear of long term effects: The fear of long term effects, of death and dying left people feeling powerless^[52].

The risks of cancer development and passing on the illness to children added to the burden of living with the physiological aspects of the illness^[38,52].

Invisible disease: A difficult aspect of living with IBD is its invisibility^[17,38,52,53]. The studies detail how this concept affected the individuals. The lack of understanding from others doubting that they were actually sick as it was not visible, added to their feelings of anger and frustration, in particular with family members^[51]. “My sister says I’m blowing this up...it’s an act...I’m trying to get attention”^[51].

Wrestling with life: Striving to thrive (“push”)

Acceptance yet fight: A common theme throughout all of the studies was the individual’s willingness and need to wrestle with their illness. Three of the studies discussed the acceptance of living with the illness yet continuing to fight it^[38,52-54]. This can be interpreted as neither a submission to the illness nor as out and out combat but more where individuals made peace with their illness. “This is how I am...to me it’s no different than saying I have a dog”^[38].

Control: The concept of control is visible in all seven studies, whether this was trying to control the illness^[50], controlling bowel urgency^[38] or losing control^[51]. Individuals fought to gain and maintain control and find a balance between what they could control and what they needed to control, for life to be acceptable^[54]. Gaining “control” had a positive impact on the individuals, recognising “performance accomplishments”^[54] and allowing them to feel “normal”^[50]. However the cost of achieving this was a large trade off which was capable of wearing the individual down and losing its ability to continue to fight, fatigue becoming a significant problem^[50]. Attempting to control their illness was their attempt to try to maintain “normality” for many people within the studies^[38,50,52,53].

Knowing my body: Participants voiced the theme of “knowing my body”, with accounts of knowing when their illness flared up better than their doctor. An increased awareness of their body led the individuals to try to identify triggers or patterns and recognise when their illness flared up. By learning about their own body the individual tried to gain some scale of control but often this concluded in them feeling helpless and misunderstood^[53]. “He stated that he knew it was not his CD even though it was the physician’s first inclination”^[38]. Wrestling with life culminates with the individual pushing to be normal, accepting their illness yet striving to thrive and survive.

Detained by the disease (“pull”)

Fear of incontinence (unpredictability, humiliation): All of the studies report the patient’s fear of incontinence and how they try to live with this^[17,38,50-54]. The fear appeared to be associated with past experiences of

actual episodes of incontinence and remembering the humiliation this produced. Actual episodes were rare but the fear remained constant. Patients felt ashamed, not only of the actual incontinence but also of their ongoing fear. Some people reported the overwhelming shame of incontinence. Shame and humiliation was even experienced within the family unit, one grandmother describing the embarrassment should her grandchildren know that she cannot make it to the bathroom^[38,51,54]. This fear of incontinence was all consuming for some patients and became a focus of living with IBD, over and above the physical symptoms. “It’s terrible, but that’s the biggest fear”^[38].

Behaviour due to fear of incontinence (avoidance):

The fear of incontinence and its unpredictability had a profound effect on the individual’s behaviour. For many this fear led to an avoidance or curtailing of daily activities and impaired individuals work, social and leisure and private functioning^[17,38,51].

Individuals used a range of coping strategies to either manage or avoid incontinence and included carrying pot-ties and spare clothes, wearing nappies and identifying bathrooms prior to any travel^[17,38]. Travelling anywhere required extra time and was dictated by the individual’s bowel frequency and control. “Planning an escape route provided a sense of security even if it was not needed”^[38].

The impact of this behaviour led to avoiding places and people. Studies describe patients only attending safe places^[50] with a dependency on toilets^[51] or avoiding public places all together^[17].

Impact of behaviour (missing out on life events, socially isolated):

The fear of incontinence, coupled with avoidance behaviour, was immensely detrimental to the individual’s QoL. They became socially isolated very easily: had limited activity with family and friends^[38]; became reclusive^[50]; and missed out on life events^[51]. The self enforced social isolation led to feelings of social inadequacy, lacking the necessary societal skills for everyday living^[51]. “I’ve just missed a whole part of my life”^[51].

Individuals expressed feeling damaged, a failure, weak and feeble with overwhelming feelings of anger, frustration and depression^[50,53]. Unable to identify a pattern or trigger for their disease reinforced all of these negative emotions^[17,38,53].

Stress was overtly discussed in five studies^[38,51-54]. Triggers for stress ranged from the illness itself to outside factors such as the ability to work and financial concerns and manifested itself in the form of fatigue and exacerbations of their disease. Lack of understanding from family members and feeling redundant in the family home^[50] left people feeling alienated from partners and family^[51], and people reported complex emotions of “letting people down”^[53].

Fatigue, tiredness and exhaustion contributed to people’s feelings of frustration, stress and powerlessness^[52]. Some people felt that fatigue was a sign of weakness^[53] and was generally misunderstood by others^[50] as it was

not evidently visible, reinforcing the invisibility of the disease.

Detained by the disease became evident as the analysis of the studies revealed that the fear of incontinence, the behaviour associated with it and the resultant enforced social isolation, resulted in “pulling” the individual back from “normal” living.

Line of argument

A line of argument was derived from the synthesis of the seven studies^[58]. The common translations from the studies were taken a step further and constructed into a new interpretation.

Line of argument synthesis: the ongoing factors identified by the qualitative studies impact on the individual's whole life with IBD leading to a compromised life: the individual pushes to be normal yet IBD pulls them back. The individual is in constant conflict, fighting to be normal with the impact of this resulting in constant tension within.

The synthesised studies revealed the fear and humiliation surrounding incontinence which resulted in severely reduced social interactions. Descriptions how the illness “intruded” into the participant's life and the constant “fight” for normality was evidenced throughout all of the studies. Phrases, including the “see-sawing of fears and hopes”, illustrate the uncertainties and contradictions of living with IBD. Importantly, the individuals describe the courage required to break the social isolation resulting from bowel symptoms. All of these aspects of living with IBD are directly related to everyday life.

DISCUSSION

The aim of this metasynthesis was to provide an interpretation of the health and social needs of patients living with IBD by synthesising qualitative studies and key issues emerged. People with IBD endure many daily challenges including stress, pain, and fatigue and fighting to maintain normality. The combined overall impact of living with IBD is the tension these patients live with. The value of metasynthesis is the interpretation of all of the synthesised studies to provide an inclusive representation of living with IBD: “Pushed and pulled: a compromised life”, people living with IBD experience a constant conflict throughout their lives, they push to be normal but IBD pulls them back.

Considering the plethora of evidence pertaining to the patient's QoL, symptom burden, and psychosocial factors related to IBD^[5,10,12,25,59,60], there are few qualitative studies directly exploring the patient's beliefs and behaviours from the patient's perspective. Only seven studies were identified, six published and one unpublished thesis, the earliest undertaken in 1996 and the latest in 2010, during a 14 year time span. The studies amount to only 86 patient accounts of living with IBD.

People diagnosed with a chronic disease must adjust to the demands of the disease as well as to the treatments for their condition^[61]. The disease may affect how

the individual perceives him or herself and their relationship with others. The shifting perspectives model of chronic illness determined that life with a chronic illness does not follow a predictable trajectory but people experience a “complex dialectic between themselves and their world”^[62]. This process of debate and argument, trying to cope with the disease is all encompassing; the individual with IBD lives in a world of disease, even when in remission.

Studies have identified the long term complications of IBD, such as bone problems and colorectal cancer^[63]. These potential long term complications heighten the individual's fear of the disease. The uncertain nature of the illness and developing cancer were concerns ranked highly for people with IBD^[4,23]. The fear of long term complications and dying are difficult discuss with others when outwardly the individual appears fine^[38,51].

The issue of control is important within all of the studies. The ability to take control and the relationship with psychological functioning has been established in the literature. Personal control may be informed by self efficacy^[64] or the Common Sense Model whereby the extent to which the individual believes that their illness is manageable and possible to control, becomes focal to their behaviour^[65]. Individuals with IBD have been found to have significantly poorer psychological health than those without IBD^[66] and the metasynthesis has illustrated that control and coping are important factors and assist the psychological well being in these individuals. Controllability and coping strategies were closely linked to knowing how their body reacted to their illness and identification of flare ups^[38], maintaining normality and acceptance of IBD within the individual's life^[50,52-54].

The unmistakable burden of the fear of incontinence, the behaviour related to this fear and the impact of this behaviour on the individual, is exposed and its ongoing effects are demonstrated much more clearly by the metasynthesis. An early study identified urgency of defecation and the fear of incontinence as factors affecting the QoL in individuals with CD^[54]. Behaviour due to fear and coping strategies, such as avoidance of public places, carrying potties when leaving the house^[38], changing working schedules^[21], have been identified in other studies, but the collective impact of this fear and behaviour reveals the true impact IBD has on the individual. The humiliation of incontinence and unpredictable nature of the disease leave the individual socially isolated and missing out on important life events. The reality that this fear and behaviour continues into disease remission compounds the stress, fatigue and debilitating nature of it.

All of the synthesised studies identified the issue of incontinence but the unmistakable burden of this is exposed and its ongoing effects are demonstrated much more clearly by the metasynthesis, supporting the value of the metasynthesis and its ability to interpret studies into new translations.

There are limitations to the metasynthesis: the low number of people with IBD included in the synthesis;

the subjective nature of the synthesis; and grouping studies from various countries with different and changing health care systems over a period of 14 years and combining them and the advent of biologic drugs. The countries have similar socio-economic systems with developed healthcare resources but differ in terms of the financial aids required to access healthcare. Over the past decade the profile of chronic disease management has increased due to the aging population and the role of health care in the management of this area has changed dramatically with greater emphasis placed on self management. Early studies may be deemed outdated. However the methodology of the metasynthesis and the accounts of living with IBD in the studies remain important to capture the phenomenon of living with IBD.

Based on our analysis, we conclude that the fear of incontinence, the behaviour related to this fear and the impact of this behaviour on the individual, are perhaps the most significant issues to emerge from the metasynthesis. The findings highlight the daily challenges and tensions that individuals with IBD face, whether their disease is in remission or not. Evidence has found the incidence and prevalence of IBD to be increasing, indicating its emergence as a global disease^[67]. Perhaps with the emergence of biologic therapies and gene identification, emphasis has been placed upon the acute aspect of IBD and the chronicity of the disease is forgotten.

The physical symptoms alone do not validate the subjective impact of living with IBD^[3]. The psychological burden of living with IBD, QoL and specific psychological co morbidities are described as “un-promoted issues”: issues that are not always addressed in the medical literature^[68]. Identification and clarity of these “un-promoted issues” can only be met by undertaking qualitative studies and health care professionals need to be aware of the influences these have on the individual when developing treatment strategies. More focused attention on the patient’s perspective of living with IBD is needed to provide patient centred care and structure health care services. The emergence of the immense impact of incontinence, fear and behaviour on the individual from this metasynthesis requires further qualitative enquiry.

COMMENTS

Background

The incidence and prevalence of inflammatory bowel disease (IBD) is increasing and it is being recognised as a global long term condition, with significant morbidity and cost. In order to provide patient centred care, an understanding of the impact of living with IBD, from the patient’s perception, is important. The Rating Form for IBD Patient Concerns and Inflammatory Bowel Disease Questionnaire are widely used measures to describe what it is like to live with IBD but these fail to capture the essence of this. There are few qualitative studies which fully demonstrate the impact of living with this condition. By using metasynthesis methodology, this study adds significant understanding of IBD and the impact of living with IBD, from the patient’s perspective.

Research frontiers

There is growing emphasis that the needs and preferences of patients must be addressed when developing and evaluating new models of care delivery. Incorporating patient preference, choice and experience is acquired through qualitative studies. Synthesising qualitative studies of IBD gives a profound insight into

the disease. Capturing this evidence can lead to a greater understating of the condition and help to tailor treatments and provide personalised care.

Innovations and breakthroughs

Recent audits from the European Federation of Crohn’s and Colitis Association has demonstrated, on a large scale, the impact IBD has on the individual’s personal, work and social life. This audit highlighted some important considerations of IBD care in Europe, however, a more immersed understanding is required. This is the first metasynthesis of IBD and provides a comprehensive insight of what it is like to live with.

Applications

The findings from this study emphasises the impact incontinence has on the individual, even in remission. The fear of incontinence, the behaviour related to this fear and the impact of this behaviour on the individual, are the most significant issues to emerge from the metasynthesis, and requires further qualitative enquiry.

Terminology

IBD is a collective term for Crohn’s disease and ulcerative colitis. Qualitative studies typically use focus groups and/or interviews to gather data. Qualitative studies, from the patient’s perspective, are used to highlight the lived experience of a phenomenon. Metasynthesis is a method of identifying and bringing together (synthesising) relevant research evidence from a variety of qualitative studies. Metasynthesis methodology seeks to expand the understanding of patient experience.

Peer review

The enclosed metasynthesis analyses the data from the literature regarding understanding the health and social care needs of patients with IBD. The paper is very well written. The authors observed that the most significant issues were fear of incontinence, the behaviour related to this fear and the impact of this behaviour on the individual. This paper adds a lot of important information on health quality of life in IBD patients and help readers to understand the IBD more.

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***Helicobacter pylori* eradication: Sequential therapy and *Lactobacillus reuteri* supplementation**

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Abstract

AIM: To evaluate the role of sequential therapy and *Lactobacillus reuteri* (*L. reuteri*) supplementation, in the eradication treatment of *Helicobacter pylori* (*H. pylori*).

METHODS: *H. pylori* infection was diagnosed in 90 adult dyspeptic patients. Patients were excluded if previously treated for *H. pylori* infection or if they were taking a proton pump inhibitor (PPI), H₂-receptor antagonist or antibiotics. Patients were assigned to receive one of the following therapies: (1) 7-d triple therapy (PPI plus clarithromycin and amoxicillin or metronidazole) plus *L. reuteri* supplementation during antibiotic treatment; (2) 7-d triple therapy plus *L. reuteri* supplementation after antibiotic treatment; (3) sequential regimen (5-d PPI plus amoxicillin therapy followed by a 5-d PPI, clarithromycin and tinidazole) plus *L. reuteri* supplementation during antibiotic treatment; and (4) sequential regimen plus *L. reuteri* supplementation after antibiotic treatment. Success-

ful eradication therapy was defined as a negative urea breath test at least 4 wk following treatment.

RESULTS: Ninety adult dyspeptic patients were enrolled, and 83 (30 male, 53 female; mean age 57 ± 13 years) completed the study. Nineteen patients were administered a 7-d triple treatment: 11 with *L. reuteri* supplementation during and 8 after therapy. Sixty-four patients were administered a sequential regimen: 32 with *L. reuteri* supplementation during and 32 after therapy. The eradication rate was significantly higher in the sequential group compared with the 7-d triple regimen (88% vs 63%, $P = 0.01$). No difference was found between two types of PPI. No difference in eradication rates was observed between patients submitted to *L. reuteri* supplementation during or after antibiotic treatment. Compliance with therapy was excellent in all patients. No difference in adverse effects was observed between the different antibiotic treatments and between patients submitted to *L. reuteri* supplementation during and after antibiotic treatment. There was a low incidence of adverse effects in all groups of patients with sequential therapy, probably due to the presence of the *L. reuteri* supplementation.

CONCLUSION: The sequential treatment regimen achieved a significantly higher eradication rate of *H. pylori* compared with standard 7-d regimen. *L. reuteri* supplementation could reduce the frequency and the intensity of antibiotic-associated side-effects.

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Key words: *Helicobacter pylori*; Probiotics; *Lactobacillus reuteri*; Sequential therapy; Gastritis; Eradication

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INTRODUCTION

Helicobacter pylori (*H. pylori*) infection is a worldwide disease causing significant morbidity. At present, the role of this infection is well known, particularly in peptic ulcer disease, gastric neoplasia (mucosa-associated lymphoid tissue-lymphoma and carcinoma), non-ulcer dyspepsia (chronic gastritis) and the possible interaction with non-steroidal anti-inflammatory drugs to damage the gastric mucosa^[1-3].

There are numerous treatment options for curing *H. pylori* infection and many are still under investigation. The eradication rate of *H. pylori* following 7-d triple treatment [proton pump inhibitor (PPI) plus clarithromycin and amoxicillin or metronidazole] is decreasing due to an increasing prevalence of bacterial resistance, poor patient compliance and the occurrence of antibiotic adverse effects^[4]. Therefore, further approaches aimed to improve standard triple therapy efficacy should be attempted. In some large studies^[5-7], a sequential regimen, i.e., simple 5-d dual (PPI plus amoxicillin) therapy followed by a 5-d triple therapy (PPI, clarithromycin and tinidazole) was more effective than 7-d triple treatment (PPI plus clarithromycin and amoxicillin or metronidazole), with few adverse effects in children, adults and elderly patients. Moreover, the Italian Working Group of the Cervia II meeting advised the use of sequential therapy as an alternative to 7-14 d triple therapy as first-line treatment^[8].

As reported by the Maastricht III Consensus Report, probiotics could also play a relevant role in the management of *H. pylori* infection by improving treatment tolerability and increasing eradication rates^[4]. Indeed, some *Lactobacilli* have been shown to possess antagonistic activity against *H. pylori*, both *in vitro* and *in vivo*^[9,10]. *L. reuteri* ATCC 55730, a probiotic of human origin, has been demonstrated to reduce adverse effects during antibiotic therapy and to increase eradication of the *H. pylori* infection^[11-13]. Further studies support that *L. reuteri* colonizes the human gastric mucosa, inhibits the binding of *H. pylori* to gastric epithelial cell lines and suppresses *H. pylori* urease activity^[14-16].

The primary end-point of our prospective study was to compare the eradication rate of 7-d triple treatment with a sequential regimen. The secondary end-point was to evaluate the role of *L. reuteri* supplementation in *H. pylori* infection.

MATERIALS AND METHODS

Patients

Between January 2008 to December 2009, 90 adult dys-

peptic outpatients aged > 18 years were consecutively referred to our Division of Gastroenterology. One hundred and five outpatients were screened for enrolment and only 15 of these were excluded. The exclusion criteria were the following: (1) the presence, at endoscopy evaluation, of an active gastro-duodenal ulcer; (2) previous treatment for *H. pylori* infection; (3) PPI, H₂-receptor antagonist or antibiotic treatment in the 4 wk before the study; and (4) a known allergy to the antibiotics used in the present study. No subjects had gastric malignancy at endoscopy.

H. pylori assessment

Patients were enrolled if *H. pylori* infection was detected. *H. pylori* infection was diagnosed based on an upper endoscopy with gastric biopsy (2 samples from the antrum and 2 samples from the corpus) or by means of the *H. pylori* stool antigen-test (SAT) or the ¹³C urea breath test (UBT). The gold standard for *H. pylori* diagnosis was an upper endoscopy with multiple gastric biopsies. All but 5 patients underwent endoscopy and, in 3 of these, *H. pylori* status was assessed by UBT and by SAT in the remaining 2 patients.

Urea breath test: Citric acid (1.5 g) as test meal and 75 mg of ¹³C-urea as water solution were given to the patients after collection of a baseline sample, obtained by blowing through a disposable plastic straw into a 20 mL container, and a further breath sample was collected 30 min later. The breath samples were considered positive if there was a greater than 5 per 1000 of ¹³CO₂ difference over baseline, according to the manufacturer's recommendations.

***H. pylori* stool antigen-test:** *H. pylori* in stool specimens was investigated by a commercial enzymatic immunoassay test (Bioscience). The enzyme immunoassay utilized for the detection of *H. pylori* antigens in human stool was the Premier Platinum HpSA Plus. The test utilizes monoclonal anti-*H. pylori* capture antibody adsorbed to microwells. Diluted patient samples and a peroxidase-conjugated polyclonal antibody were added to the wells and incubated for 1 h at room temperature. A wash was performed to remove unbound material. Substrate was added and incubated for 10 min at room temperature. Color developed in the presence of bound enzyme. Stop solution was added and the results were interpreted visually or spectrophotometrically.

Treatments

The 7-d triple therapy included a PPI 20 mg *bid* plus clarithromycin 500 mg *bid* and amoxicillin 1 g *bid* for 7 d, while the sequential regimen consisted of 5-d dual (PPI 20 mg *bid* plus amoxicillin 1 g *bid*) therapy followed by a 5-d triple therapy (PPI 20 mg *bid*, clarithromycin 500 mg *bid* and tinidazole 500 mg *bid*). The PPI (lansoprazole or pantoprazole) was continued for 30 d at a dose of 20 mg daily. A Reuflor tablet (kindly provided by Italchimici, Rome) containing *L. reuteri* (ATCC 55730; 10⁸ CFU)

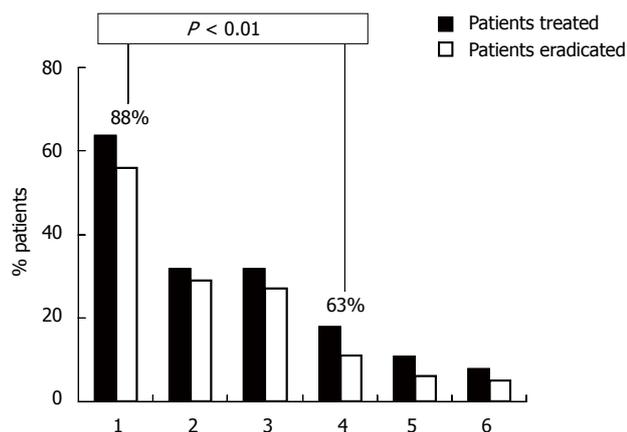


Figure 1 *Helicobacter pylori* eradication rate in the following groups of patients. 1: 10-d sequential therapy plus *Lactobacillus reuteri* (*L. reuteri*) post therapy; 2: 10-d sequential therapy plus *L. reuteri* during therapy; 3: 10-d sequential therapy; 4: 7-d standard triple therapy plus *L. reuteri* post therapy; 5: 7-d standard triple therapy plus *L. reuteri* during therapy; 6: 7-d standard triple therapy. The eradication rate was significantly higher in the sequential group compared with the 7-d triple therapy group (88% vs 63%, $P = 0.01$). No difference was found between patients submitted to *L. reuteri* supplementation during or after antibiotic treatment.

was taken once a day. Some patients received *L. reuteri* supplementation after the 7th or 10th day of antibiotic therapy, 12 h after administration of the last tablet of antibiotic. The other patients received the *L. reuteri* on the first day of antibiotic treatment.

For each therapy regimen, the PPI was prescribed 30 min before breakfast and dinner, whereas all antibiotics were given immediately after these meals.

Study groups

Consecutive patients were assigned to receive one of the following therapies: (1) 7-d triple therapy plus *L. reuteri* supplementation during the antibiotic treatment; (2) 7-d triple therapy plus *L. reuteri* supplementation after the antibiotic treatment; (3) 10-d sequential regimen plus *L. reuteri* supplementation during the antibiotic treatment; and (4) 10-d sequential regimen plus *L. reuteri* supplementation after the antibiotic treatment.

In detail, the patients enrolled between 1 January 2008 and 30 November 2008 were treated with 7-d triple therapy, while those enrolled between 1 December 2008 and 31 December 2009 were treated with sequential therapy.

Informed consent was obtained from all patients enrolled in the study. The local Ethical Committee approved the study protocol.

Follow-up

Patients were asked to return at the end of treatment to assess the compliance with therapy and to determine possible adverse effects. Compliance was defined as consumption of > 90% of the prescribed drugs. Adverse effects were evaluated using a structured questionnaire by personal interview. Bacterial eradication was checked in all patients at least 4 wk following the eradication treatment by using UBT.

Table 1 Demographic characteristics of the patient groups

Therapy	Patients	Mean age (yr)	Male/female
7-d standard triple therapy plus <i>L. reuteri</i> during therapy	11	54 ± 8	6/5
7-d standard triple therapy plus <i>L. reuteri</i> post therapy	8	59 ± 5	2/6
10-d sequential therapy plus <i>L. reuteri</i> during therapy	32	57 ± 2	7/25
10-d sequential therapy plus <i>L. reuteri</i> post therapy	32	60 ± 2	11/21

L. reuteri: *Lactobacillus reuteri*.

Statistical analysis

The data are expressed as mean ± SE. The statistical analysis was conducted by χ^2 test. $P < 0.05$ was considered statistically significant.

RESULTS

Overall, ninety adult dyspeptic patients were enrolled in the study. All but 7 patients completed the study, with one lost to follow-up and 6 noncompliant. Therefore, 83 patients (30 male, 53 female; mean age 57 ± 13 years) completed the study. Nineteen patients were administered a 7-d triple treatment: 11 with *L. reuteri* supplementation during and 8 after therapy. Sixty-four patients were administered the sequential regimen: 32 with *L. reuteri* supplementation during and 32 after therapy (Table 1).

The eradication rate was significantly higher in the sequential group compared with the 7-d triple therapy; 88% vs 63%, $P = 0.01$ (Figure 1). No difference in eradication rates was observed between patients submitted to *L. reuteri* supplementation during or after antibiotic treatment. No difference was found between the two different types of PPI (lansoprazole or pantoprazole).

Compliance and adverse effects

The reported compliance with therapy was excellent in all patients. All adverse effects, mainly mild diarrhea and abdominal pain, were self-limiting after the end of therapy. No difference in the number and type of adverse effects was observed between the different antibiotic regimens and between patients submitted to *L. reuteri* supplementation during or after antibiotic treatment.

DISCUSSION

In the last few years, with an increasing prevalence of antimicrobial resistance, the *H. pylori* cure rate with standard triple therapy has declined to unacceptable levels (i.e., 80% or less) in most countries. Two very large meta-analyses showed that standard 7-14 d triple therapies fail to eradicate *H. pylori* infection in up to 25% of patients^[17,18]. More recent data have demonstrated that triple therapy with amoxicillin, clarithromycin and a PPI has an eradication rate of only 74%-76%^[19]. Therefore, several treatment regimens have emerged for cure of

H. pylori infection. In Italy, since the prevalence of primary clarithromycin resistance is higher than 15%^[20,21], the Cervia II Working Group advised the use of 7-14 d triple therapies or a sequential therapy, as first-line treatment. To date, the efficacy of sequential therapy has been investigated in 22 trials including more than 2000 patients. The success rate of the sequential regimen was distinctly higher than that achieved by standard triple therapies^[22,23]. Zullo *et al.*^[18] found that the sequential regimen was significantly superior to either 7-d and 10-d standard triple therapies with an overall eradication rate of 93.7%, 75.9% and 79.6%, respectively. These results were confirmed in 2 other recent meta-analyses^[24,25]. It was known that a dual therapy (PPI plus amoxicillin) administered for less than 7 d was able to achieve a cure rate of up to 50%, and that the efficacy of a triple therapy (PPI, clarithromycin and tinidazole) was inversely related to the bacterial load, with higher eradication rates being achieved in those with a low bacterial density in the stomach. Because amoxicillin acts on the bacterial cell wall and damages it, the initial phase of treatment may prevent the development of efflux channels by weakening the cell wall of the bacterium. An important limitation is that data regarding the efficacy of the sequential regimen mainly came from Italian studies, and the use of metronidazole instead of tinidazole in different studies performed in other geographic areas could reduce the eradication rate^[26,27].

The primary aim of this study was to evaluate the efficacy of sequential therapy compared with standard triple therapy. We found that the eradication rate was significantly higher in the sequential group as compared with the 7-d triple therapy group (88% *vs* 63%; $P = 0.01$). These results confirm that 10-d sequential therapy is superior to standard regimens (range: 91%-96% *vs* 71%-83%), as reported by systematic reviews and meta-analyses of randomized, controlled trials comparing these 2 treatments and published until October 2008^[24,25,28]. Some prospective randomized Italian studies also more recently^[16,29,30], confirmed these results, constantly achieving very high (range: 92%-95%) eradication rates in children, adults, and elderly patients treated with sequential therapy compared with 7-d or 10-d triple treatment (range: 74%-77%).

The secondary end-point was the assessment the role of *L. reuteri* on the outcome of *H. pylori* infection. Among probiotics, it was demonstrated that *L. reuteri* colonizes the human gastric mucosa, inhibits the binding of *H. pylori* to gastric epithelial cell lines and suppresses *H. pylori* urease activity^[14-16]. Interestingly, in some studies^[12,31], monotherapy with *L. reuteri* showed a reduction in the *H. pylori* bacterial load.

In our population, all patients were assigned to receive *L. reuteri* supplementation. In the sequential therapy group, the eradication rate of *H. pylori* with this probiotic was 88%, similar to that reported in literature. In a double-blind placebo-controlled study^[16], *H. pylori*-positive adult subjects were given *L. reuteri* for 4 wk before antibiotic therapy or placebo. As with our results after the

10-d sequential regimen, the rate of *H. pylori* eradication in those who had received the probiotic was 88%.

In our study, in the 7-d therapy group, the eradication rate of *H. pylori* was 63%, greater than 53% reported by a prospective, pilot study^[32] in a subgroup of patients assigned to receive the same therapy with *L. reuteri* supplementation.

In our population, it a low incidence of adverse effects was observed in all groups of patients with sequential therapy, probably due to the presence of *L. reuteri*.

In conclusion, sequential therapy appears to be more effective than standard 7-d triple therapy, and *L. reuteri* supplementation could play a role in the eradication of *H. pylori*, but a large, double-blind, controlled study is needed to confirm these results and to explain the exact function of *L. reuteri*. Indeed, it may improve the tolerability to antibiotic therapy by decreasing adverse effects or play a primary role in the eradication of *H. pylori* infection.

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COMMENTS

Background

The *Helicobacter pylori* (*H. pylori*) cure rate following standard triple therapies is decreasing worldwide. The need for alternative treatment strategies for *H. pylori* infections has created an interest in control of this pathogen with further antibiotic protocols and probiotics.

Research frontiers

In some large studies, the sequential regimen, i.e., simple 5-d dual [proton pump inhibitor (PPI) plus amoxicillin] therapy followed by a 5-d triple therapy (PPI, clarithromycin and tinidazole), is more effective than 7-d triple treatment (PPI plus clarithromycin and amoxicillin or metronidazole). *Lactobacillus reuteri* (*L. reuteri*) ATCC 55730, a probiotic of human origin, has been demonstrated to reduce adverse effects during antibiotic therapy and to increase eradication of the *H. pylori* infection. Further studies support that *L. reuteri* colonizes the human gastric mucosa, inhibits the binding of *H. pylori* to gastric epithelial cell lines and suppresses *H. pylori* urease activity.

Innovations and breakthroughs

The *H. pylori* eradication rate was significantly higher in the sequential group compared with 7-d triple therapy. These results confirmed that 10-d sequential therapy is superior to standard regimens, as reported by systematic reviews and meta-analyses of randomized trials. Moreover, *L. reuteri* supplementation could play a role in the eradication of *H. pylori* and improve the tolerability to antibiotic therapy, despite conflicting results in previous studies.

Applications

Sequential therapy appears to be more effective than standard 7-d therapy and is a well-tolerated, promising therapy and should be recommended as first-line treatment. *L. reuteri* supplementation could play a role in the eradication of *H. pylori*, but a large, double-blind, controlled study is needed to confirm these results and to explain its exact function. It may improve the tolerability to the antibiotic therapy or play a primary role in reducing *H. pylori* infection.

Peer review

The authors investigated standard triple drug therapy treatment of *H. pylori* infection of human patients versus sequential therapy, with all therapies also including *L. reuteri* probiotic. In 83 patients, they found that eradication rates were higher in the sequential therapy group over that of the standard triple therapy group.

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Celiac disease markers in patients with liver diseases: A single center large scale screening study

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METHODS: Large-scale screening of serum antibodies against tissue transglutaminase (tTG), and deamidated gliadin using enzyme-linked immunosorbent assay and serum antibodies against endomysium using immunohistochemistry, in patients with various liver diseases ($n = 962$) and patients who underwent liver transplantation (OLTx, $n = 523$) was performed. The expression of tTG in liver tissue samples of patients simultaneously suffering from celiac disease and from various liver diseases using immunohistochemistry was carried out. The final diagnosis of celiac disease was confirmed by histological analysis of small-intestinal biopsy.

RESULTS: We found that 29 of 962 patients (3%) with liver diseases and 5 of 523 patients (0.8%) who underwent OLTx were seropositive for IgA and IgG anti-tTG antibodies. However, celiac disease was biopsy-diagnosed in 16 patients: 4 with autoimmune hepatitis type I, 3 with Wilson's disease, 3 with celiac hepatitis, 2 with primary sclerosing cholangitis, 1 with primary biliary cirrhosis, 1 with Budd-Chiari syndrome, 1 with toxic hepatitis, and 1 with non-alcoholic steatohepatitis. Unexpectedly, the highest prevalence of celiac disease was found in patients with Wilson's disease (9.7%), with which it is only rarely associated. On the other hand, no OLTx patients were diagnosed with celiac disease in our study. A pilot study of the expression of tTG in liver tissue using immunohistochemistry documented the overexpression of this molecule in endothelial cells and periportal hepatocytes of patients simultaneously suffering from celiac disease and toxic hepatitis, primary sclerosing cholangitis or autoimmune hepatitis type I.

CONCLUSION: We suggest that screening for celiac disease may be beneficial not only in patients with associated liver diseases, but also in patients with Wilson's disease.

Abstract

AIM: To study the coincidence of celiac disease, we tested its serological markers in patients with various liver diseases.

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Key words: Tissue transglutaminase; Anti-tissue transglutaminase antibodies; Autoimmune liver diseases; Wilson's disease; Celiac disease; Liver transplantation

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INTRODUCTION

Celiac disease (CLD) is a frequent, lifelong, primarily small intestinal enteropathy with an incidence of more than 1:250 which is induced in genetically susceptible individuals after ingestion of wheat gluten. The duodenal and jejunal mucosa of patients with active CLD is infiltrated by leukocytes and structurally remodeled. Villous flattening and crypt hyperplasia develop in the mucosa of these patients and cause malabsorption syndrome, diarrhea, abdominal pain, and weight loss. However, these symptoms predominate in pediatric patients (accompanied by growth retardation), whereas latent and silent forms of CLD occur more often in adult patients^[1-3].

Interestingly, a growing proportion of new cases of CLD are being diagnosed in adults and in patients with extraintestinal manifestations. CLD may affect several organs including kidney, skin, heart, and the nervous, endocrine and reproductive systems, however, liver injury is one of the most frequent extraintestinal manifestations of the disease. Although the spectrum of liver manifestations associated with CLD is particularly wide, two main forms of liver damage, namely cryptogenic and autoimmune, appear to be strictly related to this disease. The most frequent finding documenting liver damage in CLD is a cryptogenic hypertransaminasemia, observed in approximately 15%-55% of untreated patients, as an expression of mild liver dysfunction with a histological picture of nonspecific reactive hepatitis ("celiac hepatitis"). However, in a few cases a more severe liver injury, characterized by a cryptogenic chronic hepatitis or liver cirrhosis requiring liver transplantation, is present. A close association is known to exist between CLD and autoimmune liver diseases such as primary biliary cirrhosis with a prevalence of 3%-7%, autoimmune hepatitis (3%-6%), and primary sclerosing cholangitis (2%-3%). This is probably related to an association between CLD and the human leukocyte antigen (HLA)-DQ heterodi-

mer DQA1/0501/DQB1/0201 on antigen-presenting cells. In CLD, the number of gliadin-specific HLA-DQ2- or HLA-DR4-restricted T-lymphocytes expands and high titers of antibodies against gliadin and various autologous antigens are generated, which can affect the functions of many organs^[3-7].

The therapy of CLD is still based on the withdrawal of gluten and its related proteins from the diet of patients [gluten-free diet (GFD)]. After 6-12 mo on a GFD, most CLD patients experience a clinical improvement accompanied by restoration of the intestinal mucosa and a reduction in the number of gliadin and autoantigen-restricted lymphocytes. The serum concentrations of anti-gliadin antibodies and antibodies against autologous antigens are also reduced. Interestingly, mild liver dysfunction with a histological picture of nonspecific reactive hepatitis (celiac hepatitis) may improve after institution of a GFD^[8-11]. Although the withdrawal of gluten from the patients' diet improves celiac hepatitis, its influence on autoimmune hepatitis is controversial and in childhood, in which the diagnosis and the introduction of GFD are not usually delayed, it seems ineffective. However, the diet may reduce the risk of CLD complications and development of the most severe refractory CLD^[12-15].

The diagnosis of CLD is based on the histological analysis of a duodenal/jejunal biopsy and the testing of serum antibodies against gliadin and autoantibodies against endomysium or tissue transglutaminase (tTG).

This study focused on serological screening for CLD in patients with liver diseases and those who underwent liver transplantation, i.e., patients with a known higher risk of developing CLD. Moreover, we also analyzed the tissue expression and distribution of tTG in the liver of patients with various liver diseases, especially those simultaneously suffering from CLD and compared these to morphologically unaltered liver tissue.

MATERIALS AND METHODS

Patients and controls

The study enrolled patients treated in the Department of Hepatogastroenterology, Institute for Clinical and Experimental Medicine, Prague, in 2009-2010. Sera from a total of 1485 patients were tested. The tested cohorts included 962 patients with diagnosed liver diseases (mean age 55 years, range: 21-76 years) and 523 patients (mean age 49 years, range: 18-66 years) who underwent liver transplantation during 1994-2010 as a consequence of end stage of liver disease. Table 1 summarizes the baseline clinical characteristics of the patients and healthy controls included in our study. The cohort of healthy controls ($n = 300$, mean age 23 years, range: 18-45 years) was selected from the Institute of Hematology and Blood Transfusion (Prague, Czech Republic).

The diagnostic criteria for primary biliary cirrhosis included clinical symptoms, clinical chemistry, exclusion of infection with hepatitis viruses and evidence of anti-

Table 1 Patients with liver disease and patients who underwent liver transplantation

Disease	Diagnosis	n (M/F)
Liver disease	Alcoholic liver cirrhosis	152 (94/58)
	Autoimmune hepatitis type I	77 (27/50)
	Viral hepatitis B	117 (67/50)
	Viral hepatitis C	147 (82/65)
	Wilson's disease	31 (13/18)
	Primary biliary cirrhosis	32 (4/28)
	Primary sclerosing cholangitis	59 (40/19)
	Nonalcoholic steatohepatitis	23 (10/12)
	Liver steatosis	132 (77/55)
	Budd-Chiari syndrome	14 (5/9)
	Polycystic liver	10 (1/9)
	Others ¹	168 (74/94)
	OLTx	Alcoholic liver cirrhosis
Autoimmune hepatitis type I		33 (11/22)
Viral hepatitis B		33 (20/13)
Viral hepatitis C		79 (56/23)
Wilson's disease		29 (12/17)
Primary biliary cirrhosis		40 (5/35)
Primary sclerosing cholangitis		64 (47/17)
Cryptogenic liver cirrhosis		28 (16/12)
Budd-Chiari syndrome		6 (2/4)
Polycystic liver		14 (3/11)
Others ²		33 (16/17)

¹Drug-induced hepatitis, cryptogenic liver cirrhosis, hepatitis A, hepatocellular carcinoma, focal nodular hyperplasia, mild liver test abnormalities, *etc.*; ²Cryptogenic liver cirrhosis, hepatocellular carcinoma, hemochromatosis, alpha-1-antitrypsin deficiency, *etc.* M/F: Male/female; OLTx: Patients who underwent liver transplantation.

mitochondrial antibodies type M2. The diagnosis of autoimmune hepatitis was based on the scoring system devised by the International Autoimmune Hepatitis Group and International Association for the Study of the Liver^[16]. The main diagnostic criteria for alcoholic liver cirrhosis were the patient's medical history, liver histology, and exclusion of other causes of liver cirrhosis. Diagnosis of Wilson's disease was based on the recommendation of Kodama *et al*^[17], and Budd-Chiari syndrome in accordance with the concept of Fox *et al*^[18]. Patients who underwent liver transplantation were treated with standard immunosuppressive therapy following appropriate guidelines. The study was approved by the local Ethics Committee.

Serology

Diagnosics and markers for screening of CLD: All serum samples were tested for immunoglobulin (Ig) A and IgG antibodies against tTG. Individuals seropositive for IgA or IgG anti-tTG antibodies were tested for IgA or IgG (in the case of patients with IgA immunodeficiency) isotypes of antibodies against deamidated gliadin (IgA and IgG) and anti-endomysium (IgA or IgG). The final diagnosis of CLD, in individuals seropositive for these IgA or IgG antibodies, was performed by duodenal/jejunal biopsy.

Serological assays used for CLD screening: All tests were performed in the immunological laboratory of

the Institute for Clinical and Experimental Medicine according to the manufacturer's instructions. The BINDAZYME™ Anti-Tissue Transglutaminase EIA kit (the Binding Site, Birmingham, United Kingdom) and ORG 540A and ORG 540G Anti-Tissue-Transglutaminase ELISA kit (ORGENTEC Diagnostika GmbH, Mainz, Germany) were simultaneously used to test for IgA or IgG anti-tTG antibodies, IgA or IgG anti-gliadin antibodies were tested using QUANTA Lite Gliadin IgA or QUANTA Lite Gliadin IgG (INOVA Diagnostic Inc., San Diego, CA, United States), and by ELISA kits ANTI GLIADIN MGP IgA and ANTI GLIADIN MGP IgG (Binding Site). The results of the serological testing were expressed as a percentage of antibody-positive patients within individual groups. To exclude immunoglobulin deficiency, total IgA and IgG blood levels were analyzed using a routine method in all tested patients.

Detection of anti-endomysial antibodies

Anti-endomysial antibodies were routinely tested by an indirect immunofluorescence method using human umbilical cord tissue cryostat sections. The test serum samples were diluted 1:20 and 1:50. Slides were examined using a Nikon Eclipse E600 immunofluorescence microscope (Nikon, Japan). A positive result was recorded if the connective tissue surrounding the muscle cells was brightly fluorescent, forming a honeycomb pattern.

Immunohistochemistry

Analysis of tTG expression in liver tissue: The expression and distribution of tTG in the liver biopsy samples from 25 patients were analyzed and compared. Eight patients suffered from CLD simultaneously with liver diseases (2 patients with primary sclerosing cholangitis, 1 with autoimmune hepatitis type I, 1 with toxic hepatitis, 1 with Budd-Chiari syndrome, 3 with celiac hepatitis), 8 patients with liver diseases (3 with primary sclerosing cholangitis, 2 with autoimmune hepatitis type I, 1 patient with steatosis, 1 with Budd-Chiari syndrome and 1 patient with primary biliary cirrhosis), and 9 patients with liver metastasis from colorectal carcinoma where tTG expression was analyzed on histologically confirmed unaltered liver tissue at least 3 cm away from the metastasis. The patients with liver disease simultaneously suffering from CLD were seropositive for IgA antibodies against tTG, endomysium and gliadin. MARSH IIIb-c stage of jejunal mucosa was present in these patients. On the other hand, the patients suffering from liver diseases, but not from CLD, included in the analysis of tTG expression in liver were seronegative for all of the mentioned antibodies. Therefore, there was no reason to perform a small intestinal biopsy in these patients.

The tTG detection in liver tissue was performed using the immunoperoxidase staining technique, N-Histofine® Simple Stain MAX PO (MULTI, Nichirei, Japan). Detection was performed on 4 µm thick paraffin sections. Tissue sections were deparaffinated, rehydrated, and antigen retrieval was performed using heat-induced

Table 2 Immunoglobulin A anti-tissue transglutaminase seropositivity in patients with liver disease and patients who underwent liver transplantation

IgA anti-tTG seropositivity	Diagnosis	M/F	n (%)	
Liver disease	Wilson's disease	1/3	4 (12.9)	
	Autoimmune hepatitis type I	1/4	5 (6.5)	
	Primary biliary cirrhosis	0/1	1 (3.1)	
	Budd-Chiari syndrome	0/1	1 (7.1)	
	Mild hepatic liver tests abnormalities	5/2	7 (20)	
	Liver steatosis	3/1	4 (3.3)	
	Viral hepatitis B	1/0	1 (0.9)	
	Toxic hepatitis	1/0	1 (4.8)	
	Nonalcoholic steatohepatitis	0/2	2 (8.7)	
	Primary sclerosing cholangitis	1/1	2 (3.4)	
	Polycystic liver	0/1	1 (10)	
	OLTx	Wilson's disease	1/1	2 (6.9)
		Autoimmune hepatitis type I	0/2	2 (6.1)
Alcoholic liver cirrhosis		1/0	1 (0.6)	

IgA: Immunoglobulin A; tTG: Tissue transglutaminase; OLTx: Patients who underwent liver transplantation; M/F: Male/female.

epitope restoration in an EDTA buffer of pH 8.0. Endogenous peroxidase was inactivated with 0.3% H₂O₂ in 70% methanol for 30 min at room temperature. The sections were then incubated for 2 h with anti-tTG antibody CUB 7402 (Acris, Germany), diluted at 1:1200 in antibody diluent, Dako Real (Dako, Glostrup, Denmark). After a washing step, Histofine Simple Stain Max PO was added for 30 min. Tissue staining was visualized with a 3,3'-diaminobenzidine substrate chromogen solution (Dako). Slides were counterstained with hematoxylin, dehydrated, and mounted. A negative control was included by omitting the anti-tTG antibody. An isotype control was included using a nonspecific mouse IgG1 instead of specific anti-tTG antibody.

RESULTS

CLD seropositivity and final diagnosis in patients with severe liver disease

In our study, 29 of 962 adult patients with various liver diseases (3%) were seropositive for IgA anti-tTG antibodies. A summary of the data on seropositive patients is given in Table 2. Seropositivity for anti-tTG antibodies-detected by employing two different diagnostic kits-was found in five patients with autoimmune hepatitis type I, four patients with liver steatosis, four with Wilson's disease, two with primary sclerosing cholangitis, two with non-alcoholic steatohepatitis, and one each with polycystic liver disease, Budd-Chiari syndrome, primary biliary cirrhosis, toxic hepatitis, and hepatitis B. Seven patients with mild liver test abnormalities were also seropositive for anti-tTG.

Sixteen of 29 patients, i.e., those who were positive for IgA anti-tTG antibodies, were also seropositive for IgA anti-gliadin and anti-endomysial antibodies. This cohort included patients with autoimmune hepatitis type I (4), Wilson's disease (3), celiac hepatitis (3, re-

cruited from the group of anti-tTG seropositive patients with mild liver test abnormalities), primary sclerosing cholangitis (2), primary biliary cirrhosis (1), Budd-Chiari syndrome (1), toxic hepatitis (1), and non-alcoholic steatohepatitis (1). Histological analysis of duodenal or jejunal specimens confirmed the diagnosis of CLD in these 16 patients according to European Society for Paediatric Gastroenterology, Hepatology, and Nutrition criteria. MARSH IIIa-c stages of gut mucosa were observed in all 16 patients. The clinical data of these newly diagnosed patients are summarized in Table 3. After 6 mo of adherence to the GFD, all tested antibodies decreased to values occurring in healthy individuals and staging of mucosal lesions improved substantially (MARSH 0- I) in these patients. Four of these 16 patients (three with celiac hepatitis, one with non-alcoholic steatohepatitis) showed normalization of liver tests. However, no substantial clinical and laboratory improvements were observed in the other patients adequately treated for liver disease and adhering to a GFD.

Ten of the 16 newly diagnosed patients showed some non-specific symptoms of CLD (weight loss, diarrhea, abdominal pain), while 6 patients were asymptomatic for CLD.

Serum samples from the blood donors were negative for IgA anti-tTG antibodies.

CLD seropositivity in patients who underwent liver transplantation

Of the 523 patients who underwent liver transplantation, five (0.8%) were positive for IgA anti-tTG antibodies. A summary of the data on seropositive patients is given in Table 2. Anti-tTG antibody seropositivity occurred in 2 patients transplanted for autoimmune hepatitis type I, two for Wilson's disease and one transplanted for alcoholic liver cirrhosis. However, none of these patients was seropositive for IgA anti-endomysial antibodies or for IgA and IgG anti-gliadin antibodies. CLD symptoms were not observed in five patients who were seropositive for IgA anti-tTG. For that reason, small intestinal biopsy was not performed in these patients.

TTG expression in liver tissue

In liver diseases, tTG is closely related with tissue repair, fibrogenesis and inflammation^[19]. For this reason, we analyzed tTG expression in the liver tissue of twenty five patients simultaneously suffering from active CLD and liver disease, patients suffering from liver disease, but not from CLD, and patients with metastatic colorectal carcinoma.

In this pilot study, we found overexpression of tTG in the liver tissue of patients suffering from liver diseases in contrast to morphologically unaltered tissues. However, individual variability in tTG expression in the liver tissue of these patients was detected. Despite this, the overexpression of tTG was predominantly localized in endothelial cells and periportal hepatocytes and was more pronounced in the liver tissue of all patients suf-

Table 3 Patients with liver disease newly diagnosed with celiac disease

No.	Age	Gender	Diagnosis	Liver histology
1	34	F	PSC	Portal tracts with ductular reaction and minimal inflammation, features of chronic cholestasis
2	37	M	PSC	Florid ductular reaction with accompanying mild mixed inflammation and focal dark-brown granules of copper-associated protein (in orcein stain) in periportal hepatocytes
3	33	M	Wilson's d.	Macrovesicular steatosis, periportal fibrosis and periportal hepatocytes with glycogenated nuclei
4	35	F	Wilson's d.	Focal steatosis, periportal and septal fibrosis
5	36	F	Wilson's d.	Mild nonspecific hepatocellular injury with spotty hepatocyte necrosis and mononuclear portal inflammatory infiltrate, scattered apoptotic bodies and mild steatosis
6	29	M	AIH type I	Portal and lobular inflammation, periportal fibrosis
7	33	F	AIH type I	Portal and periportal inflammation, spotty necrosis
8	33	F	AIH type I	Chronic hepatitis pattern of injury with portal-based inflammation and fibrosis
9	40	F	AIH type I	Periportal interface activity and scattered hepatocyte necrosis
10	35	F	PBC	Bile duct injury with epithelioid granuloma, portal inflammation
11	32	M	Toxic hepatitis	Portal inflammation with scattered eosinophils, spotty necrosis
12	50	F	Budd-Chiari s.	Extensive centrilobular necrosis of hepatocytes
13	22	M	Celiac hepatitis	Non-specific reactive hepatitis with mild portal inflammation
14	27	M	Celiac hepatitis	Mild lobular inflammation with apoptotic bodies and hepatocyte necrosis
15	50	F	Celiac hepatitis	Mild periportal fibrosis with mild portal inflammation and focal interface activity
16	40	F	NASH	Ballooned hepatocytes, macrovesicular steatosis accentuated in zone 3 without significant liver injury

No.: Number; F: Female; M: Male; d.: Disease; PBC: Primary biliary cirrhosis; s.: Syndrome; NASH: Non-alcoholic steatohepatitis; PSC: Primary sclerosing cholangitis; AIH: Autoimmune hepatitis.

fering simultaneously from both liver diseases and active CLD in contrast to patients suffering only from liver disease. Figure 1 shows more pronounced tTG staining in the liver specimens of patients simultaneously suffering from active CLD and primary sclerosing cholangitis, toxic hepatitis, and autoimmune hepatitis type I compared to patients suffering only from primary sclerosing cholangitis and steatosis.

DISCUSSION

CLD is frequently associated with various autoimmune liver diseases such as autoimmune hepatitis, primary biliary cirrhosis and primary sclerosing cholangitis, all of which may be indications for liver transplantation. On the other hand, a controversial association exists between hepatitis B and C virus and CLD. Chronic untreated CLD of long duration may also lead to liver damage severe enough to require liver transplantation^[4,6,20-24]. Consistent with the above, reversal of hepatic failure has been described in CLD patients simultaneously suffering from liver disorders who followed a GFD^[25]. On the other hand, GFD did not always lead to complete resolution of liver damage in all patients with CLD associated with liver disease^[26-28].

This study on the occurrence of CLD and its serological markers in patients with various hepatic disorders and in those who underwent liver transplantation in the Czech Republic complements the serological screening for CLD in the general population (blood donors) and high-risk patients groups e.g., patients with osteoporosis, female infertility and some autoimmune diseases including systemic lupus erythematosus, Sjögren's syndrome and patients with connective tissue disorders in the Czech population performed by Vanciková *et al.*^[29]. In the present study, we estimated, for the first time,

the coincidence of CLD and liver diseases and the relationship between CLD and liver transplantation in the Czech population. Surprisingly, our results documented the highest incidence of CLD in patients with Wilson's disease (9.7%), followed by autoimmune hepatitis (3.9%), primary biliary cirrhosis (3.0%), and primary sclerosing cholangitis (3.4%). While a higher incidence of CLD with primary biliary cirrhosis, primary sclerosing cholangitis and autoimmune hepatitis has been well described^[26,28], the association between CLD and Wilson's disease is rare^[30-32]. The coincidence of Wilson's disease and CLD was high compared with the coincidence of CLD and osteoporosis (0.98%), female infertility (1.13%) and systemic lupus erythematosus (approximately 2.7%) in the Czech population^[29]. Our findings of a higher incidence of CLD in adult patients could be associated with the underestimation of CLD diagnosis described in the elderly^[33].

Despite the fact that Wilson's disease, which is characterized by accumulation of copper in tissue, is rarely associated with CLD^[30-32], it has been reported that copper metabolism is also impaired in CLD. In CLD patients, copper uptake from the gut is significantly reduced and levels of copper in urine are higher than in healthy individuals^[31,32].

In our study, anti-tTG seropositivity in patients who underwent liver transplantation was lower than that described in previous studies^[6]. A possible explanation for this is the exclusion from screening of 5 patients who underwent liver transplantation for primary sclerosing cholangitis (2 men, 39 years and 40 years), autoimmune hepatitis type I (woman, 25 years), viral hepatitis B (woman, 51 years) and viral hepatitis C (woman, 56 years), in whom CLD was diagnosed before the beginning of this study. All five patients who underwent liver transplantation and were seropositive for IgA anti-tTG

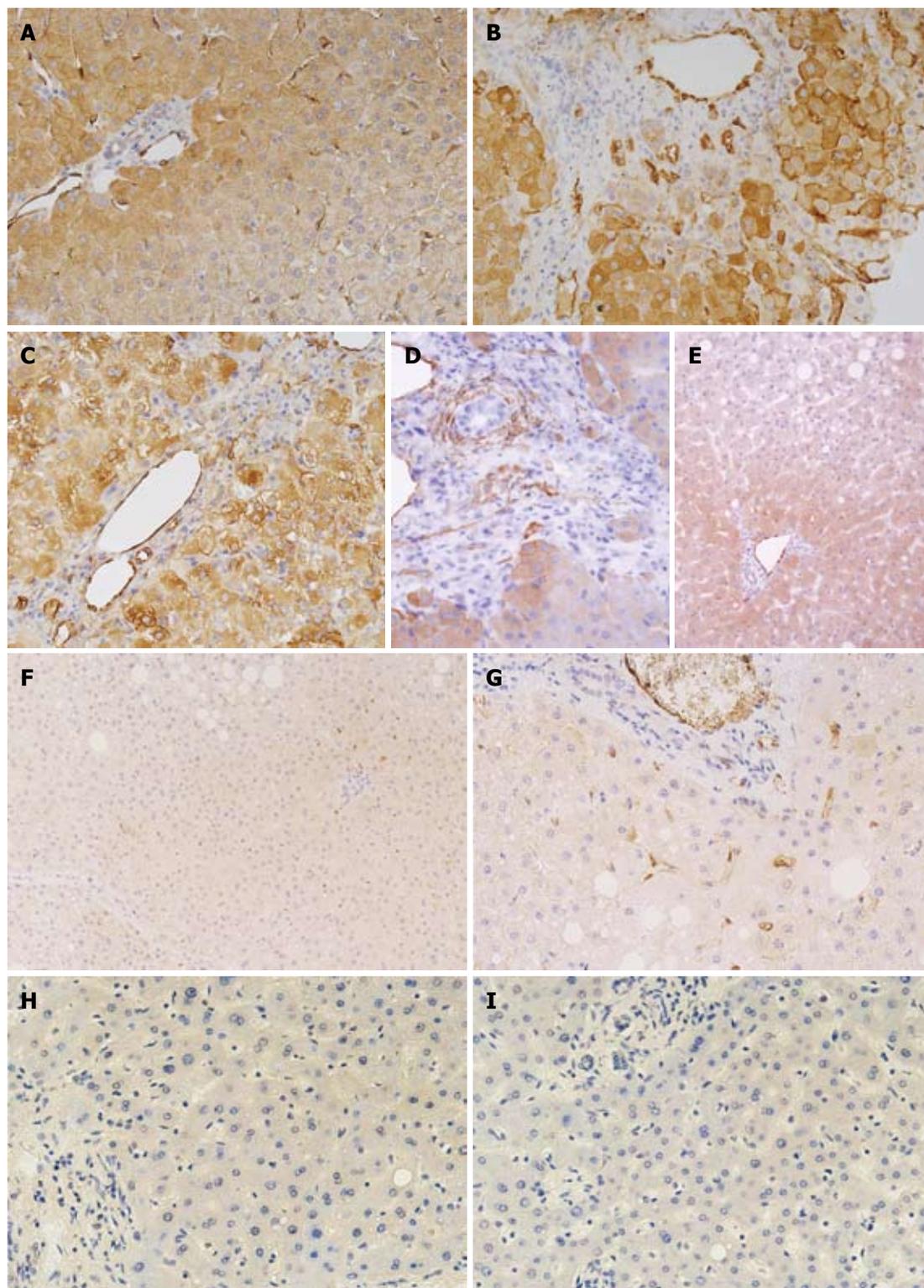


Figure 1 Tissue transglutaminase expression in liver tissue specimens from patients. A: Specimens from patients simultaneously suffering from active celiac disease and primary sclerosing cholangitis; B: Toxic hepatitis; C: Autoimmune hepatitis type I; D: Patient with primary sclerosing cholangitis without celiac disease (CLD); E: Steatosis without CLD; F, G: The basal tissue transglutaminase (tTG) expression in morphologically unaltered liver tissue surrounding colorectal adenocarcinoma metastasis; H: Negative control (no anti-tTG antibody); I: Isotype control (nonspecific mouse Immunoglobulin G1 antibody).

antibodies were also seropositive for the IgG isotype of the antibodies, but seronegative for the remaining CLD markers - antibodies against deamidated gliadin and endomysium. The antibody levels, as well as histological changes in small intestine mucosa characteristic of CLD,

could have been affected by immunosuppressive treatment complicating the diagnosis of CLD in those patients who underwent liver transplantation^[34]. This could also be the reason for the difference in seropositivity between cohorts of patients suffering from liver dis-

ease and patients who underwent liver transplantation. The putative reason for the development of anti-tTG antibodies in patients who underwent liver transplantation could be overexpression of tTG in the graft during repair and healing processes. However, our findings concerning the expression of tTG in liver and induction of anti-tTG antibodies need further investigation. Nevertheless, the testing of CLD serological markers seems to be useful in patients considered for liver transplantation.

In conclusion, we suggest that screening for CLD may be beneficial not only in groups of patients with well-known associated diseases (autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis), but also in patients with Wilson's disease, where the relationship to CLD has not been fully analyzed.

COMMENTS

Background

Celiac disease (CLD) is a frequent, lifelong, primarily small intestinal enteropathy with an incidence of more than 1:250 which is induced in genetically susceptible individuals after ingestion of wheat gluten. A growing proportion of new cases of CLD are being diagnosed in adults and in patients with extraintestinal manifestations. CLD may affect several organs including kidney, skin, heart, and the nervous, endocrine and reproductive systems, however, liver injury is one of the most frequent extraintestinal manifestations of the disease.

Research frontiers

This study on the occurrence of CLD and its serological markers in patients with various hepatic disorders and in those who underwent liver transplantation in the Czech Republic complements the serological screening for CLD in the general population (blood donors) and high-risk patients groups.

Innovations and breakthroughs

The authors suggest that screening for CLD may be beneficial not only in groups of patients with well-known associated diseases (autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis), but also in patients with Wilson's disease, where the relationship to CLD has not been fully analyzed.

Applications

Their findings on the expression of tissue transglutaminase (tTG) in liver and induction of anti-tTG antibodies need further investigation. Nevertheless, the testing of CLD serological markers seems to be useful in patients considered for liver transplantation.

Peer review

The study is a large scale serological investigation on CLD in patients with liver disease. This study first time revealed a relation between Wilson's disease and CLD, and overexpression of tTG in liver tissue.

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Clinicopathological characteristics of human epidermal growth factor receptor 2-positive Barrett's adenocarcinoma

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Abstract

AIM: To compare the clinicopathological characteristics of human epidermal growth factor receptor 2 (HER2)-positive and HER2-negative Barrett's adenocarcinoma in Japan.

METHODS: We performed immunohistochemical analysis of HER2 in 30 samples taken from patients with Barrett's adenocarcinoma and dual color *in situ* hybridization in cases showing 2+ reactions. We compared the clinicopathological characteristics of HER2-positive and HER2-negative patients.

RESULTS: HER2 positivity was identified in 8 (27%) carcinoma samples. We found that HER2 expression was associated with p53 overexpression (100% vs 52.6% in pT1 tumor; 100% vs 54.5% in all stage tumor, $P < 0.05$) and protruding lesions at the early disease stage. There was no association between the mucin phenotype of the carcinomas and prognosis. HER2 expression and low clinical stage were unexpectedly different between Barrett's adenocarcinoma patients and gastric cancer patients, but the macroscopic features may be associated with earlier diagnosis in these patients.

CONCLUSION: Our results suggest that HER2-positive Barrett's adenocarcinomas are associated with p53 overexpression and lesion protrusion at the early disease stage.

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Key words: Barrett's adenocarcinoma; Human epidermal growth factor receptor 2; p53; Mucin phenotype

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Tanaka T, Fujimura A, Ichimura K, Yanai H, Sato Y, Takata K, Okada H, Kawano S, Tanabe S, Yoshino T. Clinicopathological characteristics of human epidermal growth factor receptor 2-positive Barrett's adenocarcinoma. *World J Gastroenterol* 2012; 18(43): 6263-6268 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i43/6263.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i43.6263>

INTRODUCTION

Recent studies have shown that the incidence of Barrett's

adenocarcinoma has been increasing in Japan. Although human epidermal growth factor receptor 2 (HER2) was reported to be amplified and overexpressed in some Barrett's adenocarcinomas, the relationship between HER2 expression and patient clinicopathological characteristics has not yet been clarified.

The *HER2* gene, a proto-oncogene, is located on chromosome 17q11.2-12 and encodes the transmembrane glycoprotein receptor p185^{HER2} (or HER2), which is targeted by the humanized monoclonal antibody trastuzumab (Herceptin[®])^[1]. HER2 is amplified and overexpressed in approximately 25% of breast cancer patients, and is associated with an aggressive clinical course and poor prognosis^[2]. Recently, HER2 overexpression and amplification was detected in approximately 22% of advanced gastric cancers, and targeting of the extracellular domain of HER2 in these patients was associated with improved clinical benefits compared with chemotherapy alone in a phase III trial^[3]. Several recent studies have reported HER2 status in Barrett's adenocarcinoma; they observed a prevalence of HER2 protein overexpression or gene amplification in Barrett's adenocarcinomas ranging from 11% to 72%^[4-8]. Lack of agreement among these studies may be related to the differing sensitivities of the assay methods used to assess HER2^[9-13].

HER2 has been recognized as an important prognostic factor in breast cancer^[2]. However, the clinicopathological characteristics of HER2-positive Barrett's adenocarcinoma are controversial. In this study, we used the criteria of the trastuzumab for gastric cancer (ToGA) trial to evaluate the HER2 status of Barrett's adenocarcinomas in Japan by clarifying the clinicopathological characteristics of HER2-positive Barrett's esophageal adenocarcinomas and examining their morphological immunohistochemical characteristics.

MATERIALS AND METHODS

Patients included in the study

Samples were collected from 30 patients who visited the Okayama University Hospital between May 1998 and March 2011. Histological sections and immunohistochemical results were reviewed to confirm the diagnosis. The definition of Barrett's esophagus and Barrett's adenocarcinoma is controversial^[14,15]. In this study, the criterion for the clinical diagnosis of Barrett's adenocarcinoma was that the tumor foci were located in Barrett's mucosa, which is also referred to as the columnar-lined esophagus. The histological criterion was carcinoma that presented or was in contact with Barrett's mucosa, defined as columnar-lined mucosa with or without intestinal-type epithelium. Clinical information was obtained from the medical records of patients at Okayama University Hospital. The patients underwent a standardized informed consent procedure.

Immunohistochemistry

An automated immunostainer (Ventana Medical Systems, Tucson, AZ, United States) was used to perform all im-

munohistochemical analyses. The following monoclonal antibodies were used: p53 (DO-7; Dako, Glostrup, Denmark), MUC2 (Ccp58; Novocastra Laboratories, Newcastle upon Tyne, United Kingdom), MUC5AC (CLH2, Novocastra Laboratories, Newcastle upon Tyne, United Kingdom), MUC6 (CLH5; Novocastra Laboratories), and CD10 (56C6; Dako). For the evaluation of p53 staining, only cells with nuclear immunostaining significantly more pronounced than that of the control cells of the normal esophageal mucosa were considered positive. MUC5AC and MUC6 are markers of gastric epithelial cells, and MUC2 and CD10 are typical of the intestinal epithelial cell phenotype^[16]. Barrett's adenocarcinoma, in which more than 10% of the section area consisted of at least 1 gastric or intestinal epithelial cell phenotype, were classified as gastric (G type) or intestinal (I type) phenotypic cancers. Those which showed both gastric and intestinal phenotypes were classified as gastric and intestinal mixed phenotypic (GI type) cancers, whereas those showing neither gastric nor intestinal phenotypic expression were grouped as unclassified (N type).

HER2 testing methods and criteria

HER2 protein expression was assessed in carcinoma cells by immunohistochemistry (IHC) in paraffin-embedded 5- μ m tissue sections according to the manufacturer's instructions (Ventana I-VIEW pathway HER2/neu kit; Ventana Medical Systems). Only cell membrane staining was considered positive. Each case was analyzed by a pathologist blinded to the clinical outcome who used criteria specific to upper gastrointestinal cancer that included 2 parameters: (1) the intensity of complete, basolateral, or lateral membrane staining (0, none; 1, faint; 2, weak to moderate; and 3, strong); and (2) the percentage of cancer cells with a given staining intensity. These parameters were used to determine the IHC score according to the ToGA criteria: high (IHC 3+), strong intensity in 10% or more of the cancer cells; medium (IHC 2+), weak to moderate intensity in 10% or more; low (IHC 1+), faint intensity in 10% or more; absent (IHC 0). Dual *in situ* hybridization (DISH) using a Ventana INFORM HER2 ISH kit (Ventana Medical Systems) was used to assess *HER2* gene amplification in all IHC 2+ cases by preparing the carcinoma cells in 5 μ m tissue sections. Briefly, for each case, a parallel hematoxylin and eosin-stained slide was examined for regions of carcinoma by a pathologist. The complete tissue section was scanned by the pathologist to detect any subpopulation of amplified cells. A total of 20 representative nuclei from the invasive tumor were scored. A specimen with a HER2/centromeric enumeration probe 17 (CEP17) ratio of 2.0 or more in tumor cells was classified as HER2 amplified according to the ToGA guidelines^[17].

Definition of HER2-positive status

A case was considered HER2 positive if it was (1) IHC 3+ or (2) IHC 2+ plus after gene amplification. The remaining cases (i.e., non-amplified IHC 2+ or IHC 0-1+) were considered HER2 negative.

Table 1 Patient and tumor characteristics

Characteristics	Values
Male/female	20/10
Age, yr (mean \pm SD)	71.1 \pm 9.7
Surgical resection/endoscopic resection	14/16
Location: Siewert I / II	12/18
Tumor size, mm (mean \pm SD)	27.1 \pm 19.9
Depth of primary tumor: T1a/T1b/T2/T3/T4	13/13/1/2/1
Macroscopic appearance: 0-I / 0-II a/0-II b/0-II c/3	8/8/1/11/3
Histology: tub1/tub2	21/9
Histology tub1/tub2: mucin phenotype G/GI/I/N	7/12/8/3
p53: positive/negative	20/10

G: Gastric phenotype; GI: Gastrointestinal phenotype; I: Intestinal phenotype; N: Null type.

Statistical analysis

A χ^2 test or Fisher's exact test, depending on the sample size, was used to examine categorical variables to compare the clinical characteristics of the different groups of patients. The *t*-test was used to compare mean values. SPSS Version 14.0 (SPSS, Chicago, IL, United States) was used to analyze the data.

RESULTS

Characteristics of the studied population

The studied population consisted of 20 men and 10 women (male to female ratio = 2:1). Their ages ranged from 42 to 87 years old. Sixteen lesions were identified as protruded or superficial elevated types (type 0-I or type 0-II a), 1 lesion was identified as flat type (type 0-II b), and 11 lesions were identified as superficial depressed type (type 0-II c). Three lesions were identified as Borrmann type 3. In accordance with the World Health Organization's classification standards, 21 patients (70%) had well-differentiated tumors and 9 had moderately differentiated tumors (30%). According to the tumor-node-metastasis classification, 13 patients were pT1a, 13 were pT1b, 1 was pT2, 2 were pT3, and 1 was pT4a. All stage pT3 and pT4 patients died from the disease, but the other patients remained alive without suffering from the disease. Taking into account the combination of expression of 4 markers, the 30 cancers were divided phenotypically into 7 G, 12 GI, 8 I, and 3 N types, independent of the histological classification. Expression of p53 was demonstrated in 20 (67%) of 30 cancers. The available data for each patient are summarized in Table 1.

Expression of HER2

Of the 30 patients diagnosed with Barrett's adenocarcinoma, stained sections in 4 cases (13%) were classified as IHC 3+ (Figure 1A), 6 cases (20%) were IHC 2+ (Figure 1B), 4 cases (13%) were IHC 1+, and 16 cases (53%) were IHC 0 (Figure 1C). Criteria developed for the evaluation of upper gastrointestinal tract carcinoma were used to score the HER2-stained samples. DISH was used to assess *HER2* gene amplification in 6 IHC 2+ cases. A specimen with a *HER2*/CEP17 ratio of 2.0

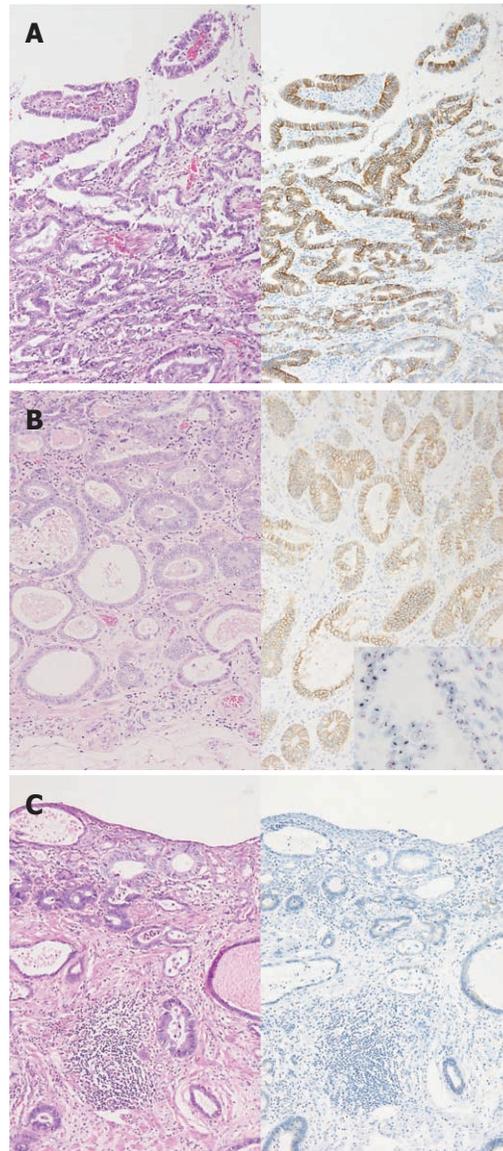


Figure 1 Immunohistochemical pattern of Barrett's adenocarcinoma. A: Immunohistochemistry (IHC) 3+ reaction in a well-differentiated tumor with papillary growth pattern; B: IHC 2+ reaction in a well-differentiated tumor with tubular pattern. Human epidermal growth factor receptor 2 (*HER2*) dual-color *in situ* hybridization amplification *HER2*/centromeric enumeration probe ratio = 3.18; C: IHC 0 reaction in a well-differentiated tumor.

or more in cancer cells was classified as *HER2* amplified (Figure 1B), consistent with the eligibility criteria for the ToGA study. *HER2* amplification was detected in 4 of IHC 2+ cases.

We defined *HER2* positivity as IHC 3+ or IHC 2+ with gene amplification, which were the characteristics of the group that derived the greatest benefit from trastuzumab in the ToGA study. The positive rate of *HER2* was 27% (Table 2).

Association of *HER2* with clinicopathological features

HER2-positive adenocarcinomas were present in 4 men and 4 women. The patients ranged in age from 52 to 80 years. Six cases were the protruded or superficially elevated types (0-I or 0-II a). Three tumors were located in

Table 2 Comparison of pT1 tumor and all stage tumor characteristics

	pT1 tumor		All stage tumor	
	HER2 +	HER2-	HER2+ (27%)	HER2- (73%)
Male/female	3/4	13/6	4/4	16/6
Age, yr (mean ± SD)	70.6 ± 9.9	71.4 ± 10.8	70.3 ± 9.2	71.4 ± 10.0
Location: Siewert I / II	3/4	11/18	3/5	9/13
Tumor size, mm (mean ± SD)	20.6 ± 17.7	23.9 ± 16.6	23.6 ± 18.5	28.3 ± 20.7
Depth of primary tumor: T1a/T1b	3/4	10/9	3/4/0/0/1	10/9/1/2/0
Macroscopic appearance: 0- I /0- II a/0- II b/0- II c	3/3/0/1	4/4/1/10	3/3/0/1/1	4/5/1/10/2
Histology: tub1/tub2	7/1	14/5	7/1	14/8
Mucin phenotype: G/GI/I/N	4/2/1/0	3/9/6/1	4/2/2/0	3/10/6/3
p53: positive/negative	7/0	10/9	8/0	12/10

The human epidermal growth factor receptor 2 (HER2)-positive cases were significantly associated with protruding lesions compared with the HER2-negative cases ($P < 0.05$), and the p53-positivity rate was more common in the HER2-positive tumors ($P < 0.05$). G: Gastric phenotype; GI: Gastrointestinal phenotype; I: Intestinal phenotype; N: Null type.

the Siewert I region and 5 in the Siewert II region. The mean tumor size was 23.6 mm (11-60 mm). Of the pT1 cases, HER2-positive cases were significantly more associated with protruding lesions compared with HER2-negative cases ($P < 0.05$) (Table 2). Seven cases had well-differentiated tumors, and 1 case had a moderately-differentiated tumor. In the mucin phenotypical analysis, 4 cases were G type, 2 cases GI type, and 2 cases were I type; there were no significant differences in mucin phenotypes between the HER2-positive and HER2-negative cases. The p53 positivity rate was higher in the HER2-positive tumors than in the HER2-negative tumors ($P < 0.05$) (Table 2). There were no prognostic differences between the HER2-positive and HER2-negative cases.

DISCUSSION

In this study, we confirmed that 27% Barrett's adenocarcinomas in Japanese patients were HER2 positive. We also found that early-stage HER2-positive Barrett's adenocarcinomas were significantly associated with protruding lesions and had a high rate of p53 positivity.

Previous studies that have examined HER2 status in Barrett's adenocarcinoma observed a prevalence of HER2 protein overexpression or gene amplification ranging from 11% to 72%^[4-8]. Except for 1 study, the lack of agreement among these studies may have been related to the differing sensitivities of the assay methods used to assess HER2. Various antibodies and probes for *in situ* hybridization were used, and the test conditions also were not standardized. In this study, the HER2 tests were performed according to the criteria of the ToGA trial, which is the standard test for HER2 status of gastric cancer. Brien *et al.*^[18] used fluorescence *in situ* hybridization to evaluate HER2 amplification and reported a significant association between amplification and poorer survival. However, in the present study, a low threshold of 4 or more signals per nucleus was used to determine HER2 amplification. On the other hand, according to the ToGA criteria, HER2-positive esophageal adenocarcinomas with Barrett's esophagus had favorable prog-

noses^[19]. The influence and criteria of HER2 expression may still be controversial. In this study, there were no prognostic differences between the HER2-positive and HER2-negative cases. Except for the pT3 and pT4 patients, most patients were early stage and remained alive without disease. Thus, the prognostic significance of HER2 expression was not clarified in this study.

Barrett's adenocarcinoma is thought to develop as a result of gastroesophageal reflux that initiates a metaplastic change in the lower esophageal epithelium. Barrett's esophagus is significant because the condition has a risk for neoplastic transformation through a metaplasia-dysplasia-carcinoma sequence. In Western populations, there has been an increase in the incidence of adenocarcinoma of the esophagus and esophagogastric junction region. In Japan, esophageal Barrett's adenocarcinomas are less common than in Western countries. However, in recent years there has been a gradual increase in the detection of both Barrett's esophagus and Barrett's adenocarcinoma^[12]. Traditionally, Barrett's adenocarcinoma has been believed to be preceded by the development of dysplasia with intestinal characteristics. Recently, Brown *et al.*^[13] and Park *et al.*^[20] validated the existence of 2 main types of dysplasia (i.e., foveolar and adenomatous) which were significantly associated with gastric and intestinal immunophenotypic markers. Khor *et al.*^[21] suggested that non-intestinal columnar metaplasia may be an unstable intermediate state at risk for neoplastic progression. In this study, we evaluated the mucin phenotype of Barrett's adenocarcinoma and found that more than half of the cases were grouped as gastric and mixed phenotypes. This result suggests the presence of a gastric pathway of carcinogenesis in Barrett's esophagus. Heterogeneity of HER2 status was seen in approximately 80% of samples with moderate or strong HER2 IHC reactivity, which was higher than that observed in breast cancer. There were only 2 diffusely strong positive cases. In our study, heterogeneity of HER2 overexpression and gene amplification appeared to represent clusters, and the intensity of IHC staining within clusters was relatively uniform. The IHC staining patterns and gene amplification ap-

peared correlated.

Seven (23%) of the 30 studied cases were the pure gastric mucin phenotype, and 12 cases were the mixed phenotype. In these cases, the background Barrett's mucosa also showed the foveolar type with or without specialized columnar epithelium (intestinal type mucosa). Carcinogenesis of complete gastric type adenocarcinomas is derived from foveolar type dysplasia with aneuploidy^[22], and intestinal type carcinomas were regarded as progressing through the metaplasia-dysplasia-carcinoma sequence, with p53 alteration^[23]. In this study, HER2-positive carcinomas showed both gastric and intestinal phenotypes. It is believed that p53 has an important role in intestinal-type Barrett's adenocarcinoma; however, p53 overexpression was observed in all HER2-positive cases regardless of mucin phenotype. These results suggest a third pathway involving abnormalities of both HER2 and p53. Gastric adenocarcinoma is also significantly correlated with HER2 positivity^[24-26]. These data suggest a possible role of p53 abnormality in the development of HER2-positive adenocarcinoma of the upper gastrointestinal tract.

The macroscopic appearance of HER2-positive Barrett's adenocarcinomas was different from that of the HER2-negative cases in early-stage disease (pT1). The frequency of protruding lesions was significantly higher in the HER2-positive cases than in HER2-negative cases. Ten cases of HER2-negative adenocarcinomas were the protruded type, 9 cases were mixed or intestinal mucin phenotypes, and only 1 case was the gastric phenotype. Thus, protruded-type lesions of the complete gastric phenotype can indicate HER2-positive status.

COMMENTS

Background

In Japan, the incidence of esophageal adenocarcinoma has been increasing, but is still very low compared with squamous cell carcinoma. The incidence of Barrett's adenocarcinoma is much higher in Western countries than in Japan. The relationship between human epidermal growth factor receptor 2 (HER2) expression and patient clinicopathological characteristics has not yet been clarified.

Research frontiers

HER2 was reported to be amplified and overexpressed in some Barrett's adenocarcinomas. The reported expression rates have varied widely due to various different methods and criteria being applied to determine HER2 expression. A practical method for determining HER2 expression has been established on the basis of the results of the trastuzumab for gastric cancer trial.

Innovations and breakthroughs

This was a retrospective study that assessed the incidence of HER2 positivity according to newly-established methods and criteria, and investigated the clinicopathological characteristics according to the new diagnostic criteria.

Applications

Since the sample size of the study was not sufficiently large, the evidence may not be robust. Even so, the authors believe that this study provides valuable data from the evaluation of HER2-positive Barrett's adenocarcinoma.

Terminology

HER2 is an important member of the epidermal growth factor receptor family that has been shown to act as an oncogene in many types of cancers.

Peer review

This is a good descriptive study in which authors analyzed the clinicopathological characteristics of HER-2 positive Barrett's adenocarcinoma. The results

suggest that HER2-positive Barrett's adenocarcinomas are associated with p53 overexpression and lesion protrusion at the early disease stage.

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Gd-EOB-DTPA-enhanced magnetic resonance imaging features of hepatic hemangioma compared with enhanced computed tomography

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Abstract

AIM: To clarify features of hepatic hemangiomas on gadolinium-ethoxybenzyl-diethylenetriaminopentaacetic acid (Gd-EOB-DTPA)-enhanced magnetic resonance imaging (MRI) compared with enhanced computed tomography (CT).

METHODS: Twenty-six patients with 61 hepatic hemangiomas who underwent both Gd-EOB-DTPA-enhanced MRI and enhanced CT were retrospectively reviewed. Hemangioma appearances (presence of peripheral nodular enhancement, central nodular enhancement, diffuse homogenous enhancement, and arteriportal shunt during the arterial phase, fill-in enhancement during the portal venous phase, and prolonged enhancement during the equilibrium phase) on Gd-EOB-DTPA-enhanced MRI and enhanced CT were evaluated.

The degree of contrast enhancement at the enhancing portion within the hemangioma was visually assessed using a five-point scale during each phase. For quantitative analysis, the tumor-muscle signal intensity ratio (SIR), the liver-muscle SIR, and the attenuation value of the tumor and liver parenchyma were calculated. The McNemar test and the Wilcoxon's signed rank test were used to assess the significance of differences in the appearances of hemangiomas and in the visual grade of tumor contrast enhancement between Gd-EOB-DTPA-enhanced MRI and enhanced CT.

RESULTS: There was no significant difference between Gd-EOB-DTPA-enhanced MRI and enhanced CT in the presence of peripheral nodular enhancement (85% vs 82%), central nodular enhancement (3% vs 3%), diffuse enhancement (11% vs 16%), or arteriportal shunt (23% vs 34%) during arterial phase, or fill-in enhancement (79% vs 80%) during portal venous phase. Prolonged enhancement during equilibrium phase was observed less frequently on Gd-EOB-DTPA-enhanced MRI than on enhanced CT (52% vs 100%, $P < 0.001$). On visual inspection, there was significantly less contrast enhancement of the enhancing portion on Gd-EOB-DTPA-enhanced MRI than on enhanced CT during the arterial (3.94 ± 0.98 vs 4.57 ± 0.64 , respectively, $P < 0.001$), portal venous (3.72 ± 0.82 vs 4.36 ± 0.53 , respectively, $P < 0.001$), and equilibrium phases (2.01 ± 0.95 vs 4.04 ± 0.51 , respectively, $P < 0.001$). In the quantitative analysis, the tumor-muscle SIR and the liver-muscle SIR observed with Gd-EOB-DTPA-enhanced MRI were 0.80 ± 0.24 and 1.28 ± 0.33 precontrast, 1.92 ± 0.58 and 1.57 ± 0.55 during the arterial phase, 1.87 ± 0.44 and 1.73 ± 0.39 during the portal venous phase, 1.63 ± 0.41 and 1.78 ± 0.39 during the equilibrium phase, and 1.10 ± 0.43 and 1.92 ± 0.50 during the hepatobiliary phase, respectively. The attenuation values in the tumor and liver parenchyma observed

with enhanced CT were 40.60 ± 8.78 and 53.78 ± 7.37 precontrast, 172.66 ± 73.89 and 92.76 ± 17.92 during the arterial phase, 152.76 ± 35.73 and 120.12 ± 18.02 during the portal venous phase, and 108.74 ± 18.70 and 89.04 ± 7.25 during the equilibrium phase, respectively. Hemangiomas demonstrated peak enhancement during the arterial phase, and both the SIR with Gd-EOB-DTPA-enhanced MRI and the attenuation value with enhanced CT decreased with time. The SIR of hemangiomas was lower than that of liver parenchyma during the equilibrium and hepatobiliary phases on Gd-EOB-DTPA-enhanced MRI. However, the attenuation of hemangiomas after contrast injection was higher than that of liver parenchyma during all phases of enhanced CT.

CONCLUSION: Prolonged enhancement during the equilibrium phase was observed less frequently on Gd-EOB-DTPA-enhanced MRI than enhanced CT, which may exacerbate differentiating between hemangiomas and malignant tumors.

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Key words: Liver; Hemangioma; Magnetic resonance imaging; Gadolinium-ethoxybenzyl-diethylenetriaminopentaacetic acid; Multidetector-row computed tomography

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INTRODUCTION

Hepatic hemangioma is the most common benign hepatic neoplasm; it occurs with a reported incidence of 0.4%-7.3% in an autopsy series^[1]. Differentiation from malignant liver tumors is very important because hemangiomas usually do not require treatment^[2]. Enhanced computed tomography (CT) and gadolinium-diethylenetriaminepentaacetic acid (Gd-DTPA)-enhanced magnetic resonance imaging (MRI) are useful modalities for the diagnosis of hepatic hemangiomas and their differentiation from malignant liver tumors^[3-7].

Gadolinium-ethoxybenzyl-diethylenetriaminopentaacetic acid (Gd-EOB-DTPA) is a new hepatobiliary contrast agent that improves the detection and characterization of focal liver lesions in clinical studies^[8-12]. This contrast agent exhibits high T1 relaxivity in the liver and shows enhancement on both early perfusion phase

images and delayed hepatobiliary phase images. Immediately after injection, Gd-EOB-DTPA is distributed into the extracellular fluid space and yields additional diagnostic information for the characterization of liver lesions, similar to performing MRI with extracellular contrast agents, such as Gd-DTPA^[13]. Hepatocellular uptake of Gd-EOB-DTPA starts approximately 90 s after injection, and Gd-EOB-DTPA is excreted equally *via* the biliary route and renal clearance^[11,12]. The enhanced liver signal intensity plateaus approximately 20 min after injection, and delayed hepatobiliary phase images have high diagnostic quality for the detection of focal liver lesions^[11].

Some studies have shown that Gd-EOB-DTPA-enhanced MRI is more useful than enhanced CT and Gd-DTPA-enhanced MRI for the detection and characterization of focal liver lesions^[8,10-12,14]. The dynamic images obtained after injection of Gd-EOB-DTPA are similar in some aspects but not the same as those images obtained using extracellular contrast agents^[11,12,14,15]. Recently, the differences in the appearances of Gd-EOB-DTPA-enhanced MRI between hemangiomas and metastases have been reported^[16,17]. To our knowledge, however, published reports regarding the findings of hemangiomas observed with Gd-EOB-DTPA-enhanced MRI have been limited^[9,16-19]. Moreover, the appearance and the degree of contrast enhancement of hepatic hemangiomas observed with Gd-EOB-DTPA-enhanced MRI compared with enhanced CT have not been elucidated. Therefore, the purpose of the present study was to clarify the features of hepatic hemangiomas observed with Gd-EOB-DTPA-enhanced MRI compared with enhanced CT.

MATERIALS AND METHODS

Patients

Institutional ethics review board approval was obtained, and informed consent was waived for this retrospective study. From February 2008 to February 2010, 432 consecutive patients underwent Gd-EOB-DTPA-enhanced MRI for the evaluation of suspected liver tumors. Among these patients, 27 were included in this study because they were suspected of having hepatic hemangiomas on three-phase enhanced CT with at least three of the following four well-documented CT characteristic findings of hepatic hemangiomas^[4-7]: relative hypoattenuation compared to normal liver on precontrast images, peripheral nodular enhancement during the arterial phase, fill-in enhancement (progressive opacification from the periphery to the center) during the portal venous phase, and prolonged enhancement (showing iso- or hyperattenuation relative to the liver) during the equilibrium phase. One patient was excluded from the study because the hemangioma did not have the typical appearance of bright signal intensity on T2-weighted MRI^[20]. Therefore, the final study group comprised 26 patients (8 men, 18 women; age range: 31-77 years;

mean age: 53.5 years) with 61 hepatic hemangiomas (size: mean, 26 mm; range 5–120 mm). Four hemangiomas in two patients were diagnosed by histological examination following surgical resections because of the risk of spontaneous rupture.

MRI technique

MRI was performed with a 3-T system (Magnetom Trio; Siemens AG, Erlangen, Germany) with a maximum gradient amplitude of 45 mT/m and a slew rate of 200 T/m·s⁻¹. The coil had four linear elements in a left-to-right direction for both the anterior and posterior components. The standard sequences performed prior to Gd-EOB-DTPA administration included T1-weighted gradient-echo, T2-weighted turbo spin-echo, and respiratory-triggered with navigator-echo technique fat-suppressed T2-weighted turbo spin-echo. Dynamic images using a three-dimensional (3D) fat-suppressed T1-weighted gradient-echo volumetric interpolated breath-hold examination (VIBE) axial series were obtained before and after intravenous contrast injection. The image parameters were as follows: repetition time/echo time, 3.06/1.12; flip angle, 10°; slice thickness, 2 mm; field of view, 350 mm × 280 mm; matrix, 256 × 224; acceleration factor, 2; number of partitions, 80; and acquisition time, 20 s. Before dynamic MRI, a test dose of 1 mL of Gd-EOB-DTPA was injected at a rate of 1 mL/s through a cubital intravenous line, and the bolus was flushed with 40 mL saline using a power injector. During the test injection, the image at the level of the celiac axis in which the aorta was enhanced the most was chosen, and its acquisition time was adopted as the peak aortic enhancement time. For dynamic MRI, 0.025 mmol/kg body weight of Gd-EOB-DTPA was intravenously administered at a flow rate of 1 mL/s, followed by a 40-mL saline solution flush. Breath-hold 3D fat-suppressed T1-weighted VIBE dynamic MRI was repeated at 10 s (arterial phase), 50 s (portal venous phase), 160 s (equilibrium phase), and 20 min (hepatobiliary phase) after the peak aortic enhancement time, which was determined by the test injection. In this study, only precontrast, arterial, portal venous, equilibrium, and hepatobiliary phase T1-weighted VIBE images were evaluated.

CT technique

Three-phase enhanced CT was performed with a 16-slice multidetector-row CT scanner (Aquilion, Toshiba Medical Systems, Tokyo, Japan). All scans were conducted from the top to the bottom of the liver with a tube voltage of 120 kVp and gantry rotation speed of 0.5 s. Unenhanced images were acquired using the following parameters: tube current, 350 mA; detector row configuration, 16 mm × 2 mm; and table increment, 30 mm/rotation. The imaging parameters for three-phase enhanced images were as follows: tube current, 440 mA; detector row configuration, 16 mm × 1 mm; and table increment, 15 mm/rotation in the cephalocaudal direction. In all patients, 2 mL/kg body weight of nonionic contrast ma-

terial with an iodine concentration of 300 mg I/mL was injected over a fixed duration of 30 s, followed by 20 mL of saline was injected at the same rate through a 20-gauge plastic intravenous catheter in an upper extremity vein. Automatic bolus tracking was employed using Sure Start software (Toshiba, Tokyo, Japan) to determine individual scan delays from the injection of the contrast material to commencement of the first pass. For bolus tracking, a series of nonhelical sequential images were obtained 8 s after contrast material administration. These images were acquired with a gantry rotation speed of 0.5 s and a low-dose radiation technique (120 kVp, 50 mA). A circular region of interest with an area of 50 pixels was placed in the aorta at the level of the celiac axis. The arterial phase scan was initiated automatically 20 s after the bolus-tracking program detected the threshold enhancement of 50 Hounsfield units in the aorta. The portal venous and equilibrium phases were obtained 70 s and 300 s, respectively, after the beginning of contrast material injection.

Image analysis

Gd-EOB-DTPA-enhanced MRI and enhanced CT images were qualitatively and quantitatively assessed. Three radiologists (Takumi K, Shindo T and Kumagae Y, with 10, 10 and 9 years of experience, respectively) with knowledge of the diagnosis of hepatic hemangioma assessed the following MRI and CT features in random order on two occasions with an interval of ≥ 2 wk: presence of peripheral nodular enhancement, central nodular enhancement, diffuse homogenous enhancement, and arteriportal shunt during the arterial phase, fill-in enhancement during the portal venous phase, and prolonged enhancement during the equilibrium phase. In the case of discrepancies between the three readers, the discrepancies were discussed during an additional reading session until a consensus was reached.

To assess the visual grades of tumor contrast enhancement relative to the surrounding liver parenchyma, the above three radiologists independently determined the degree of contrast enhancement in the enhancing portion of the hemangioma during each phase using the following five-grade scale: grade 5, a prominently greater enhancement than in the liver parenchyma; grade 4, a mildly greater enhancement than in the liver parenchyma; grade 3, an equal enhancement to the liver parenchyma; grade 2, a slightly decreased enhancement compared with the liver parenchyma; and grade 1, a prominently decreased enhancement compared with the liver parenchyma (Figure 1).

For quantitative analysis, one radiologist (Tateyama A, with 8 years of experience) who did not attend each reading session to minimize the bias of the measurements measured signal intensities of the enhancing portion of the hemangioma, liver parenchyma, and paravertebral muscle on Gd-EOB-DTPA-enhanced MRI and attenuation of the enhancing portions of the hemangioma and liver parenchyma on enhanced CT using operator-defined circular region of interests (ROIs). In

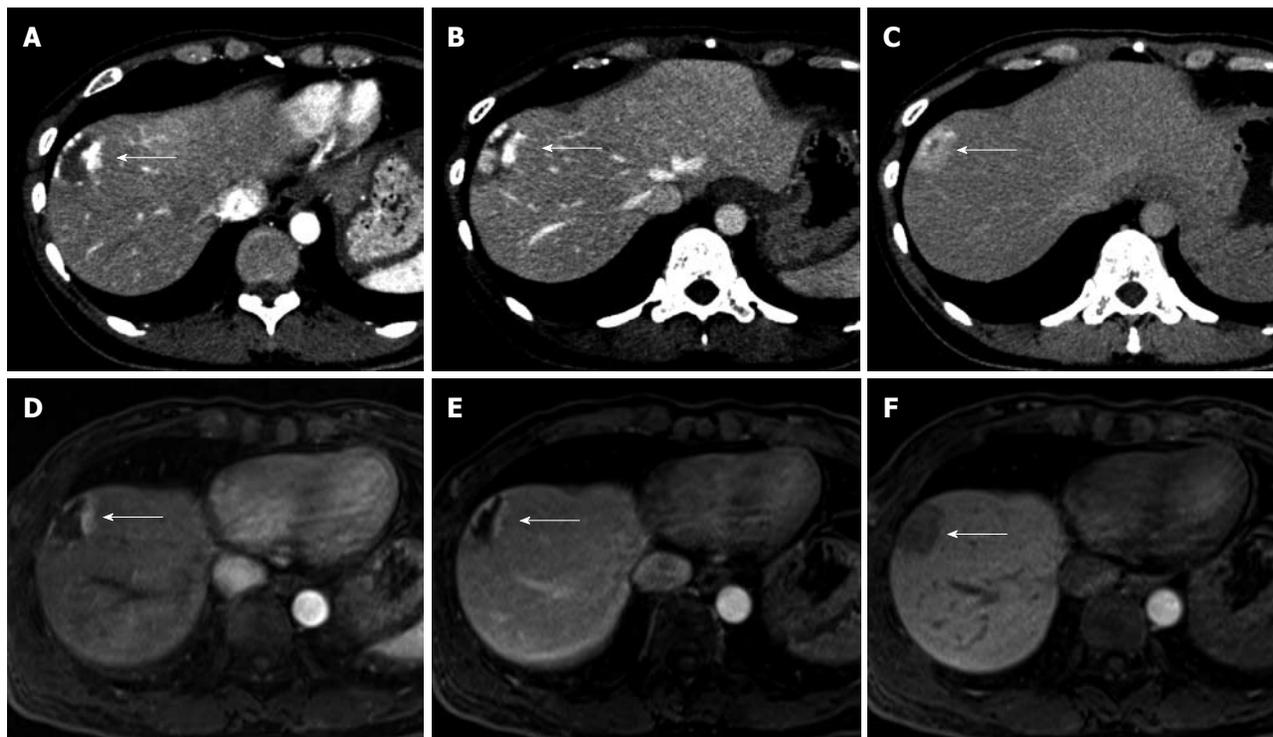


Figure 1 A 49-year-old man with hepatic hemangioma. The arterial, portal venous, and equilibrium phases on enhanced computed tomography and gadolinium-ethoxybenzyl-diethylenetriaminopentaacetic acid-enhanced magnetic resonance imaging. A: The hemangioma (arrow) shows peripheral nodular enhancement, and the enhancement degree was classified as visual grade 5 (prominently greater enhancement than in the liver parenchyma); B: The hemangioma (arrow) shows fill-in enhancement, and the enhancement degree was classified as visual grade 5 (prominently greater enhancement than in the liver parenchyma); C: The hemangioma (arrow) shows prolonged enhancement, and the enhancement degree was classified as visual grade 4 (mildly greater enhancement than in the liver parenchyma); D: The hemangioma (arrow) shows peripheral nodular enhancement, and the enhancement degree was classified as visual grade 4 (mildly greater enhancement than in the liver parenchyma); E: The hemangioma (arrow) shows fill-in enhancement, and the enhancement degree was classified as visual grade 4 (mildly greater enhancement than in the liver parenchyma); F: The hemangioma (arrow) did not show prolonged enhancement, and the enhancement degree was classified as visual grade 1 (prominently decreased enhancement compared with the liver parenchyma).

the enhancing portion of the hemangioma during each phase, the largest possible ROIs were selected to measure signal intensity and attenuation. The ROIs of the liver parenchyma at the level of the hilum of the liver were 100–200 mm² in size and were drawn in the lateral, anterior, and posterior segments, avoiding blood vessels and artifacts; the intensity and attenuation measurements were averaged. The ROIs of paravertebral muscle were 100 mm² in size. The tumor-muscle and liver-muscle signal intensity ratios (SIRs) were calculated respectively by dividing the signal intensity of the enhancing portion of the hemangioma and the liver by the signal intensity of the paravertebral muscle. The tumor-liver contrast (TLC) on Gd-EOB-DTPA-enhanced MRI was calculated with the following equation: $TLC = (\text{signal intensity of the enhancing portion} - \text{signal intensity of the liver parenchyma}) / \text{signal intensity of the paravertebral muscle}$. The TLC on enhanced CT was calculated as the difference in attenuation between the enhancing portion of the hemangioma and the liver parenchyma.

Statistical analysis

Statistical analyses were performed using SPSS 14.0 software for Windows (SPSS Version 14.0, Chicago, IL). The McNemar test was used to assess the significance

of differences in the appearances of hemangiomas between Gd-EOB-DTPA-enhanced MRI and enhanced CT. The Wilcoxon's signed rank test was used to assess the significance of differences in the visual grade of tumor contrast enhancement between Gd-EOB-DTPA-enhanced MRI and enhanced CT. To assess interobserver variability in the visual analysis of tumor contrast enhancement, the weighted κ test of concordance was applied to measure the degree of agreement between the three radiologists. Agreement was graded as poor (κ value < 0.20), moderate (≥ 0.20 and < 0.40), fair (≥ 0.40 and < 0.60), good (≥ 0.60 and < 0.80), or very good (≥ 0.80 –1). The relationship between the visual grades of tumor contrast enhancement and TLC was analyzed using the Spearman rank correlation coefficient (R_s). For all statistical analyses, $P < 0.05$ was considered significant.

RESULTS

There were no significant differences between GdEOB-DTPA-enhanced MRI and enhanced CT in the presence of peripheral nodular enhancement (85% *vs* 82%), central nodular enhancement (3% *vs* 3%), diffuse enhancement (11% *vs* 16%), or arteriportal shunt (23% *vs* 34%) during the arterial phase. There was also no difference

Table 1 Appearances and visual grades of tumor contrast enhancement of hemangiomas on gadolinium-ethoxybenzyl-diethylenetriaminopentaacetic acid-enhanced magnetic resonance imaging and enhanced computed tomography

Appearance	Tumors		P value
	Gd-EOB-DTPA-enhanced MRI	Enhanced CT	
Peripheral nodular enhancement	52 (85)	50 (82)	0.754 ¹
Central nodular enhancement	2 (3)	2 (3)	1.000 ¹
Diffuse homogenous enhancement	7 (11)	10 (16)	0.508 ¹
Arterioportal shunt	14 (23)	21 (34)	0.065 ¹
Fill-in enhancement	48 (79)	49 (80)	1.000 ¹
Prolonged enhancement	32 (52)	61 (100)	< 0.001 ¹
Visual grade			
Arterial phase	3.94 ± 0.98	4.57 ± 0.64	< 0.001 ²
Portal venous phase	3.72 ± 0.82	4.36 ± 0.53	< 0.001 ²
Equilibrium phase	2.01 ± 0.95	4.04 ± 0.51	< 0.001 ²

Data are presented as mean ± SD or *n* (%). ¹*P* value between gadolinium-ethoxybenzyl-diethylenetriaminopentaacetic acid (Gd-EOB-DTPA)-enhanced magnetic resonance imaging (MRI) and enhanced computed tomography (CT) by a McNemar test; ²*P* value between Gd-EOB-DTPA-enhanced MRI and enhanced CT by a Wilcoxon's signed rank test.

in fill-in enhancement (79% *vs* 80%, respectively) during the portal venous phase. Prolonged enhancement during the equilibrium phase was observed significantly less frequently on Gd-EOB-DTPA-enhanced MRI than on enhanced CT (52% *vs* 100%, respectively, *P* < 0.001) (Table 1).

The visual grade of tumor contrast enhancement was significantly less on Gd-EOB-DTPA-enhanced MRI than enhanced CT during the arterial phase (3.94 ± 0.98 *vs* 4.57 ± 0.64, respectively, *P* < 0.001), the portal venous phase (3.72 ± 0.82 *vs* 4.36 ± 0.53, respectively, *P* < 0.001), and the equilibrium phase (2.01 ± 0.95 *vs* 4.04 ± 0.51, respectively, *P* < 0.001) (Table 1). There was good interobserver agreement for the visual grade of tumor contrast enhancement, with weighted κ values ranging from 0.59 to 0.92.

In the quantitative analysis, the tumor-muscle SIR and the liver muscle SIR observed with Gd-EOB-DTPA-enhanced MRI were, respectively, 0.80 ± 0.24 and 1.28 ± 0.33 precontrast, 1.92 ± 0.58 and 1.57 ± 0.55 during the arterial phase, 1.87 ± 0.44 and 1.73 ± 0.39 during the portal venous phase, 1.63 ± 0.41 and 1.78 ± 0.39 during the equilibrium phase, and 1.10 ± 0.43 and 1.92 ± 0.50 during the hepatobiliary phase (Figure 2A). The attenuation values in the tumor and liver parenchyma observed with enhanced CT were, respectively, 40.60 ± 8.78 and 53.78 ± 7.37 precontrast, 172.66 ± 73.89 and 92.76 ± 17.92 during the arterial phase, 152.76 ± 35.73 and 120.12 ± 18.02 during the portal venous phase, and 108.74 ± 18.70 and 89.04 ± 7.25 during the equilibrium phase (Figure 2B). Hemangiomas demonstrated peak enhancement during the arterial phase, and both the SIR observed with Gd-EOB-DTPA-enhanced MRI and the attenuation value observed with enhanced CT decreased with time. The SIR of hemangiomas was lower than

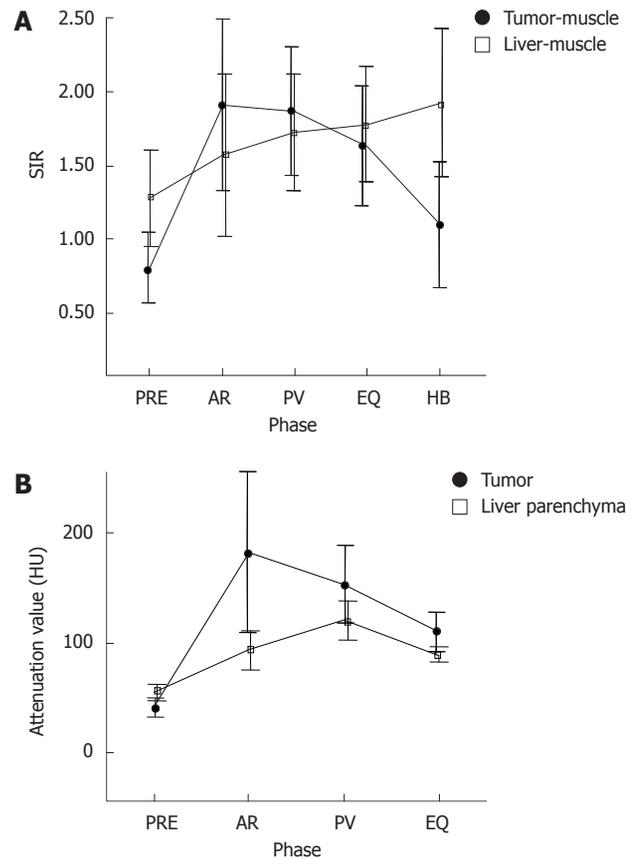


Figure 2 Temporal profiles of the signal intensity ratios on gadolinium-ethoxybenzyl-diethylenetriaminopentaacetic acid-enhanced magnetic resonance imaging and the attenuation values on enhanced computed tomography. A: Tumor-muscle and liver-muscle signal intensity ratios (SIRs) before and after gadolinium-ethoxybenzyl-diethylenetriaminopentaacetic acid (Gd-EOB-DTPA) injection; B: The attenuation values in the tumor and liver parenchyma before and after nonionic contrast material injection. Tumor-muscle SIR = signal intensity of the hemangiomas/signal intensity of the paravertebral muscle; Liver-muscle SIR = signal intensity of the liver parenchyma/signal intensity of the paravertebral muscle. PRE: Precontrast scan; AR: Arterial phase; PV: Portal venous phase; EQ: Equilibrium phase; HB: Hepatobiliary phase; HU: Hounsfield unit.

the SIR of liver parenchyma during the equilibrium and hepatobiliary phases observed with Gd-EOB-DTPA-enhanced MRI (Figure 2). The TLC observed with Gd-EOB-DTPA-enhanced MRI were -0.48 ± 0.20 precontrast, 0.35 ± 0.53 during the arterial phase, 0.15 ± 0.43 during the portal venous phase, and -0.15 ± 0.28 during the equilibrium phase. In comparison, the TLC observed with enhanced CT were -13.18 ± 7.79 precontrast, 79.9 ± 35.9 during the arterial phase, 32.6 ± 17.4 during the portal venous phase, and 19.7 ± 13.5 during the equilibrium phase. The visual assessment of tumor contrast enhancement correlated well with the TLC in the quantitative analysis of both Gd-EOB-DTPA-enhanced MRI and enhanced CT, with *R_s* values ranging from 0.45 to 0.71 (all *P* < 0.001).

DISCUSSION

Enhanced CT and Gd-DTPA-enhanced MRI findings of hepatic hemangiomas have been well documented in

many reports, and the dynamic characteristics observed using both modalities are similar^[3-7]. The typical findings of hepatic hemangioma on enhanced CT are hypoattenuation similar to the attenuation of vessels on pre-contrast images, peripheral nodular enhancement during the arterial phase, fill-in enhancement during the portal venous phase, and prolonged enhancement during the equilibrium phase^[4-7].

In previous reports, peripheral nodular enhancement during the arterial phase was identified in 55%-87% of hemangiomas^[4,5,7,21], and fill-in enhancement during the portal venous phase was observed in 59-96% of hemangiomas observed with enhanced CT^[4,5,7,22,23]. In the present study, peripheral nodular enhancement during the arterial phase and fill-in enhancement during the portal venous phase on enhanced CT were observed in 50 (82%) and 49 (80%) of 61 hemangiomas, respectively. Goshima *et al*^[6] reported that peripheral nodular enhancement with fill-in enhancement was observed in 28% who underwent Gd-EOB-DTPA-enhanced MRI. To our knowledge, no reports have compared the Gd-EOB-DTPA-enhanced MRI findings of hemangiomas with enhanced CT. In the present study, peripheral nodular enhancement was identified in 52 (85%) and fill-in enhancement was shown in 48 (79%) of 61 hemangiomas visualized with Gd-EOB-DTPA-enhanced MRI. No significant differences were observed in the presence of peripheral nodular and fill-in enhancement between Gd-EOB-DTPA-enhanced MRI and enhanced CT. In the quantitative analysis, enhanced portions of the hemangiomas demonstrated peak contrast enhancement during the arterial phase, and the contrast enhancement decreased with time on both Gd-EOB-DTPA-enhanced MRI and enhanced CT. However, tumor contrast enhancement during the arterial and portal venous phases was significantly reduced on Gd-EOB-DTPA-enhanced MRI compared with enhanced CT by both visual assessment and quantitative analysis. This result may be influenced by the lower gadolinium dose used with Gd-EOB-DTPA^[12,14].

Hemangiomas showing diffuse homogenous enhancement during the arterial phase can mimic hypervascular malignant tumors such as hepatocellular carcinomas or hypervascular metastases^[21,22,24,25]. In previous reports, diffuse homogenous enhancement during the arterial phase was observed in 8%-35% of hemangiomas on enhanced CT^[22,24] and 34% on Gd-EOB-DTPA-enhanced MRI^[16]. In the present study, diffuse homogenous enhancement during the arterial phase was observed in 10 (16%) and 7 (11%) of 61 hemangiomas on enhanced CT and Gd-EOB-DTPA-enhanced MRI, respectively. There were no significant differences between Gd-EOB-DTPA-enhanced MRI and enhanced CT in this respect.

Prolonged enhancement during the equilibrium phase is present in approximately 59%-96% of hemangiomas observed with enhanced CT^[22-24] and 37%-72% of hemangiomas observed with Gd-EOB-DTPA-enhanced

MRI^[16,17]. This difference in the presence of prolonged enhancement on Gd-EOB-DTPA-enhanced MRI may be explained by differences in case selection. In addition, on enhanced CT, once the regions in the hemangioma enhance during the arterial phase, they also show prolonged enhancement^[25]. In the present study, prolonged enhancement was observed on enhanced CT in all 61 hemangiomas, whereas Gd-EOB-DTPA-enhanced MRI showed prolonged enhancement only in 52% of the hemangiomas. The contrast enhancement was significantly reduced when using Gd-EOB-DTPA-enhanced MRI instead of enhanced CT. Ringe *et al*^[18] reported that hemangiomas appear iso- or hypointense compared to the liver parenchyma during the equilibrium and hepatobiliary phases on Gd-EOB-DTPA-enhanced MRI. They suggest that this reduction is because of Gd-EOB-DTPA uptake in the surrounding normal liver parenchyma, the lower gadolinium dose, and the shorter plasma half-life of Gd-EOB-DTPA. Hemangiomas showing diffuse homogenous enhancement during the arterial phase can be differentiated from hypervascular malignant tumors on enhanced CT and Gd-DTPA-enhanced MRI because hemangiomas show isoattenuation or hyperattenuation relative to the liver parenchyma during the equilibrium phase^[3,22,25]. In the present study, however, none of the hemangiomas with diffuse homogenous enhancement during the arterial phase showed prolonged enhancement during the equilibrium phase of Gd-EOB-DTPA-enhanced MRI. Moreover, 22 (40%) of 54 hemangiomas without diffuse enhancement during the arterial phase did not show prolonged enhancement during the equilibrium phase of Gd-EOB-DTPA-enhanced MRI. Hypovascular metastatic tumors show relatively low signal intensity during the equilibrium phase of Gd-EOB-DTPA-enhanced MRI^[9,11,16]. Therefore, the present results suggest that it may be difficult to differentiate hemangiomas from malignant tumors such as hepatocellular carcinomas and metastatic tumors on Gd-EOB-DTPA-enhanced MRI because approximately half the hemangiomas exhibited relatively low signal intensity during the equilibrium phase. Enhanced MRI with extracellular contrast agents, such as Gd-DTPA, may be more useful for the diagnosis of hepatic hemangiomas. However, Gd-EOB-DTPA-enhanced MRI is widely used for evaluating the presence of hepatocellular carcinoma or metastasis in patients with liver cirrhosis or an extrahepatic malignancy because of its higher detectability compared with Gd-DTPA-enhanced MRI^[11,12]. The differentiation of hemangioma from HCC or metastases on Gd-EOB-DTPA-enhanced MRI is frequently required in a clinical practice.

The present study has several potential limitations. First, pathological proof was obtained only for four hemangiomas and was not obtained for the majority of the lesions. Tissue biopsies were not obtained because hemangiomas are benign lesions and usually do not require invasive procedures. Therefore, case selection depended solely on imaging findings, but the typical

findings on enhanced CT and bright signal intensity on T2-weighted MRI are accepted as diagnostic for hepatic hemangioma. Second, the present study may have a potential selection bias because atypical hemangiomas that did not meet the present selection criteria were not included. Therefore, further investigation will be necessary to elucidate the appearance of atypical hemangiomas on Gd-EOB-DTPA-enhanced MRI. **Third, all the lesions were hemangiomas; other liver tumors were not included in the present study.** Therefore, this selection may bias the interpretation of the Gd-EOB-DTPA-enhanced MRI and enhanced CT findings. However, the purpose of the present study was to compare the features of hepatic hemangiomas on Gd-EOB-DTPA-enhanced with enhanced CT. Fourth, the imaging parameters, injection rate, duration of the contrast material, and scanning timing were not identical for Gd-EOB-DTPA-enhanced MRI and enhanced CT. However, it is impractical to make them uniform because they have been optimized for each imaging modality.

In conclusion, the typical findings of hemangiomas, such as peripheral nodular enhancement, central nodular enhancement, diffuse enhancement, or arterioportal shunt during the arterial phase, or fill-in enhancement during the portal venous phase, are useful for the diagnosis of hemangiomas using Gd-EOB-DTPA-enhanced MRI and enhanced CT. However, prolonged enhancement during the equilibrium phase was observed significantly less frequently on Gd-EOB-DTPA-enhanced MRI. Some hemangiomas show relatively low signal intensity during the equilibrium phase, which may mimic malignant tumors. Knowledge of Gd-EOB-DTPA-enhanced MRI findings is important for arriving at the correct diagnosis of hepatic hemangioma.

COMMENTS

Background

Hepatic hemangioma is the most common benign hepatic neoplasm, and its differentiation from malignant liver tumors is very important. The most common enhanced computed tomography (CT) and gadolinium-diethylenetriaminepentaacetic acid (Gd-DTPA)-enhanced magnetic resonance imaging (MRI) features of hepatic hemangiomas are peripheral nodular enhancement during the arterial phase, fill-in enhancement during the portal venous phase, and prolonged enhancement during the equilibrium phase. Recently, gadolinium-ethoxybenzyl-diethylenetriaminepentaacetic acid (Gd-EOB-DTPA)-enhanced MRI has been used to evaluate liver lesions.

Research frontiers

It is important to clarify the features of hepatic hemangiomas observed with Gd-EOB-DTPA-enhanced MRI. However, limited reports describe the findings of hemangiomas observed with Gd-EOB-DTPA-enhanced MRI. Moreover, the appearance and the degree of contrast enhancement of hepatic hemangiomas observed with Gd-EOB-DTPA-enhanced MRI compared with enhanced CT have not been elucidated.

Innovations and breakthroughs

The tumor-to-liver contrast enhancement during each phase was significantly lower on Gd-EOB-DTPA-enhanced MRI than enhanced CT. Prolonged enhancement during the equilibrium phase, which is an important finding for diagnosing hepatic hemangiomas, was observed less frequently on Gd-EOB-DTPA-enhanced MRI than enhanced CT.

Applications

Some hemangiomas show relatively low signal intensity during the equilibrium

phase and may mimic malignant tumors. Knowledge of Gd-EOB-DTPA-enhanced MRI findings is important for arriving at the correct diagnosis of hepatic hemangioma.

Terminology

Gd-EOB-DTPA is a new hepatobiliary contrast agent that exhibits high T1 relaxivity in the liver and shows enhancement on both early perfusion phase images and delayed hepatobiliary phase images. Gd-EOB-DTPA-enhanced MRI is more useful for the detection and characterization of focal liver lesions than enhanced CT and Gd-DTPA-enhanced MRI.

Peer review

The authors compare the liver-tumor contrasts obtained for hepatic hemangioma with hepatobiliary contrast in MRI with extracellular contrast in CT, which is of clinical interest.

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Durability of viral response after off-treatment in HBeAg positive chronic hepatitis B

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Abstract

AIM: To evaluate the durability in hepatitis B e antigen (HBeAg) positive chronic hepatitis B patients who discontinued antiviral treatment.

METHODS: A total of 48 HBeAg positive chronic hepatitis B patients who were administered nucleoside analogues and maintained virological response for ≥ 6 mo [hepatitis B virus (HBV) DNA < 300 copies/mL and HBeAg seroconversion] before cessation of treatment were enrolled between February 2007 and January 2010. The criteria for the cessation of the antiviral treatment were defined as follows: (1) achievement of virological response; and (2) duration of consolida-

tion therapy (≥ 6 mo). After treatment cessation, the patients were followed up at 3-6 mo intervals. The primary endpoint was serologic and virologic recurrence rates after withdrawal of antiviral treatment. Serologic recurrence was defined as reappearance of HBeAg positivity after HBeAg seroconversion. Virologic recurrence was defined as an increase in HBV-DNA level $> 10^4$ copies/mL after HBeAg seroconversion with previously undetectable HBV-DNA level.

RESULTS: During the median follow-up period of 18.2 mo (range: 5.1-47.5 mo) after cessation of antiviral treatment, the cumulative serological recurrence rate was 15 % at 12 mo. The median duration between the cessation of antiviral treatment and serologic recurrence was 7.2 mo (range: 1.2-10.9 mo). Of the 48 patients with HBeAg positive chronic hepatitis, 20 (41.6%) showed virological recurrence. The cumulative virologic recurrence rates at 12 mo after discontinuing the antiviral agent were 41%. The median duration between off-treatment and virologic recurrence was 7.6 mo (range: 4.3-27.1 mo). The mean age of the virological recurrence group was older than that of the non-recurrence group (46.7 ± 12.1 years vs 38.8 ± 12.7 years, respectively; $P = 0.022$). Age (> 40 years) and the duration of consolidation treatment (≥ 15 mo) were significant predictive factors for off-treatment durability in the multivariate analysis [$P = 0.049$, relative risk (RR) 0.31, 95% CI (0.096-0.998) and $P = 0.005$, RR 11.29, 95% CI (2.054-65.12), respectively]. Patients with age (≤ 40 years) who received consolidation treatment (≥ 15 mo) significantly showed durability in HBeAg positive chronic hepatitis B patients ($P = 0.014$). These results suggest that additional treatment for more than 15 mo after HBeAg seroconversion in patients who are ≤ 40 years old may be beneficial in providing a sustained virological response.

CONCLUSION: Our data suggest that HBeAg seroconversion is an imperfect end point in antiviral treat-

ment. Long-term consolidation treatment (≥ 15 mo) in younger patients is important for producing better prognosis in HBeAg positive chronic hepatitis B.

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Key words: Durability; Seroconversion; Chronic hepatitis B; Hepatitis B e antigen positive; Recurrence; Consolidation

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a major problem in medical health care with approximately 400 million people infected worldwide^[1,2]. Since lamivudine was introduced as an effective antiviral agent for chronic hepatitis B in the late 1990s, many types of nucleos(t)ide analogues (NUCs) have been developed to prevent the progression of cirrhosis and hepatocellular carcinoma^[3-6]. To assess these effects of antiviral therapy, surrogate markers such as hepatitis B e antigen (HBeAg) seroconversion or HBsAg loss have been reported^[7-12].

The current end-points of therapy are durable HBeAg seroconversion in HBeAg-positive patients^[13]. International guidelines on HBV therapy suggest that finite duration of treatment with a NUC is a reasonable option, and recommend that treatment may be stopped after HBeAg seroconversion and an additional 6-12 mo of consolidation therapy in HBeAg-positive hepatitis^[13,14]. However, it may be necessary to maintain long-term treatment for the ideal end-points such as HBsAg clearance or sustained virologic response in HBeAg-positive chronic hepatitis B due to a higher relapse rate^[15,16].

The optimum treatment durations of NUCs also remain ill defined. Several studies on the durability of HBeAg seroconversion induced by lamivudine have produced contradictory results^[16-19]. Reijnders *et al*^[20] have reported that HBeAg seroconversion is an imperfect end point in antiviral treatment. Studies investigating the durability of the response to newer agents such as entecavir and clevudine are still lacking.

The durability of virological response (HBeAg seroconversion and undetectable HBV DNA) is important

for developing a treatment strategy for chronic hepatitis B patients. In this study, we evaluated the off-treatment durability of response and the predictive factors for virologic recurrence in HBeAg positive chronic hepatitis B patients who achieved successful end point of therapy with nucleoside analogues such as entecavir and clevudine. Furthermore, we compared the durability of response between entecavir and clevudine.

MATERIALS AND METHODS

Study population

This study included 48 Korean patients with chronic hepatitis B infection (HBsAg and HBeAg positivity for at least 6 mo) who were hospitalized at the Seoul St. Mary's Hospital, Gangnam Severance Hospital, Inje University Ilsan Paik Hospital and Chung-Ang University Hospital between February 2007 and January 2010. All patients were treated with nucleoside analogue therapy such as clevudine and entecavir. When the therapy was initiated, the serum HBV DNA levels were at least 105 copies/mL and alanine aminotransferase (ALT) or aspartate aminotransferase (AST) levels were more than two times the upper limit of normal (ULN, 40 IU/L). There were no signs of either co-infection with hepatitis C virus or human immunodeficiency virus or hepatic decompensation such as ascites, variceal bleeding or hepatic encephalopathy, and treatment with (pegylated) interferon was discontinued at least 6 mo before the start of the NUC treatment.

The cessation criteria of the NUC treatment were defined as follows: (1) the achievement of a virological response; and (2) duration of consolidation therapy (≥ 6 mo). Virological response was defined as HBeAg seroconversion, HBV DNA < 300 copies/mL and normal ALT levels. The study was approved by the institutional Ethics Review Board and complied with the Declaration of Helsinki.

Follow-up evaluation

All patients were monitored at least every 3-6 mo during the antiviral treatment period. Biochemical (serum AST, ALT) and virological parameters (HBeAg, HBeAb status and quantitative HBV DNA) were assessed at every visit. Nucleoside analogue-treated patients who achieved virological response were evaluated every 3 mo. Consolidation therapy was continued for more than 6 mo after achieving virological response. In cases in which NUC treatment was discontinued, the patients were followed up at 3-6 mo intervals for a median period of 18.2 mo (range: 5.1-47.5 mo).

End points

The primary endpoint was serologic and virologic recurrence rates after withdrawal of antiviral treatment. Serologic recurrence was defined as reappearance of HBeAg positivity after HBeAg seroconversion. Virologic recurrence was defined as an increase in HBV-DNA

Table 1 Baseline characteristics of the study population

Baseline characteristics	Total (<i>n</i> = 48)
Age, yr (mean ± SD)	42.1 ± 12.9
Sex (male/female)	29/19
Pretreatment ALT level (IU/L)	198 (11-374)
Pretreatment HBV DNA level (log ₁₀ copies/mL)	8.5 (5.0-10)
Time to undetectable HBV (mo)	7.7 (1.3-39.4)
Time to HBeAg seroconversion (mo)	13.4 (0.8-39.4)
Consolidation treatment duration (mo)	10.3 (6-37.9)
Total treatment duration (mo)	26.1 (9.4-47.4)
Antiviral agent (entecavir/clevudine)	31/17
Previous NUC treatment (naïve/lamivudine)	41/7

ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; NUC: Nucleos(t)ide analogues.

level > 10⁴ copies/mL after HBeAg seroconversion with previously undetectable HBV-DNA level because a viral load of more than 10 000 copies/mL is associated with progression of liver disease^[4,21].

The secondary endpoint was to evaluate the difference in the durability between entecavir and clevudine.

Laboratory testing

Serum HBeAg and HBeAb were measured with a radioimmunoassay according to the manufacturer's protocol (Abbott Laboratories, Chicago, IL, United States). Serum HBV-DNA levels were measured with real-time PCR using Abbot Realtime HBV Quantification Kit (Abbot Molecular Inc., IL, United States).

Ethics approval

Ethics approval was provided by the institutional Ethics Review Board, The Catholic University of Korea (KC11RIMI0560).

Statistical analysis

The continuous variables are expressed as the mean ± SE or median (range) where appropriate. Durability was calculated from the date of the antiviral treatment cessation to the date of virologic recurrence or censorship. The cumulative rates of virologic recurrence, HBeAg seroreversion were estimated with the Kaplan-Meier method. The Cox's proportional hazard model was adopted to determine the predictive factors for relapse, among various variables including age, pretreatment ALT, AST level, pretreatment HBV DNA levels, consolidation treatment duration and total treatment duration. *P* < 0.05 was considered to be significant (SPSS 17, Chicago, IL).

RESULTS

Clinical characteristics of the patients

The baseline characteristics of all patients are shown in Table 1. A total of 48 patients with chronic hepatitis B who were treated with a nucleoside analogue in multi-center were included in the analysis. Of the 48 patients, 29 (60.4%) were men and the mean age was 42.1 ± 12.9

years. The pretreatment ALT level and serum HBV DNA level were 198 IU/L (11-374 IU/L) and 8.8 log₁₀ copies/mL (range: 5-10 log₁₀ copies/mL), respectively. Overall, 31 of the 48 patients with HBeAg positive hepatitis were treated with entecavir, and 17 were treated with clevudine. A total of 41 (85.4%) patients were nucleoside analog treatment naïve, whereas 7 (14.6%) patients had received prior treatment with lamivudine. All patients who were treated with lamivudine did not have any evidence of lamivudine-resistance at the initiation of entecavir or clevudine treatment. The median duration of therapy was 26.1 mo (range: 9.4-47.4 mo). Patients were followed up for a median of 45.6 mo (range: 23.6-60.2 mo); 82 % (43/48) were followed up for more than 3 years.

Serologic recurrence after HBeAg seroconversion

The median follow-up time after HBeAg seroconversion for 48 patients was 32.1 mo (range: 10.3-55.8 mo). Serologic recurrence occurred in 6 of 48 (12.5%) patients. The cumulative serologic recurrence rates at 6 mo, 12 mo and 18 mo after NUC induced HBeAg seroconversion were 0%, 7% and 15%, respectively. The median duration between the cessation of antiviral treatment and serologic recurrence was 7.2 mo (range: 1.2-10.9 mo). In 1 case, serologic recurrence occurred less than 6 mo after the cessation of NUC treatment, and in 5/6 (83%) cases, serologic recurrence occurred within 12 mo after off-treatment (Figure 1A).

Virologic recurrence after HBeAg seroconversion and undetectable HBV DNA in HBeAg positive hepatitis

Virologic recurrence occurred in 20 of 48 patients (41.6%) after HBeAg seroconversion. The cumulative virologic recurrence rates at 6 mo, 12 mo and 24 mo after discontinuing the antiviral agent were 38%, 41% and 60%, respectively (Figure 1B). Of these 20 patients, 6 reverted to chronic HBeAg-positive chronic hepatitis B, and 14 progressed to HBeAg negative chronic hepatitis B. One of 6 patients with HBeAg reversion (16.6%) showed HBV DNA levels of 10-100 copies/L. Six of 14 patients with HBeAg seronegativity (42.8%) showed HBV DNA levels of more than 100 copies/L (Table 2). The median duration between off-treatment and virologic recurrence was 7.6 mo (range: 4.3-27.1 mo) in HBeAg positive hepatitis. Seventeen cases (85%) of virologic recurrence occurred within 12 mo after off-treatment.

Durable response

In HBeAg positive chronic hepatitis, only 28 (58.3%) had a durable response, as defined by the continued absence of HBeAg and HBV DNA levels less than 10 copies/L, during a median follow-up period of 18.2 mo (range: 5.1-47.5 mo). In all patients, NUC therapy was discontinued after HBeAg seroconversion occurred and at least 6 mo of consolidation therapy. The clinical features and laboratory data including age, sex, pretreatment ALT, HBV DNA titer, duration of consolidation

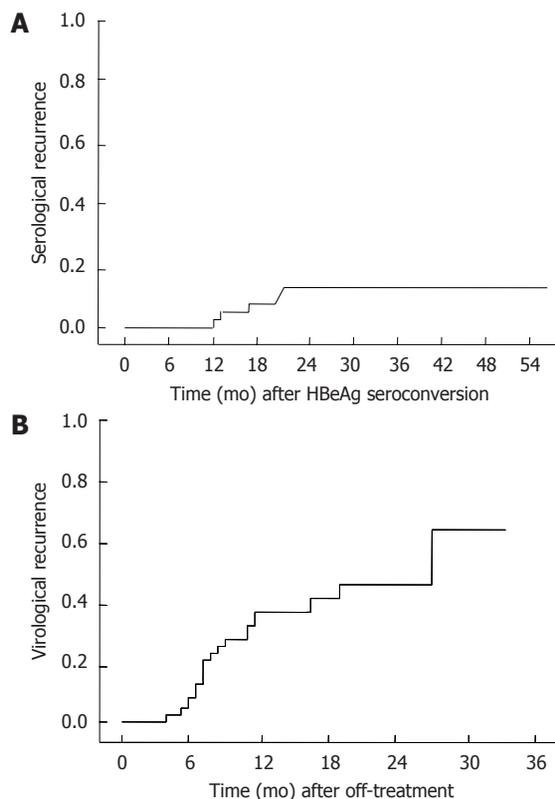


Figure 1 Serologic recurrence and virologic recurrence after off-treatment in patients with hepatitis B e antigen positive hepatitis. A: Serologic recurrence after off-treatment in patients with hepatitis B e antigen (HBeAg) positive hepatitis who achieved the successful end point of virologic response; B: Virologic recurrence after off-treatment in HBeAg positive hepatitis.

treatment, total treatment duration, and duration of treatment before the HBV DNA levels were undetectable were compared between the virologic recurrence and the non-recurrence group (Table 3). The mean age of the recurrence group was older than that of the non-recurrence group (46.7 ± 12.1 years *vs* 38.8 ± 12.7 years, respectively; $P = 0.022$).

Univariate analysis was performed to determine the predictive factors of durability. The variables including age, sex, pretreatment ALT, level, pretreatment HBV DNA levels, time to undetectable HBV DNA, time to HBeAg seroconversion, consolidation treatment duration and total treatment duration. Age (≤ 40 years) and the duration of consolidation treatment (≥ 15 mo) were significant predictive factors for off-treatment durability ($P = 0.017$, $P = 0.046$, respectively). In the multivariate analysis using the Cox's proportional hazard model, the factors of age (≤ 40 years) and prolonged-consolidation treatment ≥ 15 mo were also significant predictive factors of virologic recurrence in HBeAg positive chronic hepatitis B patients [$P = 0.049$, 95% CI (0.096-0.998); $P = 0.005$, 95% CI (2.054-65.12)]. Serologic or virologic recurrence did not show a significant difference between clevudine and entecavir treatment (Table 4).

Using the two predictive factors including age (≤ 40 years) and prolonged-consolidation treatment ≥ 15 mo

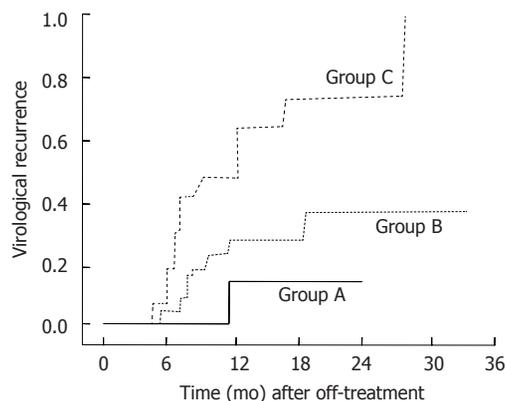


Figure 2 Using the two predictive factors for virologic recurrence (age > 40 yr *vs* ≤ 40 yr and consolidation treatment duration < 15 mo *vs* ≥ 15 mo). Patients were categorized into 3 groups. Group A: Patients with aged ≤ 40 yr and consolidation treatment duration ≥ 15 mo; Group B: Patients with either aged ≤ 40 yr or consolidation treatment duration ≥ 15 mo; Group C: Patients with aged > 40 yr and consolidation treatment for < 15 mo. P values were derived from the log-rank test (group A *vs* group B, $P = 0.291$; group A *vs* group C, $P = 0.014$, respectively).

Table 2 Summary of patients who showed serologic and virologic recurrence

Patient No.	Age (yr)	Sex	Serologic recurrence (HBeAg reversion)	Virologic recurrence (copies/L)	ALT level at relapse (U/L)
1	28	Female	Yes	97 600	139
2	24	Female	Yes	645 000 000	236
3	42	Male	Yes	120 759	71
4	46	Male	Yes	980 000	45
5	47	Male	Yes	254 251 078	89
6	48	Female	Yes	320 630	287
7	46	Male	No	42 900	42
8	32	Female	No	20 916	32
9	67	Male	No	10 083	38
10	42	Male	No	41 982	65
11	46	Male	No	86 000	40
12	66	Female	No	4 521 266	131
13	38	Male	No	587 000	43
14	67	Female	No	10 032	31
15	62	Female	No	637 898	28
16	50	Male	No	43 719	98
17	51	Female	No	32 033	101
18	42	Male	No	86 6197	243
19	53	Male	No	6 158 288	54
20	37	Female	No	116 000 000	436

ALT: Alanine aminotransferase; HBeAg: Hepatitis B e antigen.

patients could be classified into 3 groups: patients who had both of the good predictors (age ≤ 40 years and consolidation treatment ≥ 15 mo, group A); patients with either ≥ 40 years old or underwent consolidation treatment ≥ 15 mo (group B), and patients with neither of the two good predictors (group C). Among these 3 groups, group C showed a significantly higher recurrence rate (Figure 2). These results suggest that additional treatment for more than 15 mo after HBeAg seroconversion in patients who are ≤ 40 years old may be beneficial in providing a sustained virologic response.

Table 3 Clinical characteristics in virological recurrence and non-recurrence

	Virological recurrence (<i>n</i> = 20)	Virological non-recurrence (<i>n</i> = 28)	<i>P</i> value
Age, yr (mean ± SD)	46.7 ± 12.1	38.8 ± 12.7	0.022
Sex (male/female)	11/9	29/19	0.561
Pretreatment ALT level (IU/L)	199 (40-374)	189 (11-364)	0.491
Pretreatment HBV DNA level (log ₁₀ copies/mL)	7.7 (5.1-10)	7 (5.0-9.1)	0.125
Time to undetectable HBV (mo)	6.7 (2.3-39.4)	9.1 (1.3-25.0)	0.490
Time to HBeAg seroconversion (mo)	13.9 (0.8-39.4)	12.7 (1.4-31.2)	0.744
Consolidation treatment duration (mo)	10.3 (6-23.9)	11.7 (6-37.9)	0.517
Total treatment duration (mo)	25.6 (9.6-47.4)	27.0 (9.4-43.3)	0.358
Antiviral agent (entecavir/clevudine)	15/5	16/12	0.236

ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen.

Table 4 Multivariate analysis on predictive factors of virological recurrence after withdrawal of antiviral treatment

	Regression coefficient	Standard error	<i>P</i> value	RR (95% CI)
Age (≤ 40 yr vs > 40 yr)	-1.17	0.596	0.049	0.310 (0.096-0.998)
Sex (male vs female)	-0.938	0.527	0.075	0.391 (0.139-1.099)
Pretreatment ALT level (< 2 × ULN vs ≥ 2 × ULN)	-2.11	1.135	0.063	0.121 (0.013-1.121)
Pretreatment HBV DNA level (< 108 vs ≥ 108)	-0.27	0.533	0.612	0.763 (0.268-2.170)
Time to undetectable HBV (< 6 mo vs ≥ 6 mo)	-0.237	0.635	0.709	0.789 (0.227-2.739)
Time to HBeAg seroconversion (< 12 mo vs ≥ 12 mo)	1.417	0.846	0.094	4.126 (0.786-21.66)
Consolidation treatment (< 15 mo vs ≥ 15 mo)	2.425	0.87	0.005	11.299 (2.054-65.12)
Total treatment duration (< 24 mo vs ≥ 24 mo)	0.001	0.732	0.999	1.001 (0.238-4.207)
Antiviral agent (entecavir vs clevudine)	-1.529	0.861	0.076	0.217 (0.040-1.172)

HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; ULN: Upper limit of normal; ALT: Alanine aminotransferase; RR: Relative risk.

DISCUSSION

The end points of chronic hepatitis B treatment, independent of HBeAg status, are the sustained suppression of HBV replication, the remission of liver disease and the prevention of hepatocellular carcinoma^[22,23]. Clinical studies have reported that rapid and sustained suppression of viral load is an important factor in the prediction of the virological response. It was reported that entecavir had the potential for a very rapid and effective antiviral response and clevudine-induced viral response was durable after discontinuation of treatment^[24]. It was hypothesized that entecavir would induce a more durable suppression of viral load compared with that of clevudine after discontinuation of treatment.

We studied the durability of off treatment for chronic HBV infection among entecavir-treated and clevudine-treated patients who achieved “virological response”. Current international guidelines suggest that treatment with NUC can be discontinued after 6-12 mo of consolidation therapy after HBeAg seroconversion^[14].

Consistent with these recommendations, NUC treatment was discontinued in all patients after 6 mo of consolidation treatment. During follow-up after the discontinuation of NUC therapy, only 20 (41.6%) of the 48 patients showed a durable response, which was defined as HBeAg seroconversion and HBV DNA levels less than 10 copies/L. Moreover, a sustained response was achieved in patients who discontinued therapy after a

consolidation therapy period of at least 15 mo regardless of the type of antiviral drug.

In our study, both serologic and virological recurrence after HBeAg seroconversion and undetectable HBV DNA were considered. Serologic recurrence showed 5 of 6 patients (83%) and virological recurrence occurred 17 of 20 patients (85%) within 12 mo after discontinuing the antiviral agent. This recurrence rate was consistent with a previous study^[20]. These results showed longer treatment durations of nucleoside analogs may contribute to the suppression of viral replication and the clearance of infected hepatocytes, but HBV cannot be completely eradicated. Therefore, HBeAg seroconversion and consolidation therapy for more than 6 mo might be an imperfect end point in antiviral treatment.

Patients who were ≤ 40 years old also showed independently significant associations with a sustained response based on the multivariate analysis. Younger patients had a more sustained durability. This result was similar to that reported by Chien *et al*^[25] who demonstrated that Taiwanese patients who were < 36 years of age had a more sustained response. This difference in durability based on age may be related to immune system activity^[26]. It is assumed that younger patients were more likely to be sufficiently immunocompetent to maintain viral suppression, which contributed to a higher rate of off-treatment durability. While age appears to be directly related to durability in this study, further research is necessary to more clearly define the cutoff age.

This study has some limitations in a retrospective study. First, a limited number of patients were treated with either entecavir or clevudine monotherapy; Second, HBsAg loss in a more stable remission of chronic hepatitis B compared with HBeAg seroconversion was not analyzed because the follow-up period was relatively short. However, we intended to analyze the predictive factors of sustained virologic response in HBeAg positive chronic hepatitis B patients who discontinued successful antiviral therapy that was considered as high potency antiviral agent more than lamivudine. Our results might suggest that the high recurrence rates after achieving sustained virologic response (HBeAg seroconversion and undetectable HBV DNA) showed no difference of durability regardless of the type of oral high potency antiviral agent.

In conclusion, this study shows that HBeAg seroconversion and consolidation therapy for more than 6 mo are an imperfect end point in antiviral treatment. Long-term consolidation treatment (≥ 15 mo) in younger patients is important to produce better prognosis in HBeAg positive chronic hepatitis B.

COMMENTS

Background

The durability of the virologic response is important to plan the strategy of treatment in chronic hepatitis B. The present study evaluated the off-treatment durability of response and the predictive factors for virologic recurrence in hepatitis B e antigen (HBeAg) positive chronic hepatitis B patients who achieved the successful end point of nucleotide analogue therapy.

Research frontiers

This study showed that HBeAg seroconversion is an imperfect end point in antiviral treatment. Long-term consolidation treatment (≥ 15 mo) in younger patients is important for producing better prognosis in HBeAg positive chronic hepatitis B.

Innovations and breakthroughs

The high recurrence rates after achieving sustained virologic response (HBeAg seroconversion and undetectable hepatitis B virus DNA) showed no difference of durability regardless of the type of oral high potency antiviral agent.

Applications

The study results suggest that long-term consolidation treatment (≥ 15 mo) after achieving virological response is important for maintaining better sustained virological response in HBeAg positive chronic hepatitis B.

Terminology

The criteria for the cessation of treatment were defined as follows: (1) achievement of virological response; and (2) duration of consolidation therapy (≥ 6 mo). Virological response defined HBeAg seroconversion, HBV DNA < 300 copies/mL and normal alanine aminotransferase levels. Virologic recurrence was defined as an increase of HBV-DNA level > 10 copies/L after HBeAg seroconversion with previously undetectable HBV-DNA level because a viral load of more than 10 copies/L is associated with progression of liver disease.

Peer review

This study can be considered as a preliminary report of the comparison between entecavir and clevudine therapy in chronic HBV in relation to the behaviour of HBeAg positive and viral load. It was well designed and conducted but a continuation of this study must be done with a greatest number of patients under a longest time of therapy before discontinuation.

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Bispectral index monitoring as an adjunct to nurse-administered combined sedation during endoscopic retrograde cholangiopancreatography

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Abstract

AIM: To determine whether bispectral index (BIS) monitoring is useful for propofol administration for deep sedation during endoscopic retrograde cholangiopancreatography (ERCP).

METHODS: Fifty-nine consecutive patients with a variety of reasons for ERCP who underwent the procedure at least twice between 1 July 2010 and 30 November 2010. This was a randomized cross-over study, in which each patient underwent ERCP twice, once with BIS monitoring and once with control monitoring. Whether BIS monitoring was done during the first or second ERCP procedure was random. Patients were intermittently administered a mixed regimen including

midazolam, pethidine, and propofol by trained nurses. The nurse used a routine practice to monitor sedation using the Modified Observer's Assessment of Alertness/Sedation (MOAA/S) scale or the BIS monitoring. The total amount of midazolam and propofol used and serious side effects were compared between the BIS and control groups.

RESULTS: The mean total propofol dose administered was 53.1 ± 32.2 mg in the BIS group and 54.9 ± 30.8 mg in the control group ($P = 0.673$). The individual propofol dose received per minute during the ERCP procedure was 2.90 ± 1.83 mg/min in the BIS group and 3.44 ± 2.04 mg in the control group ($P = 0.103$). The median value of the MOAA/S score during the maintenance phase of sedation was comparable for the two groups. The mean BIS values throughout the procedure (from insertion to removal of the endoscope) were 76.5 ± 8.7 for all 59 patients in using the BIS monitor. No significant differences in the frequency of $< 80\%$ oxygen saturation, hypotension (< 80 mmHg), or bradycardia (< 50 beats/min) were observed between the two study groups. Four cases of poor cooperation occurred, in which the procedure should be stopped to add the propofol dose. After adding the propofol, the procedure could be conducted successfully (one case in the BIS group, three cases in the control group). The endoscopist rated patient sedation as excellent for all patients in both groups. All patients in both groups rated their level of satisfaction as high (no discomfort). During the post-procedural follow-up in the recovery area, no cases of clinically significant hypoxic episodes were recorded in either group. No other postoperative side effects related to sedation were observed in either group.

CONCLUSION: BIS monitoring trend to slightly reduce the mean propofol dose. Nurse-administered propofol

sedation under the supervision of a gastroenterologist may be considered an alternative under anesthesiologist.

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Key words: Conscious sedation; Bispectral index monitoring; Pancreatic neoplasm; Endoscopic retrograde cholangiopancreatography

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INTRODUCTION

Electroencephalography (EEG)-guided sedation has been used by anesthesiologists to achieve optimal titration of sedatives^[1]. Bispectral index (BIS) monitoring is an EEG-based method that quantifies the depth of anesthesia by analyzing the EEG and uses a complex algorithm to generate an index score, providing an objective measurement of the level of consciousness in sedated patients^[1]. The value of BIS monitoring as an adjunct to endoscopic sedation has been tested in a limited number of endoscopic studies^[2-6]. The EEG-guided method was originally evaluated for facilitation of sedation in endoscopic retrograde cholangiopancreatography (ERCP)^[7-10].

Propofol can rapidly and easily induce deep sedation, and the depth of sedation can be adjusted^[11]. The Division of Drug Risk Evaluation in the United States has stated that “doses of propofol should be kept as low as effectively possible and patients who are sedated with propofol should be monitored properly”^[12]. A combination of propofol and midazolam significantly reduces the total propofol amount required and consequently reduces the risk of apnea; however, recovery time is prolonged as compared with propofol alone^[8]. According to Athens international statements^[12] and the American Society of Anesthesiologists Physical Status Classification (ASA grade), patients of classes I, II and often III can be safely sedated to the level of conscious sedation by nurses qualified in cardiopulmonary resuscitation for esophagogastroduodenoscopy and colonoscopy, but there are no data for sedation by nurses during ERCP.

In this study, we hypothesized that if we employed BIS monitoring to achieve the desired level of deep sedation using the minimal doses of propofol, then the risk of respiratory depression would be reduced. In

particular, a combination of propofol and midazolam, administered by trained nurses with BIS monitoring, was used to evaluate the usefulness of this sedation method during ERCP.

We determined whether BIS monitoring is a useful adjunct technique for the administration of propofol titrated for deep sedation, as measured by differences in the dose of propofol administered during ERCP.

MATERIALS AND METHODS

This study was approved by the hospital ethics committee, and informed consent was obtained from participating patients. Fifty-nine consecutive patients with a variety of reasons for ERCP and who underwent the procedure at least twice between 1 July 2010 and 30 Nov 2010 were prospectively included in this study. Associated medical illnesses were graded according to ASA grade^[13]. Exclusion criteria were age < 20 years, critical illness (ASA grade IV or V), pregnancy, chronic use of benzodiazepines or opiates, and history of allergy to eggs. No patient was excluded after randomization.

All procedures were therapeutic and were performed by one gastroenterologist (Jung MK, who has performed > 500 ERCP procedures per annum for 5 years). Trained three nurses administered the sedative agents and carried out the anesthetic protocol, which was generated by an anesthesiologist (Jeon YH, who has performed > 1000 general anesthesia procedures per annum for 15 years). This was a randomized cross-over study, in which each patient underwent ERCP twice, once with BIS monitoring and once with control monitoring. Whether BIS monitoring was done during the first or second ERCP procedure was random (Figure 1). The endoscopist, trained nurse, radiologist, and assistant nurse had access to the randomization scheme when the patient was admitted to the endoscopy suite. The independent research fellow (Park HG) who performed all pre- and post-procedural assessments was blinded to the randomization scheme.

The sedation end point was conscious sedation^[14]. Patient received the fixed dose midazolam, propofol and pethidine before the procedure. Trained nurse reassessed the sedation grade using BIS monitor or the modified Observer's Assessment of Alertness/Sedation (MOAA/S) scale every minute^[3]. Patients were intermittently administered a mixed regimen including midazolam, pethidine, and propofol by trained nurse. The end point of sedation was a MOAA/S score of 1. For the BIS monitoring group, the nurse was instructed to use BIS as the primary end point for titration of propofol and to target the BIS value to between 65 and 80. Patients were moved to the recovery area immediately after the procedure if their vital signs were stable. All patients were routinely kept in the hospital for at least 24 h after the ERCP procedure. If patients awoke or were hyperactive during sedation, this was considered a significant adverse event. And the procedure should be stopped to add the propofol dose. After adding the propofol, patient could be controlled. the procedure could be conducted successfully.

BIS monitoring

All patients were continuously monitored for heart rate, oxygen saturation (pulse oximetry), and blood pressure (automated blood pressure cuff, serial measurements every 3 min).

Baseline vital signs were recorded immediately before the procedure. All patients were given supplemental intranasal oxygen (5 L/min). Respiratory depression was considered significant when oxygen saturation was < 80% for > 15 s with oxygen supplementation. A drop in systolic arterial blood pressure below 80 mmHg or a heart rate below 50 beats/min was considered a significant adverse event. All patients were monitored in the recovery area by electrocardiography, pulse oximetry, and blood pressure recording. All patients were monitored for BIS scores using the BIS VIEW device (BIS Monitoring System) and a specific BIS Quatro Sensor (Aspect Medical System, Newton, MA, United States). During the procedure, BIS scores were monitored every 3 min.

Statistical analysis

The total amount of midazolam, propofol used and serious side effects between the BIS and control groups were compared. Continuous data were compared using the paired Student's *t*-test. Categorical variables were tested using the χ^2 test. The criterion for statistical significance was $P < 0.05$. SPSS Version 15 (SPSS, Chicago, IL, United States) was used for analyses. Data are presented as the mean \pm SD.

RESULTS

Baseline characteristics of the 59 patients are presented in Table 1. The mean total propofol dose administered was 53.1 ± 32.2 mg in the BIS group and 54.9 ± 30.8 mg in the control group ($P = 0.673$; Table 2). The individual propofol dose administered per minute during the ERCP procedure was 2.90 ± 1.83 mg/min in the BIS group and 3.44 ± 2.04 mg/min in the control group ($P = 0.103$). The median value of the MOAA/S score during the maintenance phase of sedation was comparable between the two groups. The mean BIS values throughout the procedure (from insertion to removal of the endoscope) were 76.5 ± 8.7 .

No significant differences in the frequency of < 80% oxygen saturation, hypotension (< 80 mmHg), or bradycardia (< 50 beats/min) were observed between the two study groups. Four cases of poor cooperation occurred, in which the procedure should be stopped to add the propofol dose. After adding the propofol, the procedure could be conducted successfully (one case in the BIS group, three cases in the control group).

The endoscopist rated the patient sedation as excellent for all the patients in both groups. All patients in both groups rated their level of satisfaction as high (no discomfort). During the post-procedural follow-up in the recovery area, no cases of clinically significant hypoxic episodes were recorded in either group. No other

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

Characteristics	Value
Gender	
Male	36 (61)
Female	23 (39)
Etiology	
Acute idiopathic pancreatitis	3 (5.1)
Biloma	1 (1.7)
Common bile duct stone	34 (57.6)
Cholangiocarcinoma	10 (16.9)
Chronic pancreatitis	4 (6.8)
Intraductal papillary mucinous neoplasm	2 (3.4)
Pancreatic cancer	5 (8.5)

Table 2 Comparative results of paired examinations

	BIS	Control	<i>P</i> value
Midazolam (mg)	1.64 \pm 0.87	1.61 \pm 0.70	0.788
Propofol (mg)	53.1 \pm 32.2	54.9 \pm 30.8	0.673
Procedure duration (min)	21.0 \pm 10.5	18.6 \pm 9.6	0.187
Propofol dose/min (mg/min)	2.90 \pm 1.83	3.44 \pm 2.04	0.103
Procedure failed cases	1	3	0.309
Poor cooperation	2	2	
Mean BIS score	76.5 \pm 8.7	NA	

NA: Not available; BIS: Bispectral index.

postoperative side effects related to sedation were observed in either group.

DISCUSSION

Sedation and analgesia reduce pain, discomfort, and stress in patients undergoing unpleasant and prolonged procedures such as ERCP and contribute to better patient tolerance and compliance^[15]. Moreover, sedation and analgesia reduce the danger of injuries during ERCP due to a lack of cooperation by the patient and facilitate the endoscopist's task^[16]. Since the first report of endoscopic cannulation of major papilla in 1968^[17], ERCP has evolved from being a simple diagnostic procedure to becoming a therapeutic procedure with increased duration and complexity, requiring a high degree of patient cooperation. Reports have indicated that complications such as duodenal perforation and pancreatitis result as a consequence of poor patient cooperation manifested by restlessness and anxiety during the procedure^[18]. Successful ERCP procedures have been performed with the patient either moderately or deeply sedated or under general anesthesia. Patel *et al*^[19] reported that even when the target level of sedation was moderate, deep sedation episodes of all sedation occurred in 35% of ERCP patients. ERCP is thus recognized as an independent risk factor of deep sedation.

Propofol is a lipophilic anesthetic agent with rapid distribution and elimination times, and it does not have a cumulative effect after infusion. Its therapeutic spectrum is much narrower than that of midazolam, so careful

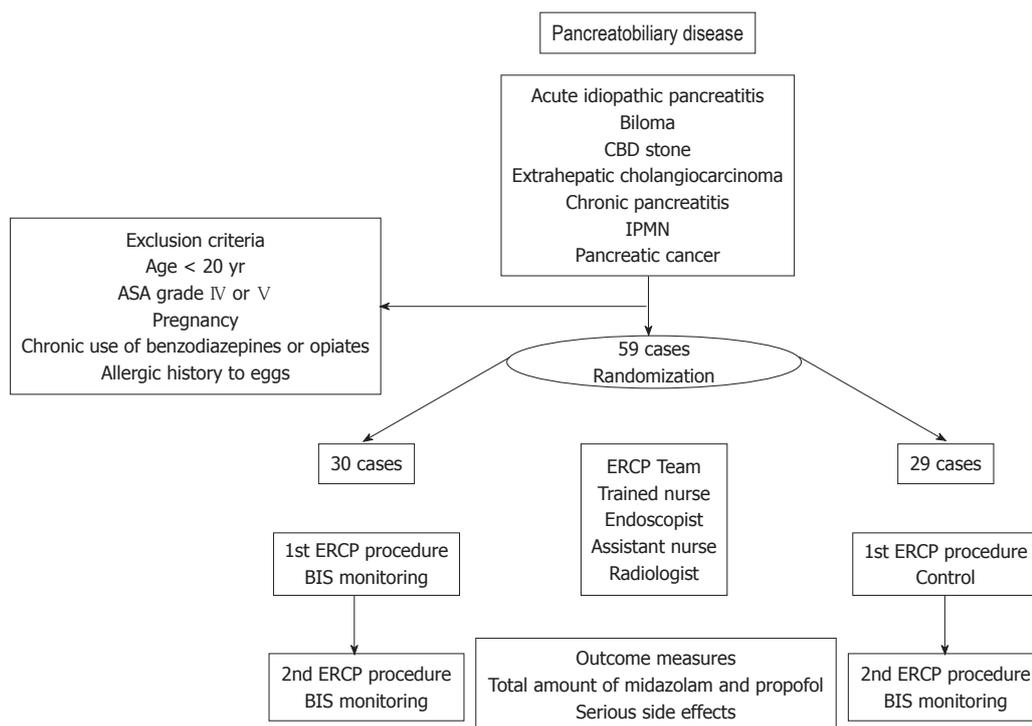


Figure 1 Flow chart of the enrolled patients. ASA grade: American Society of Anesthesiologists Physical Status Classification; BIS: Bispectral index; CBD: Common bile duct; ERCP: Endoscopic retrograde cholangiopancreatography; IPMN: Intraductal papillary mucinous neoplasm.

monitoring is much more demanding to differentiate between moderate and deep sedation and general anesthesia. Propofol has been evaluated in a variety of regimens in ERCP and has been shown to provide the same or superior sedation quality as midazolam with the advantage of better patient cooperation and shorter recovery time^[20-24]. One large multicenter study from North America demonstrated that the leading cause of death from ERCP was cardiopulmonary complications^[25]. In a large audit of upper endoscopy procedures from the United Kingdom, cardiopulmonary complications resulted in mortality in one in 2000 procedures^[26]. Sedation-related complications were attributed to high doses of sedatives and inadequate monitoring. In a retrospective analysis, Sharma *et al*^[27] showed that the incidence of cardiopulmonary complications in ERCP (2.1%) was almost double that during colonoscopy (1.1%) and more than triple that during upper endoscopy (0.6%).

The computer-generated BIS ranges from 0 (coma) to 100 (fully awake) and reflects the level of sedation regardless of a patient's clinical characteristics and the type of sedative drug used. The main aim of this study was to determine whether BIS monitoring could be a useful adjunct technique to the administration of propofol that was titrated to achieve conscious sedation, as measured by a difference in the propofol dose administered during ERCP. The primary outcome observed was a slightly lower mean dose of propofol in the group of patients who were deeply sedated with the use of BIS monitoring.

As the primary end point, we chose the amount of propofol given, because we believe that this will be of significant interest for endoscopists. More specifically,

our guiding hypothesis was that if we achieved the desired level of sedation using a minimal dose of propofol with BIS monitoring, then the risk of respiratory depression would be reduced.

The method of sedation during ERCP procedures involves a formulation of benzodiazepines in combination with opioids and propofol. The level of sedation is a continuum, and deep sedation is logically associated with an increased frequency of inadequate ventilation or airway obstruction^[28].

The Practice Committee of the American Society for Gastrointestinal Endoscopy has stated that “the use of EEG monitoring may have a role in the future for the delivery of sedation during selected endoscopic procedures”^[29]. EEG monitoring, which is a more complex technique than BIS monitoring with respect to interpretation, enables more effective titration of the propofol dose for sedation during ERCP^[10]. More specifically, Wehrmann *et al*^[10] found that the mean propofol dose was significantly lower in the group of patients sedated with EEG-guided monitoring as compared with that of the control group. The results from a previous colonoscopy study suggested that BIS monitoring may be useful in preventing over-sedation as well as in reducing the propofol dose during the maintenance phase of the sedation^[2]. A randomized controlled trial published 2 years later by the same group did not confirm these suggestions^[3]. This may be explained by the notably shorter time required for a colonoscopy and, in particular, the especially shorter maintenance phase during a colonoscopy, during which over-sedation is less likely to occur. BIS monitoring may be valuable during ERCP

during which patients are sedated with the conventional regimen of benzodiazepines plus opioids^[7]. More specifically, in that study the total dose of midazolam was significantly lower in the BIS group as compared with the control group^[7]. In a recent study, BIS monitoring during propofol sedation during endoscopic submucosal dissection did not lead to a reduction in the dose of propofol required but did lead to higher satisfaction scores from the patients and endoscopists^[30]. In this study, BIS monitoring during midazolam and propofol sedation during ERCP was safe and effective when sedatives were intermittently infused by well-trained nurses.

The role of BIS monitoring for conscious sedation targeted to the moderate level has not been established^[4]. BIS monitoring is less accurate for detecting deep sedation episodes during endoscopy where a particular level of conscious sedation is desired^[14,19]. In contrast, a meta-analysis of ambulatory surgery studies showed that the use of BIS monitoring significantly reduces the amount of anesthetic administered by 19%^[31]. Furthermore, titrating propofol with BIS monitoring during balanced anesthesia can decrease the amount of propofol required^[32]. Moreover, BIS monitoring may prevent awareness during general anesthesia^[33], although conflicting data have been reported^[34].

There are some limitations to our study. First, the nurse was not blinded to the absence or presence of BIS monitoring during ERCP. It was impossible for this study to be scheduled in a blinded fashion. Second, it is well established that BIS values lag behind actual sedation scores during induction of sedation as well as during recovery^[2]. However, it is noteworthy that all patients in the BIS group were deeply sedated during the maintenance phase (BIS score of 65-80).

In conclusion, BIS monitoring trend to slightly reduce the mean propofol dose, when the BIS index is used as the primary target for sedation during ERCP procedures. Approaches such as nurse-administered propofol sedation under the supervision of a gastroenterologist may be considered an alternative under the anesthesiologist.

COMMENTS

Background

Electroencephalography (EEG)-guided sedation has been used by anesthesiologists to achieve optimal titration of sedatives. Bispectral index (BIS) monitoring is an EEG-based method that quantifies the depth of anesthesia by analyzing the EEG and uses a complex algorithm to generate an index score, providing an objective measurement of the level of consciousness in sedated patients.

Research frontiers

Successful endoscopic retrograde cholangiopancreatography (ERCP) procedures have been performed with the patient either moderately or deeply sedated or under general anesthesia. Even when the target level of sedation was moderate, deep sedation episodes of all sedation occurred in 35% of ERCP patients. ERCP is thus recognized as an independent risk factor of deep sedation.

Innovations and breakthroughs

BIS monitoring trend to slightly reduce the mean propofol dose. Nurse-administered propofol sedation under the supervision of a gastroenterologist may be considered an alternative under anesthesiologist.

Peer review

The authors studied the usefulness of BIS monitor in the sedation of ERCP. The findings reported are of clinical importance.

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Effects of telbivudine and entecavir for HBeAg-positive chronic hepatitis B: A meta-analysis

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Abstract

AIM: To compare the effects of telbivudine (LDT) and entecavir (ETV) in treatment of hepatitis B e antigen (HBeAg)-positive chronic hepatitis B by meta-analysis.

METHODS: We conducted a literature search using PubMed, MEDLINE, EMBASE, the China National Knowledge Infrastructure, the VIP database, the Wanfang database and the Cochrane Controlled Trial Register for all relevant articles published before April 1, 2012. Randomized controlled trials (RCTs) comparing LDT with ETV for treatment of HBeAg-positive chronic hepatitis B were included. The data was analyzed with Review Manager Software 5.0. We used relative risk (RR) as an effect measure, and reported its 95% CI. Meta-analysis was performed using either a fixed-effect or random-effect model, based on the absence or presence of significant heterogeneity. Two reviewers assessed the risk of bias and extracted data independently and in duplicate. The analysis was executed using the main outcome parameters including hepatitis

B virus (HBV) DNA undetectability, alanine aminotransferase (ALT) normalization, HBeAg loss, HBeAg seroconversion, drug-resistance, and adverse reactions. Meta-analysis of the included trials and subgroup analyses were conducted to examine the association between pre-specified characteristics with the therapeutic effects of the two agents.

RESULTS: Thirteen eligible trials (3925 patients in total) were included and evaluated for methodological quality and heterogeneity. In various treatment durations of 4 wk, 8 wk, 12 wk, 24 wk, 36 wk, 48 wk, 52 wk, 60 wk and 72 wk, the rates of HBV DNA undetectability and ALT normalization in the two groups were similar, without statistical significance. At 4 wk and 8 wk of the treatment, no statistical differences were found in the rate of HBeAg loss between the two groups, while the rate in the LDT group was higher than in the ETV group at 12 wk, 24 wk, 48 wk and 52 wk, respectively (RR 2.28, 95% CI 1.16, 7.03, $P = 0.02$; RR 1.45, 95% CI 1.16, 1.82, $P = 0.001$; RR 1.45, 95% CI 1.11, 1.89, $P = 0.006$; and RR 1.86, 95% CI 1.04, 3.32, $P = 0.04$). At 4 wk, 8 wk, 60 wk and 72 wk of the treatment, there were no significant differences in the rate of HBeAg seroconversion between the two groups, while at 12 wk, 24 wk, 48 wk and 52 wk, the rate in the LDT group was higher than in the ETV group (RR 2.10, 95% CI 1.36, 3.24, $P = 0.0008$; RR 1.71, 95% CI 1.29, 2.28, $P = 0.0002$; RR 1.86, 95% CI 1.36, 2.54, $P < 0.0001$; and RR 1.87, 95% CI 1.21, 2.90, $P = 0.005$). The rate of drug-resistance was higher in the LDT group than in the ETV group (RR 3.76, 95% CI 1.28, 11.01, $P = 0.02$). In addition, no severe adverse drug reactions were observed in the two groups. And the rate of increased creatine kinase in the LDT group was higher than in the ETV group (RR 5.58, 95% CI 2.22, 13.98, $P = 0.0002$).

CONCLUSION: LDT and ETV have similar virological and biomedical responses, and both are safe and well tolerated. However, LDT has better serological response and higher drug-resistance.

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Key words: Telbivudine; Entecavir; Hepatitis B e antigen-positive chronic hepatitis B; Randomized controlled trials; Meta-analysis

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INTRODUCTION

Chronic hepatitis B (CHB) infection is a major health problem affecting over 350 million people worldwide^[1,2]. CHB can lead to various life-threatening conditions, such as liver failure, liver cirrhosis (LC) and hepatocellular carcinoma (HCC)^[3]. Hepatitis B virus (HBV) covalent closed circular DNA (cccDNA) is the main cause of the sustainability of the hepatitis virus, and it is difficult to completely eliminate it^[4]. So the primary therapeutic goal is to sustain viral suppression. Current anti-viral medication includes interferon [interferon-alpha (IFN- α), and pegylated (PEG) IFN- α] and nucleosides or nucleoside analogues [entecavir (ETV), adefovir dipivoxil, telbivudine (LDT), and lamivudine]^[5]. Recent studies have shown that LDT and ETV are the strongest nucleoside analogues. LDT (β -L-2'-deoxythymidine) is an orally bioavailable L-nucleoside. It can effectively suppress HBV DNA replication, and has a higher rate of hepatitis B e antigen (HBeAg) seroconversion than other current oral antiviral agents^[6]. However, its drug-resistance remains high^[7]. ETV is a new generation nucleoside analogues. It has the advantage of higher rate of HBV DNA suppression, low drug-resistance and high safety, especially in lamivudine-resistant CHB patients^[8]. But the rates of HBeAg loss and seroconversion are very low in ETV group, which is difficult to meet the withdrawal standards. There are few systematic reviews about the comparison of LDT and ETV. Therefore, we conducted a meta-analysis of the randomized controlled trials (RCTs) using the Cochrane methodology to explore the efficacy of LDT and ETV for clinical treatment of HBeAg-positive chronic hepatitis B.

MATERIALS AND METHODS

Literature search

We searched PubMed, MEDLINE, EMBASE, China

National Knowledge Infrastructure, the VIP database, the Wanfang database and the Cochrane Controlled Trial Register for articles published up to April 1, 2012, using the following keywords: "HBeAg-positive chronic hepatitis B", "telbivudine", "entecavir", and "RCTs". The reference lists of eligible studies were also searched.

Inclusion criteria

The following inclusion criteria were used: (1) RCTs; (2) Articles studying HBeAg-positive chronic hepatitis B patients, according to diagnostic standards in "China guidelines for HBV management (2010)"^[9]; (3) Studies comparing LDT (600 mg/d) with ETV (0.5 mg/d); and (4) The main outcome parameters included virological, biochemical, and serological responses [HBV DNA undetectability, alanine aminotransferase (ALT) normalization, HBeAg loss, HBeAg seroconversion, drug-resistance, and adverse reactions]. Virological response was defined as attainment of undetectable levels of HBV DNA. Determined by quantitative polymerase chain reaction, the threshold of detection was 1000 copies/mL or less in each corresponding study (Table 1). Biochemical response was defined as normalization of ALT levels to below the upper limit of normal (< 40 IU/mL). HBeAg loss was defined as HBeAg levels < 1.0 S/CO, HBeAg seroconversion was defined as HBeAg loss and the presence of anti-HBeAg, determined by microparticle enzyme immunoassay or enzyme-linked immunosorbent assay.

Exclusion criteria

The following exclusion criteria were used: (1) Nonrandomized controlled trials (NRCTs); (2) Insufficient analytical information regarding treatment schedule, follow-up, and outcomes; (3) Patients receiving interferon, nucleosides or nucleotides for CHB within 6 mo; (4) Patients coinfecting with hepatitis A, C, D and E virus, cytomegalovirus, or human immunodeficiency virus; (5) Patients with liver failure, HCC, and liver-related complications caused by alcoholism, autoimmune disease, and cholestasis; and (6) Pregnant and breastfeeding patients.

Data extraction

Data extraction was assessed independently by two reviewers (Song LY and Zhang SR). Discrepancies were solved through discussions between the reviewers or by a third person. Systematic Reviews of Interventions Version 5.0.2 (Cochrane Collaboration, Oxford, United Kingdom) was used to assess risk of bias (adequate sequence generation, allocation concealment, blinding, incomplete outcome data addressed, free of selective reporting and free of other bias)^[10]. Basic information obtained from each eligible trial included study design, patient characteristics, number of two groups, treatment duration and related study results. Data were reviewed to eliminate duplicate reports of the same trial.

Statistical analysis

We used Review Manager Software 5.0 (Cochrane Collab-

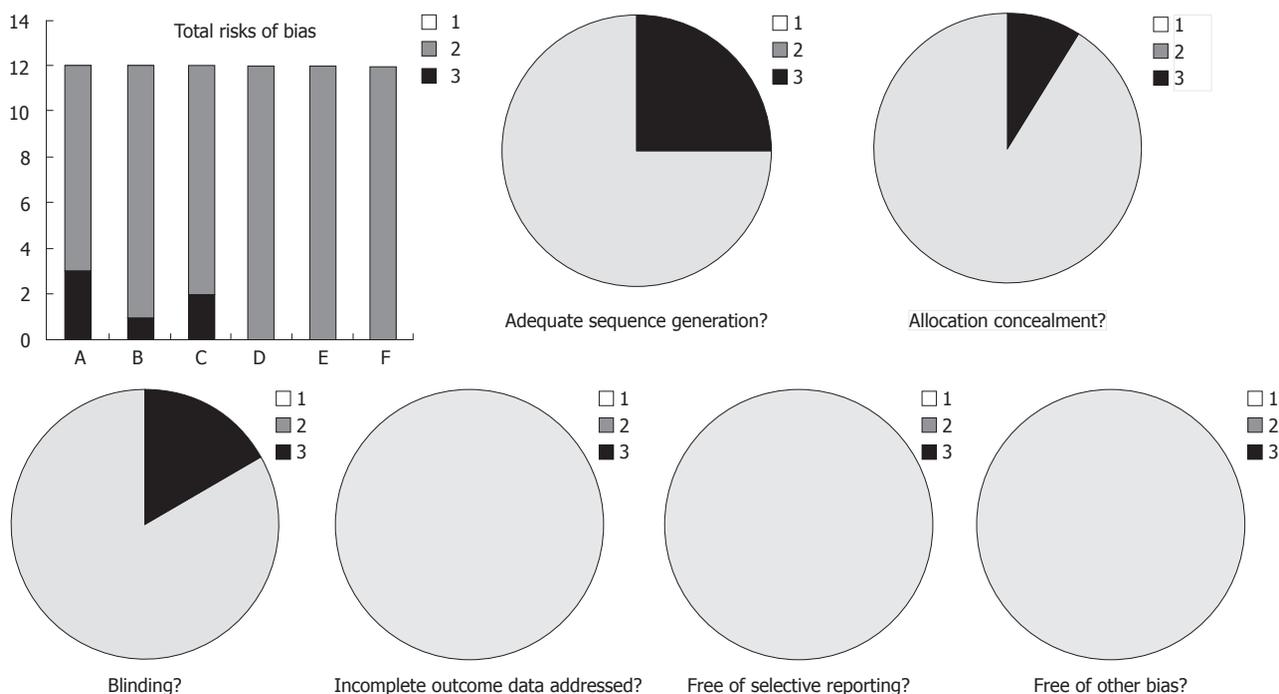


Figure 1 Risk of bias in included trials. A: Adequate sequence generation; B: Allocation concealment; C: Blinding; D: Incomplete outcome data addressed; E: Free of selective reporting; F: Free of other bias. 1: No (high risk of bias); 2: Unclear; 3: Yes (low risk of bias).

oration, Oxford, United Kingdom) for the data analysis. For dichotomous data, we used relative risk (RR) as an effect measure, and reported its 95% CI. Meta-analysis was performed using either a fixed-effect or random-effect model, based on the absence or presence of significant heterogeneity.

Statistical heterogeneity between trials was evaluated by χ^2 and I^2 analysis. The fixed-effect method was used in the absence of statistically significant heterogeneity ($P \geq 0.1$), and the random-effect method was used when the heterogeneity test was statistically significant ($P < 0.1$). $P < 0.05$ was regarded as statistically significant. Subgroup analysis was performed to examine the association between pre-specified characteristics (treatment duration) and the therapeutic effect, sensitivity analysis was made to estimate result stability, and funnel plots were used to assess publication bias if more than five trials were included^[11].

RESULTS

Characteristics and quality of studies

We initially identified 1165 abstracts, and after evaluating the full texts, we included 13 trials (12 in Chinese and one in English)^[12-24] based on the pre-specified criteria. A total of 3925 patients were included: 1987 treated with LDT and 1938 treated with ETV. Table 1 shows the characteristics of the 13 trials. All these studies showed baseline comparability, 9 of them reported the baseline of two groups in detail^[13-15,17,19,20,22-24], the other 4 presented no significant differences in gender, age and duration of treatment between the two groups^[12,16,18,21]. Three described the methods of randomization in detail^[13,14,24],

nine reported randomization, but did not describe the method of randomization in detail^[12,15,17-23], one reported allocation concealment^[24] and two presented blinding method^[22,24]. None of the trials referred to incomplete outcome data addressed, free of selective reporting, and free of other bias. Various risks of bias in the 13 trials. In addition, none of the trials reported mortality, life quality and liver cancer incidence are shown in Figure 1.

HBV DNA undetectability

All the trials reported the rate of HBV DNA undetectability. χ^2 and I^2 analyses showed no heterogeneity ($\chi^2 = 35.37$, $P = 0.74$, $I^2 = 0\%$); therefore, we used the fixed-effect method to analyze the data. The results showed that in various treatment durations of 4 wk, 8 wk, 12 wk, 24 wk, 36 wk, 48 wk, 52 wk, 60 wk and 72 wk, there were no statistical differences in the rate of HBV DNA undetectability between the two groups (RR 1.04, 95% CI 0.72, 1.49, $P = 0.85$; RR 0.98, 95% CI 0.74, 1.28, $P = 0.86$; RR 1.01, 95% CI 0.89, 1.15, $P = 0.83$; RR 1.06, 95% CI 0.99, 1.14, $P = 0.12$; RR 1.03, 95% CI 0.86, 1.37, $P = 1.24$; RR 1.02, 95% CI 0.95, 1.09, $P = 0.63$; RR 0.95, 95% CI 0.86, 1.05, $P = 0.29$; RR 1.02, 95% CI 0.83, 1.24, $P = 0.88$; and RR 0.95, 95% CI 0.80, 1.12, $P = 0.54$) (Figure 2A).

ALT normalization

Eleven trials reported the rate of ALT normalization^[12,13,15-20,22-24]. χ^2 and I^2 analysis showed no heterogeneity ($\chi^2 = 32.22$, $P = 0.51$, $I^2 = 0\%$). At various treatment durations of 4 wk, 8 wk, 12 wk, 24 wk, 36 wk, 48 wk, 52 wk, 60 wk and 72 wk, there were no statistical differences in the rate of ALT normalization between the two groups (RR 1.08, 95% CI 0.81, 1.43, $P = 0.59$; RR 1.05, 95% CI 0.77, 1.43, $P = 0.77$;

Table 1 Characteristics of included trials

Trials	Sample size (n)		Mean age (yr)		Regimen (mg/d)		Duration (wk)	Observationtime (wk)	Outcome parameters	HBV DNA undetectability (copy/mL)
	LDT	ETV	LDT	ETV	LDT	ETV				
Zhao <i>et al</i> ^[12]	36	36	34.30		600	0.5	48	24, 48	ABDE	-
Zhu <i>et al</i> ^[13]	30	30	28.00 ± 9.10	31.80 ± 7.10	600	0.5	24	12, 24	ABCDE	1000
Zhou <i>et al</i> ^[14]	52	63	46.30 ± 9.00		600	0.5	48	12, 24, 36, 48	ACD	-
Xu <i>et al</i> ^[15]	30	30	32.70 ± 10.60	33.60 ± 8.80	600	0.5	24	12, 24, 48	ABCDF	1000
Ye <i>et al</i> ^[16]	46	46	32.20		600	0.5	48	12, 24, 48	ABCDE	100
Zhang <i>et al</i> ^[17]	75	65	31.93 ± 7.96		600	0.5	72	8, 12, 24, 52, 72	ABDEF	500
Liu ^[18]	20	20	33.50		600	0.5	48	4, 12, 24, 48	ABCDF	1000
Zhao <i>et al</i> ^[19]	42	39	33.56 ± 10.25		600	0.5	60	8, 12, 24, 48, 60	ABD	1000
Shi <i>et al</i> ^[20]	40	40	30.50 ± 7.11	31.50 ± 7.95	600	0.5	24	12, 24	ABCD	500
Yu <i>et al</i> ^[21]	92	85			600	0.5	48	4, 8, 12, 24, 48	ACD	500
Huang <i>et al</i> ^[22]	90	90	28.80 ± 9.80		600	0.5	52	52	ABCDE	500
Ding <i>et al</i> ^[23]	30	30	37.20 ± 7.96	36.10 ± 7.12	600	0.5	48	4, 8, 12, 24, 36, 48	ABCDEF	1000
Zheng <i>et al</i> ^[24]	65	66	31.60 ± 8.70	33.50 ± 9.10	600	0.5	24	12, 24	AFCDF	500

A: Hepatitis B virus DNA undetectability; B: Alanine aminotransferase normalization; C: Hepatitis B e antigen loss; D: Hepatitis B e antigen seroconversion; E: Drug-resistance; F: Increased creatine kinase. LDT: Telbivudine; ETV: Entecavir; HBV: Hepatitis B virus.

RR 1.05, 95% CI 0.94, 1.16, $P = 0.40$; RR 1.00, 95% CI 0.93, 1.08, $P = 0.91$; RR 0.95, 95% CI 0.67, 1.34, $P = 0.78$; RR 1.01, 95% CI 0.92, 1.11, $P = 1.08$; RR 0.94, 95% CI 0.86, 1.02, $P = 0.14$; RR 0.96, 95% CI 0.77, 1.19, $P = 0.69$; and RR 0.98, 95% CI 0.84, 1.13, $P = 0.76$) (Figure 2B).

HBeAg loss

Ten trials reported the rate of HBeAg loss^[13-16,18,20-24]. χ^2 and I^2 analyses found no heterogeneity ($\chi^2 = 38.84$, $P = 0.04$, $I^2 = 36\%$). At 4 wk and 8 wk of the treatment, no statistical differences in the rate of HBeAg loss were observed between the two groups (RR 2.89, 95% CI 0.31, 27.23, $P = 0.35$; and RR 1.50, 95% CI 0.50, 4.46, $P = 0.47$). At 12 wk, 24 wk, 48 wk and 52 wk, the rate of HBeAg loss was higher in the LDT group than in the ETV group, and the difference between two groups was statistically significant (RR 2.28, 95% CI 1.16, 7.03, $P = 0.02$; RR 1.45, 95% CI 1.16, 1.82, $P = 0.001$; RR 1.45, 95% CI 1.11, 1.89, $P = 0.006$; RR 1.86, 95% CI 1.04, 3.32, $P = 0.04$) (Figure 2C).

HBeAg seroconversion

All the trials reported the rate of HBeAg seroconversion. χ^2 and I^2 analyses showed no heterogeneity ($\chi^2 = 22.15$, $P = 0.85$, $I^2 = 0\%$). At 4 wk, 8 wk, 60 wk and 72 wk of the treatment, the rate of HBeAg seroconversion in the two groups was similar, and no statistical significances were observed (RR 2.34, 95% CI 0.55, 9.92, $P = 0.25$; RR 1.55, 95% CI 0.77, 3.12, $P = 0.22$; RR 1.56, 95% CI 0.91, 2.67, $P = 0.1$). However, at 12 wk, 24 wk, 48 wk and 52 wk, the rate of HBeAg loss was higher in the LDT group than in the ETV group, with statistically significant difference between two groups (RR 2.1, 95% CI 1.36, 3.24, $P = 0.0008$; RR 1.71, 95% CI 1.29, 2.28, $P = 0.0002$; RR 1.86, 95% CI 1.36, 2.54, $P < 0.0001$; RR 1.87, 95% CI 1.21, 2.90, $P = 0.005$) (Figure 2D).

Drug-resistance

Six trials reported drug-resistance^[12,13,16,17,22,23]. χ^2 and I^2 analyses showed no heterogeneity ($\chi^2 = 0.63$, $P = 0.96$,

$I^2 = 0\%$). The rate of drug-resistance was higher in the LDT group than in the ETV group, and the difference between two groups was statistically significant (RR = 3.76, 95% CI 1.28, 11.01, $P = 0.02$) (Figure 2E).

Adverse reactions

Ten trials reported on the adverse reactions^[12-18,20,23,24]. No severe adverse reactions were observed in both groups. Common adverse reactions in the two groups included influenza-like symptoms such as fever, headache, fatigue, muscular stiffness, gastrointestinal upset such as nausea and diarrhea, alopecia and rash. Five of the trials reported the rate of increased creatine kinase (CK)^[15,17,18,23,24]. χ^2 and I^2 analyses showed no heterogeneity ($\chi^2 = 1.06$, $P = 0.94$, $I^2 = 0\%$). The rate of increased CK was higher in the LDT group than in the ETV group, the difference being statistically significant (RR 5.58, 95% CI 2.22, 13.98, $P = 0.0002$). But the increased CK recovered without any intervention, and did not influence the anti-HBV treatment (Figure 2F).

Statistical analysis

Meta-analysis was performed based on the rate of HBeAg seroconversion, using the fixed-effect model, and the minimum sample size trials were excluded^[18]. Odds ratio (OR) of all sensitivity analyses was higher than 1 and statistically significant ($P < 0.05$) (Table 2).

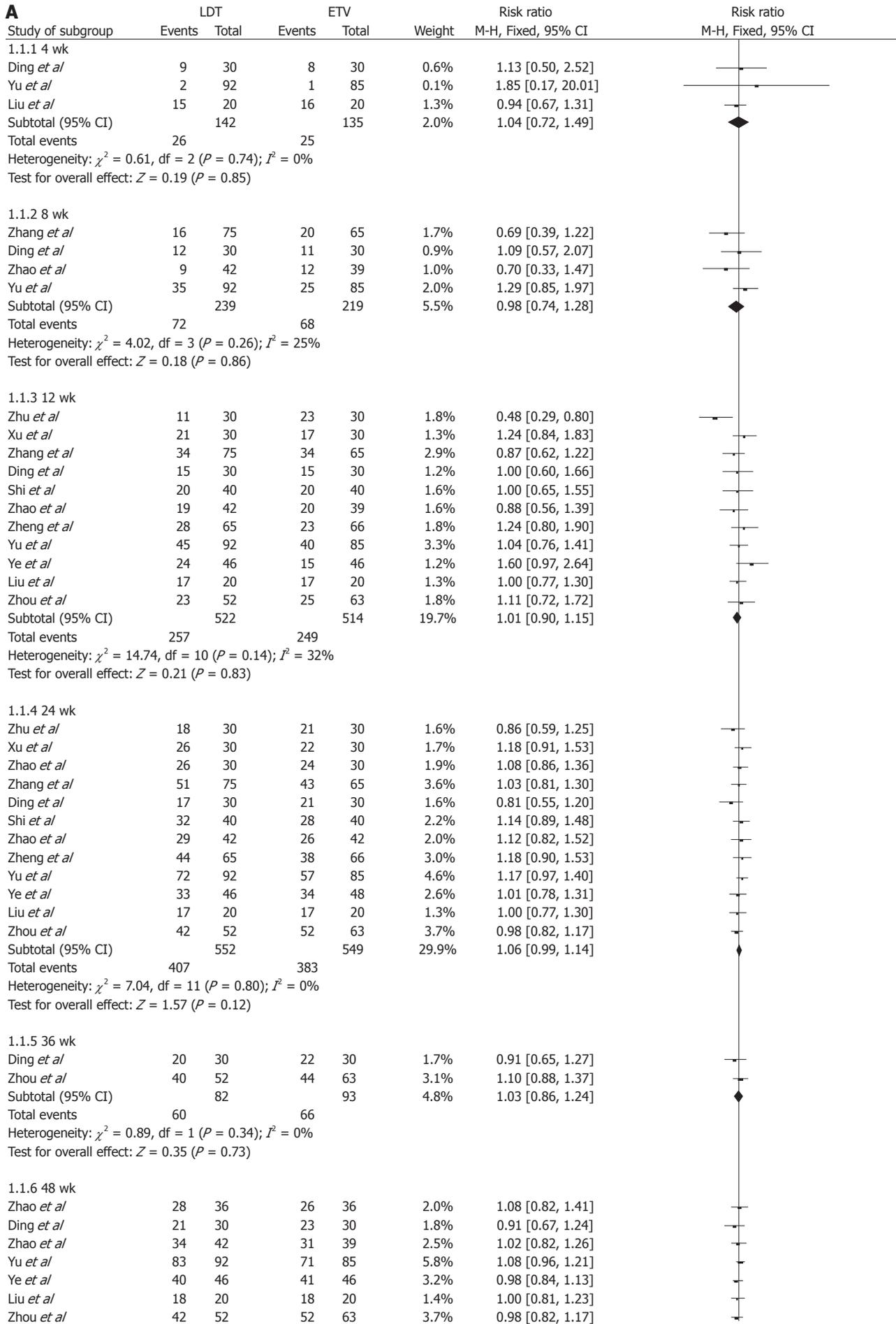
Funnel plots

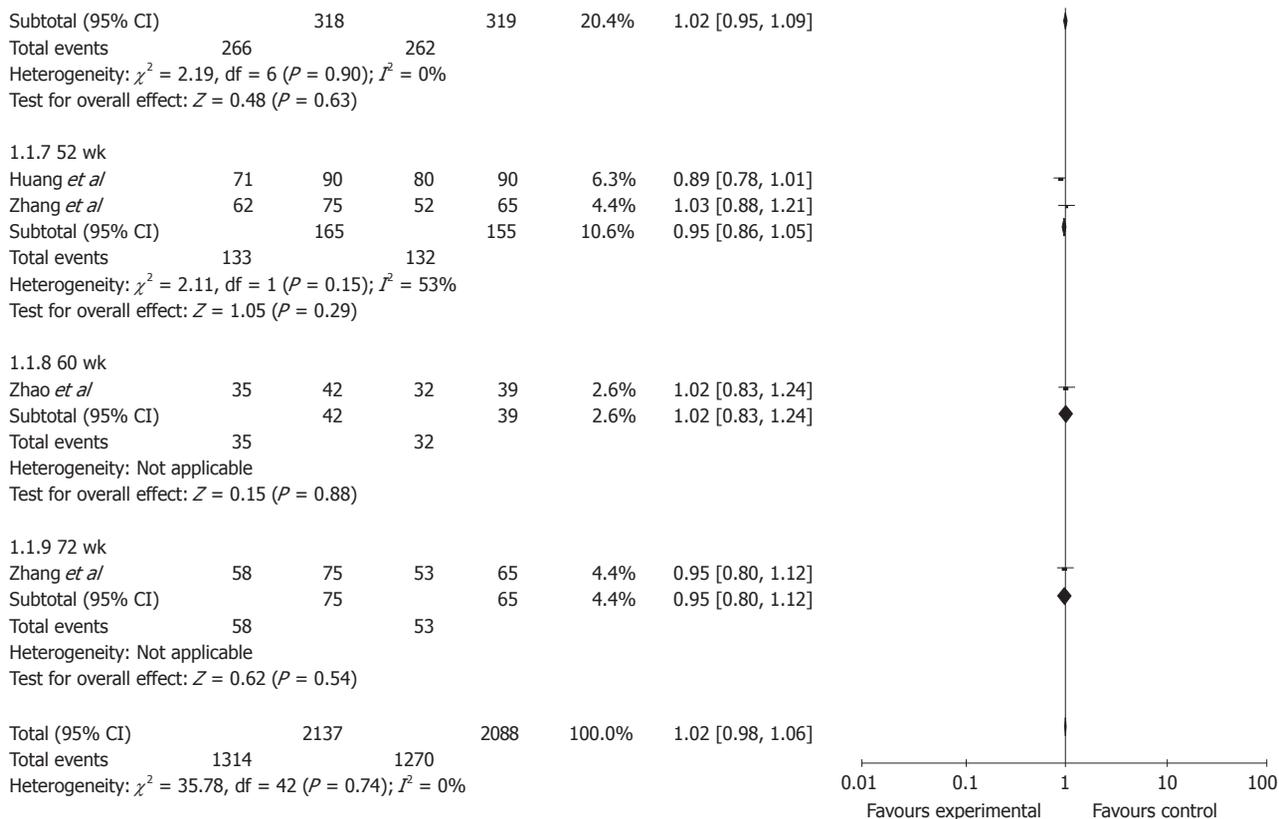
Funnel plots were performed based on the rate of HBV DNA undetectability. The results showed that funnel plots were symmetric and suggested that there was no publication bias (Figure 3).

DISCUSSION

The RCTs comparing LDT with ETV for patients with HBeAg-positive chronic hepatitis B were included, and meta-analyses on virology, serology, biochemical respons-

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Study of subgroup	LDT		ETV		Weight	Risk ratio	Risk ratio
	Events	Total	Events	Total		M-H, Fixed, 95% CI	M-H, Fixed, 95% CI
1.2.1 4 wk							
Ding <i>et al</i>	8	30	7	30	0.7%	1.14 [0.47, 2.75]	
Liu <i>et al</i>	19	20	18	20	1.8%	1.06 [0.88, 1.26]	
Subtotal (95% CI)		50		50	2.5%	1.08 [0.81, 1.43]	
Total events	27		25				
Heterogeneity: $\chi^2 = 0.08$, $df = 1$ ($P = 0.78$); $I^2 = 0\%$							
Test for overall effect: $Z = 0.53$ ($P = 0.59$)							
1.2.2 8 wk							
Zhang <i>et al</i>	27	75	23	65	2.4%	1.02 [0.65, 1.59]	
Ding <i>et al</i>	13	30	12	30	1.2%	1.08 [0.59, 1.97]	
Zhao <i>et al</i>	15	42	13	39	1.3%	1.07 [0.59, 1.95]	
Subtotal (95% CI)		147		134	4.9%	1.05 [0.77, 1.43]	
Total events	55		48				
Heterogeneity: $\chi^2 = 0.03$, $df = 2$ ($P = 0.98$); $I^2 = 0\%$							
Test for overall effect: $Z = 0.30$ ($P = 0.77$)							
1.2.3 12 wk							
Zhu <i>et al</i>	16	30	22	30	2.0%	0.73 [0.49, 1.08]	
Xu <i>et al</i>	15	30	18	30	1.8%	0.83 [0.53, 1.32]	
Zhang <i>et al</i>	56	75	43	65	4.5%	1.13 [0.91, 1.40]	
Ding <i>et al</i>	15	30	16	30	1.6%	0.94 [0.57, 1.53]	
Shi <i>et al</i>	21	40	24	40	2.4%	0.88 [0.59, 1.29]	
Zhao <i>et al</i>	30	42	27	39	2.7%	1.03 [0.78, 1.37]	
Zheng <i>et al</i>	56	65	38	65	3.7%	1.47 [1.17, 1.85]	
Ye <i>et al</i>	23	46	25	46	2.5%	0.92 [0.62, 1.36]	
Liu <i>et al</i>	19	20	18	20	1.8%	1.06 [0.88, 1.26]	
Subtotal (95% CI)		378		365	23.0%	1.05 [0.94, 1.16]	
Total events	251		231				
Heterogeneity: $\chi^2 = 14.80$, $df = 8$ ($P = 0.06$); $I^2 = 46\%$							
Test for overall effect: $Z = 0.84$ ($P = 0.40$)							
1.2.4 24 wk							
Zhu <i>et al</i>	22	30	27	30	2.6%	0.81 [0.64, 1.04]	
Xu <i>et al</i>	23	30	27	30	2.6%	0.85 [0.68, 1.07]	
Zhao <i>et al</i>	28	36	26	36	2.5%	1.08 [0.82, 1.41]	
Zhang <i>et al</i>	57	75	44	65	4.6%	1.12 [0.91, 1.39]	
Ding <i>et al</i>	16	30	18	30	1.8%	0.89 [0.57, 1.39]	

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Shi <i>et al</i>	31	40	30	40	2.9%	1.03 [0.81, 1.32]
Zhao <i>et al</i>	32	42	29	39	2.9%	1.02 [0.80, 1.32]
Zheng <i>et al</i>	51	65	49	66	4.8%	1.06 [0.87, 1.28]
Ye <i>et al</i>	39	46	40	46	3.9%	0.97 [0.83, 1.15]
Liu <i>et al</i>	18	20	17	20	1.7%	1.06 [0.84, 1.34]
Subtotal (95% CI)		414		402	30.5%	1.00 [0.93, 1.08]
Total events	317		307			
Heterogeneity: $\chi^2 = 7.01$, $df = 9$ ($P = 0.64$); $I^2 = 0\%$						
Test for overall effect: $Z = 0.11$ ($P = 0.91$)						

1.2.5 36 wk

Ding <i>et al</i>	20	30	21	30	2.1%	0.95 [0.67, 1.34]
Subtotal (95% CI)		30		30	2.1%	0.95 [0.67, 1.34]
Total events	20		21			
Heterogeneity: Not applicable						
Test for overall effect: $Z = 0.28$ ($P = 0.78$)						

1.2.6 48 wk

Zhao <i>et al</i>	30	36	28	36	2.7%	1.07 [0.85, 1.35]
Ding <i>et al</i>	23	30	24	30	2.4%	0.96 [0.73, 1.25]
Zhao <i>et al</i>	33	42	31	39	3.2%	0.99 [0.79, 1.24]
Ye <i>et al</i>	43	46	42	46	4.1%	1.02 [0.91, 1.15]
Liu <i>et al</i>	18	20	18	20	1.8%	1.00 [0.81, 1.23]
Subtotal (95% CI)		174		171	14.1%	1.01 [0.92, 1.11]
Total events	147		143			
Heterogeneity: $\chi^2 = 0.50$, $df = 4$ ($P = 0.97$); $I^2 = 0\%$						
Test for overall effect: $Z = 0.24$ ($P = 0.81$)						

1.2.7 52 wk

Huang <i>et al</i>	75	90	86	90	8.4%	0.87 [0.79, 0.97]
Zhang <i>et al</i>	62	75	52	65	5.5%	1.03 [0.88, 1.21]
Subtotal (95% CI)		165		155	13.9%	0.94 [0.86, 1.02]
Total events	137		138			
Heterogeneity: $\chi^2 = 3.29$, $df = 1$ ($P = 0.07$); $I^2 = 70\%$						
Test for overall effect: $Z = 1.46$ ($P = 0.14$)						

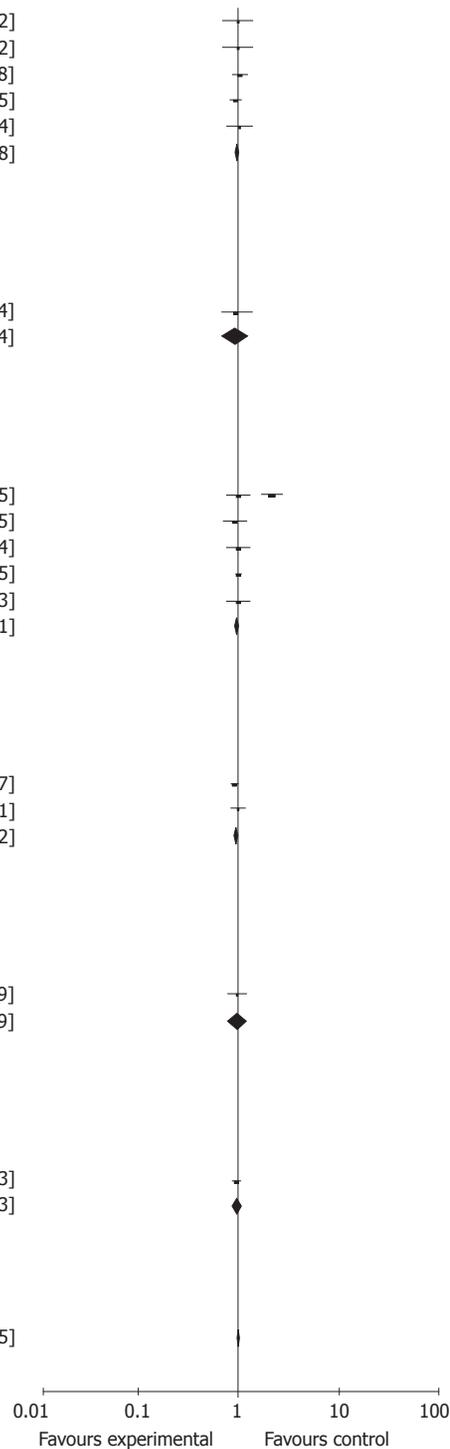
1.2.8 60 wk

Zhao <i>et al</i>	33	42	32	39	3.3%	0.96 [0.77, 1.19]
Subtotal (95% CI)		42		39	3.3%	0.96 [0.77, 1.19]
Total events	33		32			
Heterogeneity: Not applicable						
Test for overall effect: $Z = 0.39$ ($P = 0.69$)						

1.2.9 72 wk

Zhang <i>et al</i>	62	75	55	65	5.8%	0.98 [0.84, 1.13]
Subtotal (95% CI)		75		65	5.8%	0.98 [0.84, 1.13]
Total events	62		55			
Heterogeneity: Not applicable						
Test for overall effect: $Z = 0.31$ ($P = 0.76$)						

Total (95% CI)		1475		1411	100.0%	1.01 [0.96, 1.05]
Total events	1049		1000			
Heterogeneity: $\chi^2 = 32.22$, $df = 33$ ($P = 0.51$); $I^2 = 0\%$						
Test for overall effect: $Z = 0.23$ ($P = 0.82$)						
Test for subgroup differences: Not applicable.						



C

Study of subgroup	LDT		ETV		Weight	Risk ratio M-H, Random, 95% CI	Risk ratio M-H, Random, 95% CI
	Events	Total	Events	Total			
1.3.1 4 wk							
Ding <i>et al</i>	1	30	0	30	0.5%	3.00 [0.13, 70.83]	
Yu <i>et al</i>	1	92	0	85	0.5%	2.77 [0.11, 67.19]	
Liu <i>et al</i>	0	20	0	20		Not estimable	
Subtotal (95% CI)		142		135	0.9%	2.89 [0.31, 27.23]	
Total events	2		0				
Heterogeneity: $\tau^2 = 0.00$; $\chi^2 = 0.00$, $df = 1$ ($P = 0.97$); $I^2 = 0\%$							
Test for overall effect: $Z = 0.93$ ($P = 0.35$)							
1.3.2 8 wk							
Ding <i>et al</i>	2	30	1	30	0.8%	2.00 [0.19, 20.90]	
Yu <i>et al</i>	6	92	4	85	2.6%	1.39 [0.40, 4.74]	
Subtotal (95% CI)		122		115	3.4%	1.50 [0.50, 4.46]	
Total events	8		5				

Heterogeneity: $\tau^2 = 0.00$; $\chi^2 = 0.07$, $df = 1$ ($P = 0.79$); $I^2 = 0\%$
 Test for overall effect: $Z = 0.73$ ($P = 0.47$)

1.3.3 12 wk

Zhu <i>et al</i>	6	30	14	30	4.7%	0.43 [0.19, 0.96]
Xu <i>et al</i>	11	30	5	30	4.0%	2.20 [0.87, 5.57]
Ding <i>et al</i>	5	30	3	30	2.2%	1.67 [0.44, 6.36]
Shi <i>et al</i>	12	40	2	40	2.0%	6.00 [1.43, 25.11]
Zheng <i>et al</i>	13	65	2	66	2.0%	6.60 [1.55, 28.10]
Yu <i>et al</i>	27	97	4	85	3.5%	5.91 [2.16, 16.22]
Ye <i>et al</i>	3	46	0	46	0.5%	7.00 [0.37, 131.81]
Liu <i>et al</i>	3	20	0	20	0.5%	7.00 [0.38, 127.32]
Subtotal (95% CI)		358		347	19.5%	2.86 [1.16, 7.03]
Total events	80		30			

Heterogeneity: $\tau^2 = 1.10$; $\chi^2 = 25.62$, $df = 7$ ($P = 0.0006$); $I^2 = 73\%$
 Test for overall effect: $Z = 2.29$ ($P = 0.02$)

1.3.4 24 wk

Zhu <i>et al</i>	8	30	10	30	5.0%	0.80 [0.37, 1.74]
Xu <i>et al</i>	14	30	6	30	4.7%	2.33 [1.04, 5.25]
Ding <i>et al</i>	7	30	5	30	3.4%	1.40 [0.50, 3.92]
Shi <i>et al</i>	18	40	13	40	7.2%	1.38 [0.79, 2.43]
Zheng <i>et al</i>	24	65	19	66	8.0%	1.28 [0.78, 2.10]
Yu <i>et al</i>	44	92	27	85	9.7%	1.51 [1.03, 2.20]
Ye <i>et al</i>	10	46	3	46	2.6%	3.33 [0.98, 11.33]
Liu <i>et al</i>	4	20	2	20	1.7%	2.00 [0.41, 9.71]
Subtotal (95% CI)		353		347	42.3%	1.45 [1.16, 1.82]
Total events	129		85			

Heterogeneity: $\tau^2 = 0.00$; $\chi^2 = 5.83$, $df = 7$ ($P = 0.56$); $I^2 = 0\%$
 Test for overall effect: $Z = 3.22$ ($P = 0.001$)

1.3.6 48 wk

Ding <i>et al</i>	10	30	6	30	4.3%	1.67 [0.69, 4.00]
Yu <i>et al</i>	47	92	35	85	10.5%	1.24 [0.90, 1.71]
Ye <i>et al</i>	20	46	10	46	6.3%	2.00 [1.05, 3.79]
Liu <i>et al</i>	8	20	2	20	2.0%	4.00 [0.97, 16.55]
Zhou <i>et al</i>	8	52	7	63	3.8%	1.38 [0.54, 3.56]
Subtotal (95% CI)		240		244	27.0%	1.45 [1.11, 1.89]
Total events	93		60			

Heterogeneity: $\tau^2 = 0.00$; $\chi^2 = 4.06$, $df = 4$ ($P = 0.40$); $I^2 = 2\%$
 Test for overall effect: $Z = 2.75$ ($P = 0.006$)

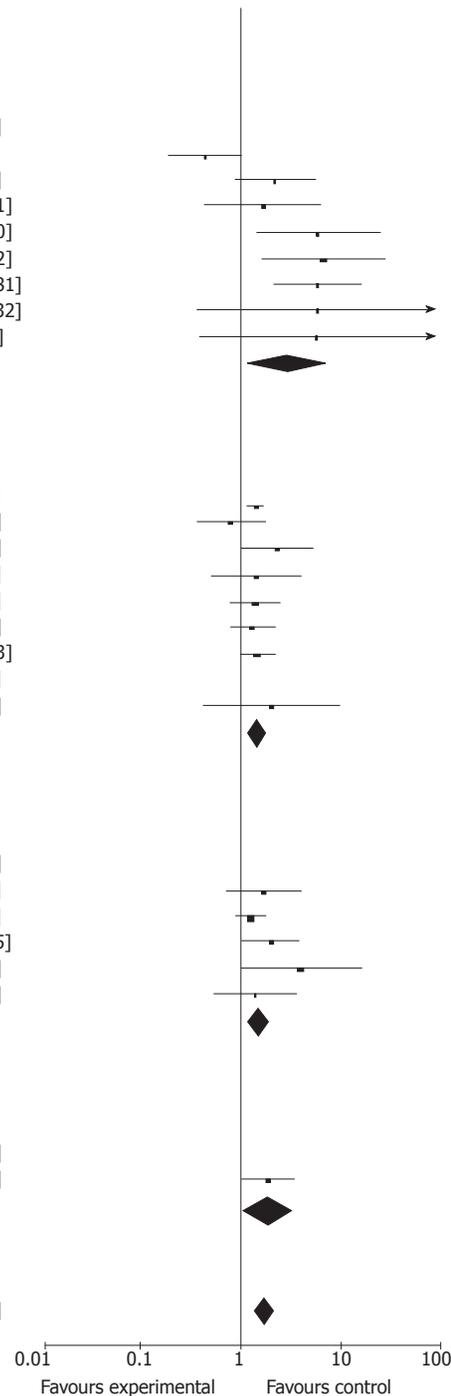
1.3.7 52 wk

Huang <i>et al</i>	26	90	14	90	7.0%	1.86 [1.04, 3.32]
Subtotal (95% CI)		90		90	7.0%	1.86 [1.04, 3.32]
Total events	26		14			

Heterogeneity: Not applicable
 Test for overall effect: $Z = 2.09$ ($P = 0.04$)

Total (95% CI)		1305		1278	100.0%	1.68 [1.35, 2.09]
Total events	338		194			

Heterogeneity: $\tau^2 = 0.09$; $\chi^2 = 38.84$, $df = 25$ ($P = 0.04$); $I^2 = 36\%$
 Test for overall effect: $Z = 4.66$ ($P < 0.00001$)



D

Study of subgroup	LDT		ETV		Weight	Risk ratio M-H, Fixed, 95% CI	Risk ratio M-H, Fixed, 95% CI
	Events	Total	Events	Total			
1.4.1 4 wk							
Yu <i>et al</i>	0	92	0	85		Not estimable	
Liu <i>et al</i>	0	20	0	20		Not estimable	
Subtotal (95% CI)		112		105		Not estimable	
Total events	0		0				
Heterogeneity: Not applicable Test for overall effect: Not applicable							
1.4.2 8 wk							
Zhang <i>et al</i>	3	75	1	65	0.6%	2.60 [0.28, 24.39]	
Zhao <i>et al</i>	2	42	1	39	0.5%	1.86 [0.18, 19.68]	
Yu <i>et al</i>	1	92	0	85	0.3%	2.77 [0.11, 67.19]	
Subtotal (95% CI)		209		189	1.4%	2.34 [0.55, 9.92]	
Total events	6		2				
Heterogeneity: $\chi^2 = 0.06$, $df = 2$ ($P = 0.97$); $I^2 = 0\%$ Test for overall effect: $Z = 1.16$ ($P = 0.25$)							

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1.4.3 12 wk

Zhu <i>et al</i>	4	30	11	30	5.8%	0.36 [0.13, 1.01]
Xu <i>et al</i>	8	30	2	30	1.1%	4.00 [0.92, 17.30]
Zhang <i>et al</i>	6	75	3	65	1.7%	1.73 [0.45, 6.66]
Shi <i>et al</i>	8	40	2	40	1.1%	4.00 [0.90, 17.68]
Zhao <i>et al</i>	3	42	2	39	1.1%	1.39 [0.25, 7.90]
Zheng <i>et al</i>	9	65	2	66	1.0%	4.57 [1.03, 20.34]
Yu <i>et al</i>	21	92	5	85	2.7%	3.88 [1.53, 9.83]
Ye <i>et al</i>	0	46	0	46		Not estimable
Liu <i>et al</i>	0	20	0	20		Not estimable
Subtotal (95% CI)		440		421	14.5%	2.10 [1.36, 3.24]
Total events	59		27			

Heterogeneity: $\chi^2 = 15.69$, $df = 6$ ($P = 0.02$); $I^2 = 62\%$
 Test for overall effect: $Z = 3.36$ ($P = 0.0008$)

1.4.4 24 wk

Zhu <i>et al</i>	8	30	6	30	3.2%	1.33 [0.53, 3.38]
Xu <i>et al</i>	12	30	6	30	3.2%	2.00 [0.86, 4.63]
Zhao <i>et al</i>	7	36	6	36	3.2%	1.17 [0.43, 3.13]
Zhang <i>et al</i>	12	75	6	65	3.4%	1.73 [0.69, 4.36]
Shi <i>et al</i>	11	40	7	40	3.7%	1.57 [0.68, 3.64]
Zhao <i>et al</i>	6	42	4	39	2.2%	1.39 [0.42, 4.57]
Zheng <i>et al</i>	16	65	9	66	4.7%	1.81 [0.86, 3.79]
Yu <i>et al</i>	26	92	14	85	7.7%	1.72 [0.96, 3.06]
Ye <i>et al</i>	7	46	2	46	1.1%	3.50 [0.77, 15.96]
Liu <i>et al</i>	2	20	0	20	0.3%	5.00 [0.26, 98.00]
Subtotal (95% CI)		476		457	32.6%	1.71 [1.29, 2.28]
Total events	107		60			

Heterogeneity: $\chi^2 = 2.52$, $df = 9$ ($P = 0.98$); $I^2 = 0\%$
 Test for overall effect: $Z = 3.71$ ($P = 0.0002$)

1.4.5 48 wk

Zhao <i>et al</i>	10	36	7	36	3.7%	1.43 [0.61, 3.34]
Ding <i>et al</i>	8	30	5	30	2.6%	1.60 [0.59, 4.33]
Zhao <i>et al</i>	15	42	9	39	4.9%	1.55 [0.77, 3.12]
Yu <i>et al</i>	37	92	18	85	9.9%	1.90 [1.18, 3.07]
Ye <i>et al</i>	12	46	4	46	2.1%	3.00 [1.04, 8.62]
Liu <i>et al</i>	4	20	0	20	0.3%	9.00 [0.52, 156.91]
Zhou <i>et al</i>	3	52	3	63	1.4%	1.21 [0.26, 5.75]
Subtotal (95% CI)		318		319	25.0%	1.86 [1.36, 2.54]
Total events	89		46			

Heterogeneity: $\chi^2 = 2.97$, $df = 6$ ($P = 0.81$); $I^2 = 0\%$
 Test for overall effect: $Z = 3.89$ ($P < 0.0001$)

1.4.6 52 wk

Huang <i>et al</i>	25	90	13	90	6.9%	1.92 [1.05, 3.52]
Zhang <i>et al</i>	23	75	11	65	6.2%	1.81 [0.96, 3.43]
Subtotal (95% CI)		165		155	13.1%	1.87 [1.21, 2.90]
Total events	48		24			

Heterogeneity: $\chi^2 = 0.02$, $df = 1$ ($P = 0.89$); $I^2 = 0\%$
 Test for overall effect: $Z = 2.80$ ($P = 0.005$)

1.4.7 60 wk

Zhao <i>et al</i>	15	42	9	39	4.9%	1.55 [0.77, 3.12]
Subtotal (95% CI)		42		39	4.9%	1.55 [0.77, 3.12]
Total events	15		9			

Heterogeneity: Not applicable
 Test for overall effect: $Z = 1.22$ ($P = 0.22$)

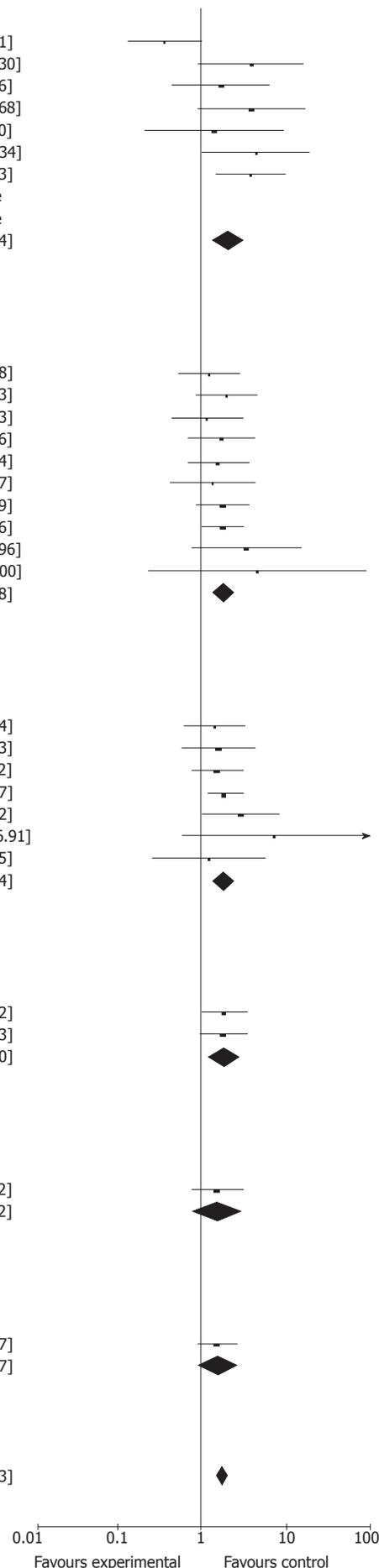
1.4.8 72 wk

Zhang <i>et al</i>	27	75	15	65	8.5%	1.56 [0.91, 2.67]
Subtotal (95% CI)		75		65	8.5%	1.56 [0.91, 2.67]
Total events	27		15			

Heterogeneity: Not applicable
 Test for overall effect: $Z = 1.62$ ($P = 0.10$)

Total (95% CI)		1837		1750	100.0%	1.81 [1.55, 2.13]
Total events	351		183			

Heterogeneity: $\chi^2 = 22.15$, $df = 30$ ($P = 0.85$); $I^2 = 0\%$
 Test for overall effect: $Z = 7.29$ ($P < 0.00001$)
 Test for subgroup differences: Not applicable.



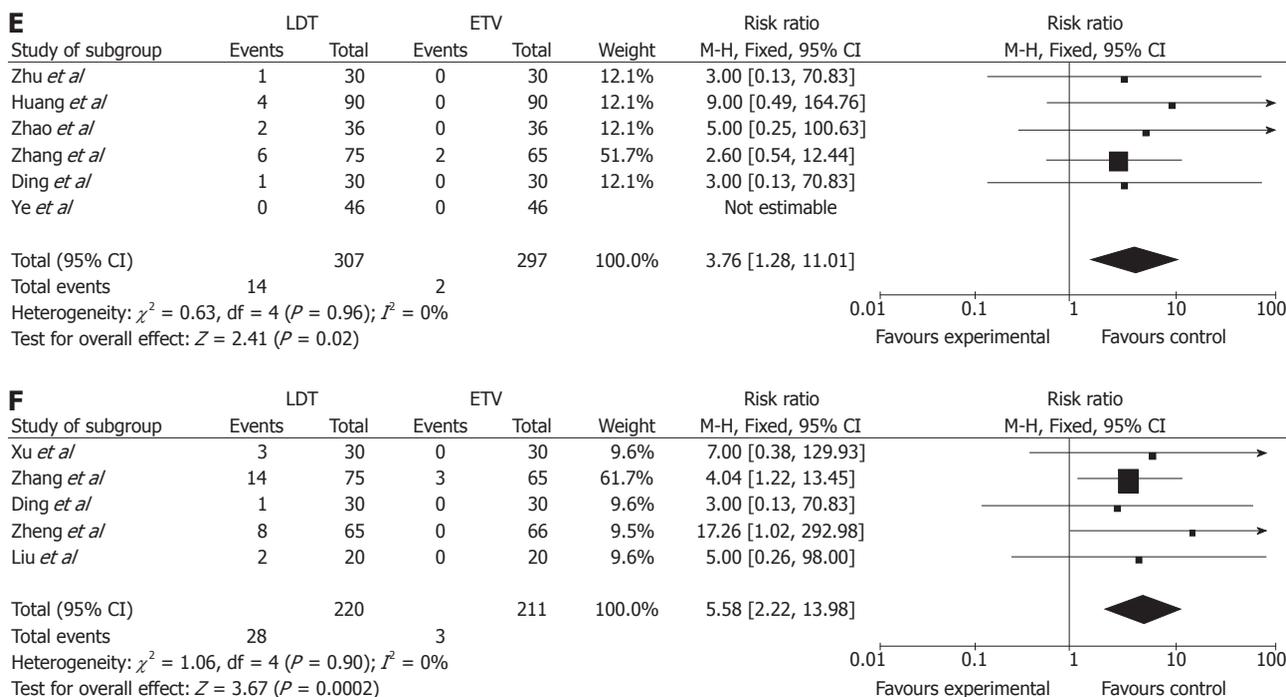


Figure 2 Meta-analysis of the two groups. A: Hepatitis B virus DNA undetectability; B: Alanine aminotransferase normalization; C: Hepatitis B e antigen (HBeAg) loss; D: HBeAg seroconversion; E: Drug-resistance; F: Increased creatine kinase (CK). ETV: Entecavir; LDT: Telbivudine.

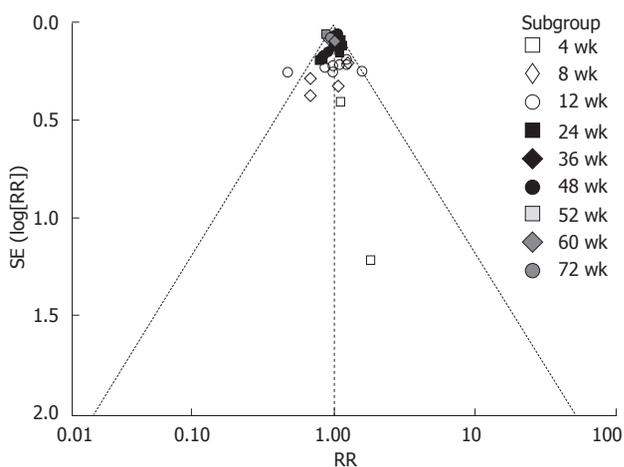


Figure 3 Funnel plots of the two groups in hepatitis B virus DNA undetectability. RR: Relative risk;

es, drug-resistance and adverse reactions were performed to examine the association between pre-specified characteristics (treatment duration) and the therapeutic effect of the two drugs.

HBV DNA level is a primary prognostic marker for the treatment of patients with CHB^[25,26]. The early and sustained suppression of HBV DNA replication is associated with improved long-term rates of virological, serological and biochemical responses. Rapidly and effectively suppressing HBV DNA replication can decrease the incidence of liver cirrhosis (LC), HCC and drug-resistance^[27,28]. The results of the meta-analysis showed that in various treatment durations (4 wk, 8 wk, 12 wk, 24 wk, 36 wk, 48 wk, 52 wk, 60 wk and 72 wk), there were no statistical differences in the rate of HBV DNA undetect-

Table 2 Sensitivity analysis		
Index	Total HBeAg loss	
	OR (95% CI)	P value
Excluding the minimum sample size trials ^[18]	1.64 (1.31, 2.05)	< 0.00 010
Using random-effect model	1.68 (1.35, 2.09)	< 0.00 001
Using fixed-effect model	1.69 (1.46, 1.97)	< 0.00 001

OR: Odds ratio; HBeAg: Hepatitis B e antigen.

ability between the two groups. This suggested that both LDT and ETV have rapid and effective anti-viral activity and the result is similar with a large sample size study^[29]. In addition, there was also no significant difference in the rate of ALT normalization between the two drugs.

HBeAg is a protein expressed by *pre-C* gene. HBeAg loss occurs with the rise of immunomodulatory effect which can suppress HBV DNA replication. HBeAg seroconversion has been established as a key marker of treatment response and is associated with improved clinical outcomes. It is one of the significant withdrawal standards for HBeAg-positive patients and suggests that patients can obtain sustained immune response^[30]. The results of the meta-analysis showed that at 4 wk and 8 wk of the treatment, the rates of HBeAg loss and HBeAg seroconversion were similar, with no statistical difference between the two groups, while at 12 wk, 24 wk, 48 wk and 52 wk, the rate was higher in the LDT group than in the ETV group, the difference being statistically significant. At 60 wk and 72 wk, there was no significant difference in the rate of HBeAg seroconversion between the two groups. These results suggested that the rates of HBeAg loss and HBeAg seroconversion in the short-term and medium-

term treatment were higher in the LDT group than in the ETV group. So LDT can be used as a primary drug for HBeAg-positive patients. However, its long-term efficacy needs to be further explored.

The higher rate of HBeAg seroconversion during LDT treatment might be associated with the potential immunomodulatory effect of LDT. CHB is a viral as well as an immunological disease. Specific immune function is impaired in the patients with CHB. Many studies suggested that LDT promoted T-helper 1 cytokine and CD4+/CD8+ cell production, but only downregulated programmed death ligand 1, regulatory T cell and T-helper 2 cytokine production^[31-33]. These immunomodulatory effects increase the rate of HBeAg seroconversion.

ETV has a high genetic barrier to resistance^[34-36]. The meta-analysis (Figure 2E) showed that the rate of drug-resistance was higher in the LDT group (4.69%) than in the ETV group (0.75%), the difference being statistically significant between the two groups. ETV has a lower drug-resistance than LDT and it is preferred for long-term anti-HBV activity.

The meta-analysis (Figure 2F) showed no severe adverse reactions in the two groups. Although the rate of increased CK in the LDT group was higher than in the ETV group, CK can recover without any intervention, and does not influence the anti-HBV treatment. These results suggest that both LDT and ETV are safe and well tolerated.

COMMENTS

Background

Chronic hepatitis B (CHB) infection is a major health problem affecting over 350 million people worldwide. CHB can lead to a number of life-threatening conditions such as liver failure, liver cirrhosis and hepatocellular carcinoma. Recent studies have shown that telbivudine (LDT) and entecavir (ETV) are the strongest nucleoside analogues in the treatment of CHB. But there are few systematic reviews about the comparison of LDT and ETV.

Research frontiers

LDT is an orally bioavailable L-nucleoside. It can rapidly and effectively suppress HBV DNA replication, but it has a higher drug-resistance. ETV is a new generation nucleoside analogues. It has the advantage of a higher rate of HBV DNA suppression, low drug-resistance and high safety, especially in lamivudine-resistant CHB patients. But the rate of hepatitis B e antigen (HBeAg) loss and HBeAg seroconversion was very low, which is difficult to meet the withdrawal standards.

Innovations and breakthroughs

There are few systematic reviews about the efficacy of LDT and ETV in the CHB treatment. The authors conducted a meta-analysis of the included randomized controlled trials using the Cochrane methodology and explored the efficacy of LDT and ETV for clinical treatment of HBeAg-positive chronic hepatitis B.

Applications

The results of this meta-analysis suggest that LDT and ETV have similar virological and biomedical response, and both are safe and well tolerated. However, LDT has better serological response and higher rate of drug-resistance.

Peer review

This study reviewed 13 trials comparing the effects of telbivudine and entecavir for patients with chronic HBeAg-positive chronic hepatitis B infection. Based on their analyses, the authors conclude that LDT and ETV exert an effective antiviral effect on HBV. Regarding the undetectability and ALT normalization, there was no big difference between the two drugs. The analysis was carefully performed, and the results were clearly presented and summarized, which provided valuable advice for clinical treatment of CHB.

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Adjuvant probiotics improve the eradication effect of triple therapy for *Helicobacter pylori* infection

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Abstract

AIM: To investigate whether the addition of probiotics can improve the eradication effect of triple therapy for *Helicobacter pylori* (*H. pylori*) infection.

METHODS: This open randomized trial recruited 234 *H. pylori* positive gastritis patients from seven local centers. The patients were randomized to one-week standard triple therapy (omeprazole 20 mg *bid*, clarithro-

mycin 500 mg *bid*, and amoxicillin 1000 mg *bid*; OCA group, $n = 79$); two weeks of pre-treatment with probiotics, containing 3×10^7 *Lactobacillus acidophilus* per day, prior to one week of triple therapy (POCA group, $n = 78$); or one week of triple therapy followed by two weeks of the same probiotics (OCAP group, $n = 77$). Successful eradication was defined as a negative C13 or C14 urease breath test four weeks after triple therapy. Patients were asked to report associated symptoms at baseline and during follow-up, and side effects related to therapy were recorded. Data were analyzed by both intention-to-treat (ITT) and per-protocol (PP) methods.

RESULTS: PP analysis involved 228 patients, 78 in the OCA, 76 in the POCA and 74 in the OCAP group. Successful eradication was observed in 171 patients; by PP analysis, the eradication rates were significantly higher ($P = 0.007$ each) in the POCA (62/76; 81.6%, 95% CI 72.8%-90.4%) and OCAP (61/74; 82.4%, 95% CI 73.6%-91.2%) groups than in the OCA group (48/78; 61.5%, 95% CI 50.6%-72.4%). ITT analysis also showed that eradication rates were significantly higher in the POCA (62/78; 79.5%, 95% CI 70.4%-88.6%) and OCAP (61/77; 79.2%, 95% CI 70%-88.4%) groups than in the OCA group (48/79; 60.8%, 95% CI 49.9%-71.7%), ($P = 0.014$ and $P = 0.015$). The symptom relieving rates in the POCA, OCAP and OCA groups were 85.5%, 89.2% and 87.2%, respectively. Only one of the 228 patients experienced an adverse reaction.

CONCLUSION: Administration of probiotics before or after standard triple therapy may improve *H. pylori* eradication rates.

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Key words: *Helicobacter pylori*; Probiotic; Eradication

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INTRODUCTION

Eradication therapy has been widely used since *Helicobacter pylori* (*H. pylori*) was recognized as a major cause of peptic ulcers, gastric atrophy and stomach neoplasms^[1]. However, the classic one-week triple therapy, consisting of a proton pump inhibitor (PPI), clarithromycin and amoxicillin, has become less effective, with eradication rates as low as 50%-70%^[2,3], especially in areas with a high prevalence of clarithromycin resistance^[4]. For example, in Shanghai from 2000 to 2009, the resistance rates of *H. pylori* to clarithromycin and levofloxacin increased from 8.6% to 20.7% and from 10.3% to 32.5%, respectively, whereas the resistance rates to metronidazole remained stable at 40%-50%^[5]. In addition, the high rates of antibiotic-associated side-effects may result in poor patient compliance^[6]. Administration of probiotics to adults^[7,8] and children^[9,10] has been reported to improve *H. pylori* eradication rates and to reduce the side-effects of PPI-based eradicating therapies. However, the timing of probiotic administration relative to the triple therapy has not been well characterized. In most studies, probiotics were started immediately after the start of triple therapy and were administered for one^[11] to four^[12] weeks. Bacteria of the *Lactobacillus* family were shown to inhibit *H. pylori* colonization of the stomach^[13,14] and the binding of *H. pylori* to its glycolipid receptors^[15]. To better investigate the effects of probiotics, in the present work we examined whether probiotics administered before or after eradication therapy to *H. pylori*-infected individuals could better enhance the eradication rate.

Lactobacillus acidophilus (*L. acidophilus*) is a common bacterium that resides in the human gastrointestinal tract and is added to food and milk. Compared with other *Lactobacillus* species, *L. acidophilus* has shown promising effects in the treatment of *H. pylori*^[16,17]. We therefore used a compound probiotic, with *L. acidophilus* as the predominant bacterium. In this multicenter, open and randomized trial, we assessed whether probiotics, administered before or after *H. pylori* eradication therapy, could enhance eradication rates. We found that both approaches improved eradication rates, although the rate was somewhat higher in patients administered probiotics after than before triple therapy.

MATERIALS AND METHODS

Patients

The patient population consisted of individuals, aged

Table 1 Demographic characteristics of *Helicobacter pylori* infected patients in probiotic pre-treated, post-treated and standard therapy groups (per protocol set, *n* = 228)

	POCA (<i>n</i> = 76)	OCAP (<i>n</i> = 74)	OCA (<i>n</i> = 78)	<i>P</i> value
Age, yr	44.9 ± 13.8	48.2 ± 12.2	48.0 ± 13.3	0.227 ¹
Sex (male %)	32 (42.1)	26 (35.1)	33 (42.3)	0.594 ²
<i>H. pylori</i> test (pre-therapy)				0.949 ²
¹³ C-UBT	13 (17.1)	10 (13.5)	14 (17.9)	
¹⁴ C-UBT	23 (30.3)	24 (32.4)	21 (26.9)	
RUT	34 (44.7)	34 (45.9)	34 (43.6)	
HE	6 (7.9)	6 (8.1)	9 (11.5)	
<i>H. pylori</i> test (post-therapy)				0.879 ²
¹³ C-UBT	38 (50.0)	34 (45.9)	38 (48.7)	
¹⁴ C-UBT	38 (50.0)	40 (54.1)	40 (51.3)	
Mean follow-up time, d	58.2 ± 15.5	44.9 ± 12.3	43.2 ± 11.0	< 0.001 ¹

Data are presented as mean ± SD or *n* (%). ¹Student's *t* test; ²Fisher's exact test. *H. pylori*: *Helicobacter pylori*; UBT: Urea breath test; RUT: Rapid urease test; HE: Hematoxylin and eosin stain; POCA: Probiotic pre-treated group; OCAP: Probiotic post-treated group; OCA: Standard therapy group.

18-65 years, newly diagnosed with gastritis or dyspepsia and infected with *H. pylori*. *H. pylori* infection was determined by rapid urease tests (RUT) during endoscopy; by pathologic examination of antrum specimens; or by C13 or C14 urea breath tests (UBT)^[18]. The method used by each participating center is shown in Table 1. Candidates for inclusion were screened from November 2008 to July 2009. Exclusion criteria were: (1) neoplasm or peptic ulcer with or without complications, or gastroesophageal reflux disease; (2) previous failure of *H. pylori* eradication or a history of gastric surgery; (3) consumption of acid-inhibitors, bismuth compounds, antibiotics, or probiotics during the previous 4 wk; and (4) known allergy to antibiotics or probiotics.

Study design

The enrolled patients were randomized 1:1:1 into three groups (Figure 1). One group received standard triple therapy, consisting of omeprazole 20 mg *bid*, clarithromycin 500 mg *bid*, and amoxicillin 1000 mg *bid* for 7 d (OCA group). The second group received two weeks of pre-treatment with probiotics, containing 3×10^7 *L. acidophilus* per day, prior to one week of triple therapy (POCA group), and the third group received one week of triple therapy followed by two weeks of the same probiotics (OCAP group).

Due to uncertainties about the effects of the addition of probiotics, we estimated sample size by the non-inferiority method. Assuming eradication rates of 90% and 85% in probiotic-combined and OCA groups ($\alpha = 0.05$ and $\beta = 0.2$), respectively, at least 70 individuals per group would be required. We calculated a final sample size of 240, including 30 patients at each center and 60 at the leading institute. The randomization number was produced by SPSS 18.0 software with a block of three and assigned to each center.

Each probiotic tablet (Yi Jun Kang®, He Li Pharm. Co. Ltd. China), weighing 0.5 g, contained 5×10^6

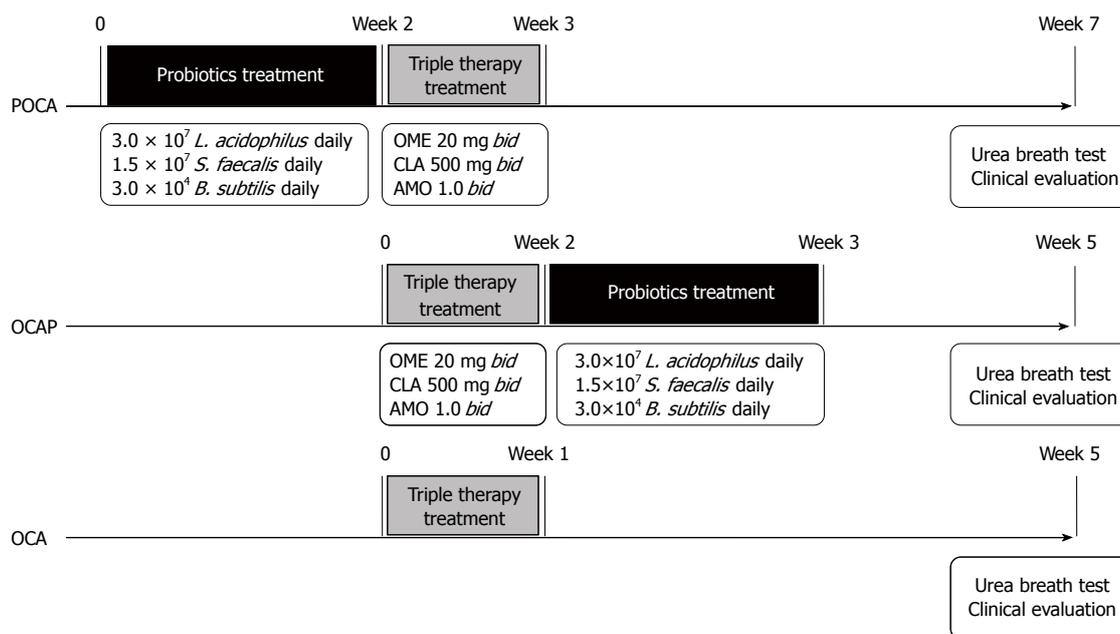


Figure 1 Flow chart of the study. OME: Omeprazole; CLA: Clarithromycin; AMO: Amoxicillin; POCA: Probiotic pre-treated group; OCAP: Probiotic post-treated group; OCA: Standard therapy group.

Table 2 *Helicobacter pylori* eradication results in the participating centers

Center	UBT	n	<i>H. pylori</i> eradication rate (%)			
			POCA	OCAP	OCA	Total
01	C13	52	83.3 (15/18)	80.0 (12/15)	52.6 (10/19)	71.2
02	C13	29	80.0 (8/10)	90.0 (9/10)	88.9 (8/9)	86.2
03	C14	30	70.0 (7/10)	50.0 (5/10)	30.0 (3/10)	50.0
04	C13	29	80.0 (8/10)	88.9 (8/9)	70.0 (7/10)	79.3
05	C14	30	100 (10/10)	70.0 (7/10)	60.0 (6/10)	76.7
06	C14	29	88.9 (8/9)	100 (10/10)	40.0 (4/10)	75.9
07	C14	29	66.7 (6/9)	100 (10/10)	100 (10/10)	89.7

H. pylori: *Helicobacter pylori*; UBT: Urea breath test; POCA: Probiotic pre-treated group; OCAP: Probiotic post-treated group; OCA: Standard therapy group.

L. acidophilus, 2.5×10^6 *Streptococcus faecalis* (*S. faecalis*) and 5×10^3 *Bacillus subtilis* (*B. subtilis*). Patients were instructed to take two of these tablets 30 min after meal, three times a day. Boxes containing a sufficient number of these tablets for the study period were provided to each patient by the probiotics producer.

Patient compliance was evaluated by counting the number of tablets returned, with an error rate lower than 5% considered acceptable. Four weeks after the completion of *H. pylori* eradication therapy, *H. pylori* status was assessed using a C13 or C14 based UBT (Table 2), with complete eradication defined as < 4.0 dpm and < 100 dpm, respectively.

The study was performed in accordance with good clinical practice and the guidelines of the Declaration of Helsinki. All patients provided a written informed consent and the study protocol was approved by the Ethics Committee of Changhai Hospital (CHEC2008-041).

Symptoms and safety evaluation

All patients were asked to report associated symptoms at baseline and during follow-up, including abdominal pain, acid regurgitation, heartburn, nausea, vomiting, abdominal distension and diarrhea. Any side effect related to therapy was recorded and analyzed.

Statistical analysis

Data were computerized and analyzed with SPSS 18.0 software (IBM Corporation, NY, United States). The intention-to-treat (ITT) population consisted of all randomized patients, whereas the per-protocol (PP) population consisted of subjects who completed the entire study without any major protocol violations. The baseline demographic and clinical characteristics of the ITT and PP populations, and of the three groups of randomized patients in each population, were compared using Student's *t* tests and Fisher's exact tests, as warranted. Eradication rates in the three groups were compared by Fisher's exact tests. The eradication rate and 95% confidence intervals in each group were calculated for both the PP and ITT populations. All statistical tests were two-sided, with a 5% level of significance.

RESULTS

We enrolled 234 *H. pylori* positive subjects. Of them, 228 were included in the PP population, with 76, 74 and 78 subjects in the POCA, OCAP and OCA groups, respectively (Figure 2). Two patients were randomized in error, including one patient aged 81 years randomized to OCAP and one patient with a bleeding duodenal ulcer randomized to POCA. One subject in the OCAP group was reassessed by RUT. Two patients showed a

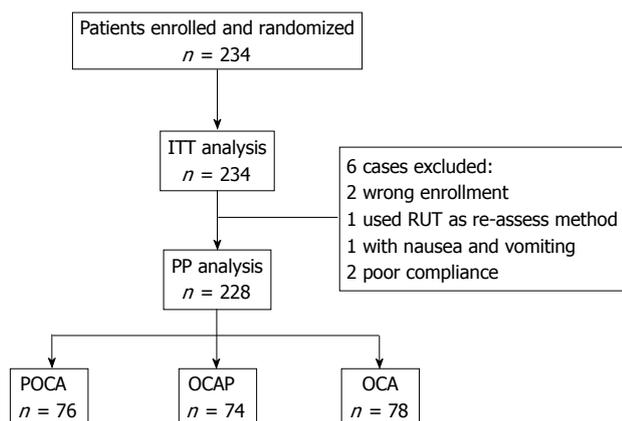


Figure 2 Flow diagram showing numbers of patients enrolled and missed for per protocol and intention-to-treat analyses. ITT: Intention-to-treat; PP: Per-protocol; RUT: Rapid urease test; POCA: Probiotic pre-treated group; OCAP: Probiotic post-treated group; OCA: Standard therapy group.

poor compliance with treatment, one each in the OCAP and OCA groups. One patient in the POCA group complained of nausea and vomiting on the fourth day of triple therapy and discontinued the study. The baseline demographic and clinical characteristics of the 228 enrolled patients are shown in Table 1. There were no significant differences in mean age, sex distribution, and distribution of *H. pylori* detecting methods. *H. pylori* was initially assessed by RUT in 102 (44.7%) patients, by UBT in 105 (46.1%) and by pathology in 21 (9.2%). C13 UBT was used for the second assessment of *H. pylori* in about 50% of patients, and C14 UBT in the other 50%. The mean follow-up time was significantly longer in the POCA (58.2 d) than in the OCAP and OCA (44 d) groups, due to the study design and treatment protocol (Figure 1).

Helicobacter pylori eradication

Four weeks after the completion of triple therapy, *H. pylori* test on C13 or C14 UBT was negative in 171 (75%) of the 228 patients. There were significant differences among the groups, however. PP analysis showed that the eradication rates were significantly higher in the POCA (81.6%, 95% CI: 72.8%-90.4%) and OCAP (82.4%, 95% CI: 73.6%-91.2%) groups than in the OCA (61.5%, 95% CI: 50.6%-72.4%) group ($P = 0.007$ each, Figure 3). ITT analysis showed that, compared with the OCA group (48/79; 60.8%, 95% CI: 49.9%-71.7%), success rates were significantly higher in the POCA (62/78; 79.5%, 95% CI: 70.4%-88.6%, $P = 0.014$) and OCAP (61/77; 79.2%, 95% CI: 70%-88.4%, $P = 0.015$) groups. Further analysis showed that probiotic supplementation was associated with a higher eradication rate in all centers except for Centers 2 and 7 (Table 2).

Symptoms and safety assessment

The symptom relieving rates in the POCA, OCAP and OCA groups were 85.5% (65/76), 89.2% (66/74) and 87.2% (68/78), respectively. Only one of the 228 (0.4%)

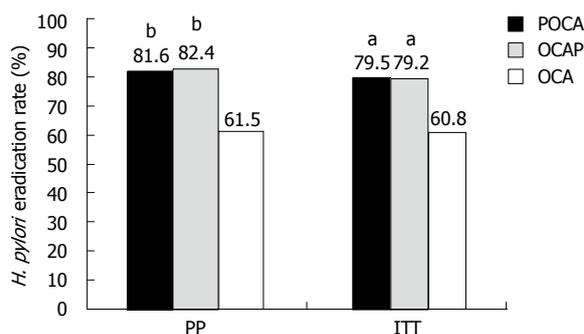


Figure 3 *Helicobacter pylori* eradication rates in groups treated with triple therapy, with or without probiotics. ^a $P < 0.05$, ^b $P < 0.01$ vs the standard therapy group (OCA group) by Fisher's exact test. *H. pylori*: *Helicobacter pylori*; POCA: Probiotic pre-treated group; OCAP: Probiotic post-treated group; PP: Per protocol; ITT: Intention-to-treat.

patients experienced an adverse effect. This patient, randomized to the POCA group, experienced severe vomiting on the fourth day of antibiotic treatment. Side-effect rates did not differ significantly among the three groups.

DISCUSSION

To our knowledge, this study is the first to show that administration of probiotics, either before or after traditional triple therapy, enhances *H. pylori* eradication rates. We found, in particular, that probiotic treatment after triple therapy significantly increased the eradication rate. Other than the studies, in which probiotics were started at the same time as PPI-based therapy, only one study has found that four weeks of pre-treatment with *Lactobacillus* and *Bifidobacterium*-containing yogurt resulted in a higher *H. pylori* eradication rate than quadruple therapy alone (85% vs 71%, $P < 0.05$)^[19]. That study, however, assessed second-line therapy for *H. pylori*, whereas we evaluated first-line treatment. We found that pre-treatment with *L. acidophilus*, *S. faecalis* and *B. subtilis* for two weeks prior to triple therapy improved the eradication rate, from 60.8% to 79.5%. Pretreatment with probiotics may decrease *H. pylori* load despite antimicrobial resistance, thus improving the efficacy of eradication therapy^[19].

Pretreatment with probiotics has been found to significantly reduce the *H. pylori* colonization rate in mice, from 100% to 50% ($P = 0.02$)^[20] and to reduce inflammation in the gastric antrum. In humans, four weeks of treatment with *Lactobacillus reuteri* ATCC 55730 reduced *H. pylori* load and decreased the occurrence of dyspeptic symptoms^[21]. Although our results confirm previous findings^[22], that pretreatment with suitable probiotics could benefit *H. pylori* infected patients, the optimal treatment period (two or four weeks) and optimal dose of probiotics have not been determined.

It is not clear whether simultaneously administered probiotics will be destroyed by anti-*H. pylori* drugs. Although pre-treatment with probiotics may constitute a solution, it results in a prolonged course of therapy, two

weeks longer than the routine method. It is also unclear whether pre-treatment with probiotics could reduce the rates of antibiotic-related side effects to those observed with combined therapy^[23-26]. Large clinical trials are required to compare the side-effect reduction rates of patients administered probiotics before and during eradication therapy.

Another possible regimen is to treat patients with probiotics after the completion of eradication therapy. Interestingly, we found that this regimen significantly increased the *H. pylori* eradication rate compared with eradication therapy alone, from 60.8% to 79.2%. To compare the outcome, we performed repeat UBT in our OCAP group four weeks after triple therapy (two weeks after probiotic therapy). Probiotics may inhibit residual *H. pylori* and mask UBT tests as 'false negatives', similar to the effects of PPIs. Further long-term studies are needed to determine if *H. pylori* eradication is true and permanent.

The recommended species and number of bacteria in probiotics have not been determined^[27,28]. An 833-fold higher dose of *L. acidophilus* (2.5×10^{10} /d) than ours, when added to triple therapy containing esomeprazole, amoxicillin, and clarithromycin, failed to increase the eradication rate observed with triple therapy alone (an eradication rate, 83.9% *vs* 80.6%, $P = 0.74$)^[29]. This may have been due to the relatively small number of subjects, the short duration of probiotic administration (8 d) and the use of a single species of bacteria. Probiotic mixtures appear to be effective against a wide range of end points, including treatment of *H. pylori* infection, with multi-strain probiotics showing greater efficacy than single strains, including strains that are components of the mixtures themselves^[30]. *H. pylori* may be less sensitive to single than to multiple probiotics, similar to resistance to antibiotics.

The probiotic mixture we tested is easily acquired in China and has been used widely to treat diarrhea and inflammatory bowel disease. If our findings are confirmed, Chinese *H. pylori* infected patients may benefit from probiotic treatment.

Due to the low incidence of side effects we observed, we could not evaluate whether probiotics reduce the adverse effects of antibiotics. However, safety evaluation was not a primary end point of this study. Other limitations include the lack of blinding; the absence of a placebo group; the lack of standardization of the *H. pylori* assay method; the short follow-up period; and the lack of confirmation of *H. pylori* load by biopsy or culture. Nevertheless, this study is the first to provide information on the timing of probiotics relative to routine eradication therapy in *H. pylori* infected patients. We found that both pre- and post-treatment with probiotics increased the eradication rates of triple therapy, with post-treatment being more effective than pre-treatment.

In conclusion, our results suggest that either pre- or post-administration of probiotics may improve the *H. pylori* eradication effect of standard triple therapy.

COMMENTS

Background

Eradication therapy has been widely used since *Helicobacter pylori* (*H. pylori*) was first recognized as a major cause of gastric diseases. Classic triple therapy, including a proton pump inhibitor (PPI) and the antibiotics clarithromycin and amoxicillin, has become less effective over time. Addition of probiotics may improve the eradication rates of triple therapy, but the optimal timing of probiotics remains unclear.

Research frontiers

Pretreatment with probiotics has been shown to reduce *H. pylori* colonization in animals and humans.

Innovations and breakthroughs

This is the first study to show that both pre- and, particularly, post-treatment with probiotics increased the eradication rates of triple therapy. These results suggest that mixed probiotics may improve the *H. pylori* eradication effect of standard triple therapy with fewer adverse effects.

Applications

These results provide a guide for better use of probiotics, in combination with triple therapy, for the clinical treatment of *H. pylori*-infected patients. This new regimen may be particularly applicable to *H. pylori* eradication in areas of high resistance to antibiotics.

Peer review

This study was designed to determine whether the administration of probiotics, before or after standard triple therapy, could improve *H. pylori* eradication rates. Interestingly, both regimens were effective in improving the efficacy of standard triple therapy. The study is well designed and the results are interesting.

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Effects of warm ischemia time on biliary injury in rat liver transplantation

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Abstract

AIM: To investigate the effect of different secondary warm ischemia time (SWIT) on bile duct injury in liver-transplanted rats.

METHODS: Forty-eight male inbred Sprague-Dawley rats were randomly assigned into four groups: a sham-operation group and three groups with secondary biliary warm ischemia time of 0 min, 10 min and 20 min. A rat model of autologous liver transplantation under ether anesthesia was established, and six rats were killed in each group and blood samples and the median lobe of the liver were collected for assay at 6 h and 24 h after hepatic arterial reperfusion.

RESULTS: With prolongation of biliary warm ischemia time, the level of vascular endothelial growth factor-A was significantly decreased, and the value at 24 h was

higher than that at 6 h after hepatic arterial reperfusion, but with no significant difference. The extended biliary SWIT led to a significant increase in bile duct epithelial cell apoptosis, and a decrease in the number of blood vessels, the bile duct surrounding the blood vessels and bile duct epithelial cell proliferation in the early postoperative portal area. Pathologic examinations showed that inflammation of the rat portal area was aggravated, and biliary epithelial cell injury was significantly worsened.

CONCLUSION: A prolonged biliary warm ischemia time results in aggravated injury of the bile duct and the surrounding vascular plexus in rat autologous orthotopic liver transplantation.

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Key words: Bile duct; Liver; Transplantation; Warm ischemia; Rat

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Zhu XH, Pan JP, Wu YF, Ding YT. Effects of warm ischemia time on biliary injury in rat liver transplantation. *World J Gastroenterol* 2012; 18(43): 6308-6314 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i43/6308.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i43.6308>

INTRODUCTION

Orthotopic liver transplantation (OLT) has proven to be a successful treatment choice for patients with end-stage chronic or acute liver failure. However, biliary complications remain a significant source of morbidity and have

been associated with a mortality rate ranging from 8% to 15%^[1,2]. With improvement in surgical techniques, the incidence of anastomotic biliary strictures was markedly reduced, whereas non-anastomotic biliary strictures (NAS) have become a predominant biliary complication of liver allografts^[3]. Diffuse NAS after OLT have been termed as ischemic-type biliary complications, ischemic cholangitis and ischemic cholangiopathy. NAS remains the most challenging type of biliary complication as it is frequently therapy-resistant and often associated with long-term consequences^[4,5].

The cause of NAS is multifactorial, and ischemia/reperfusion injury of the biliary epithelium is considered as one of the major causes^[6]. Warm ischemic time in donors after cardiac death (DCD), in addition to subsequent cold ischemia-reperfusion injury, is believed to result in increased damage to biliary epithelial cells^[7,8]. The most commonly used procedure for revascularization of the liver graft in clinical practice is initial portal reperfusion and subsequent reconstruction of the hepatic artery. Compared with liver cells, the bile duct epithelial cells experience an extra ischemic process from portal venous recanalization to hepatic arterial recanalization, which is defined as the “secondary warm ischemia time (SWIT) in the biliary tract” or “relative warm ischemia time in the biliary tract”. This is a special phase of biliary warm ischemia in the graft. Because warm ischemia time in the harvesting of donor livers after cardiac death is inevitable, more and more studies have shifted their focus to the effect of SWIT on bile duct injury^[9].

The terminal branches of the hepatic artery are represented by either the extra- or intra-hepatic peribiliary arterial plexus (PBP). The function of the intrahepatic biliary tree is linked to its vascular supply sustained by PBP^[10]. Alterations of intrahepatic bile duct mass are associated with the architectural changes in the PBP. In this study, we investigated the impact of different SWITs on the bile duct and PBP in a rat autologous liver transplantation model.

MATERIALS AND METHODS

Animals and experimental groups

Forty-eight male inbred SD rats weighing 220–250 g were purchased from the Animal Center of Yangzhou University (Yangzhou, China). The rats were housed and fed at the Animal Center of Drum Tower Hospital for at least 7 d before transplantation for acclimatization to the environment. All rats were provided with standard laboratory chow and water and housed in accordance with institutional animal care policies. The rats were fasted for 8 h, but allowed free access to water before being used in the study.

The following experimental protocol was approved by the Animal Care and Use Committee of the Drum Tower Hospital and conformed to Guide for the Care and Use of Laboratory Animals from National Institutes of Health.

A rat model of autologous liver transplantation was

established using the technique of Wang *et al.*^[11] under ether anesthesia. Rats were randomly assigned into four groups according to the SWIT: a sham-operation group and three groups with the biliary SWIT of 0 min, 10 min and 20 min. In the sham-operation group (group I), the liver was mobilized without cold or warm ischemia-reperfusion injury to exclude the influence of surgery. In group II with no SWIT, simultaneous reperfusion was performed through the portal vein and hepatic artery after cold perfusion. In groups III and IV with SWIT of 10 min and 20 min, hepatic arterial perfusion was performed for 10 min and 20 min, respectively, after portal venous reperfusion.

Sample collection

At 6 h and 24 h after hepatic arterial reperfusion, 6 rats were killed in each group, and blood samples were collected via the infrahepatic vena cava, and the median lobe of liver was obtained for assay. The serum was separated and stored at -70 °C until analysis. After washing with cold saline solution, the liver samples were stored immediately in liquid nitrogen until analysis.

Assessment of vascular endothelial growth factor-A level

Vascular endothelial growth factor-A (VEGF-A) plasma levels of the samples collected at 6 h and 24 h after hepatic arterial reperfusion were determined with enzyme-linked immunosorbent assay kits (Ruiqi Biotechnology Co. Ltd, Shanghai, China) according to the manufacturer's instruction.

Bile ducts and blood vessels in portal area

The liver specimens were fixed with 10% formalin and embedded in paraffin. The liver tissues were cut into sequential slices of 2.5 mm. The bile ducts were immunolocalized by CK19 polyclonal antibody (Boside Biotechnology Co. Ltd, Wuhan, China), and the blood vessels were tagged with rabbit factor VIII-related antigen (Boaoseng Biotechnology Co. Ltd, Beijing, China). In the portal area, the number of bile ducts, blood vessels, and bile ducts with and without blood vessels were counted.

Assessment of cholangiocyte proliferation with proliferating-cell nuclear antigen immunolabeling

The number of proliferating-cell nuclear antigen (PCNA)-positive cells was used to evaluate cholangiocyte proliferation with immunohistochemistry. After rehydration, the silane-coated slides were treated with 0.3% H₂O₂ in methyl alcohol for 15 min and then briefly washed in phosphate buffer saline. They were then incubated overnight at 4 °C, with a 1:100 dilution of anti-PCNA monoclonal antibody, and subsequently incubated with Envision Plus Sunpoly-H III HRP rabbit/mouse kit (Boshide Biotech Co. Ltd., Wuhan, China) for 30 min at 37 °C. Finally, the sections were counterstained with hematoxylin and coverslipped. After staining, sections were analyzed in a coded fashion under a light microscope.

Table 1 Effect of different secondary warm ischemia times on vascular endothelial growth factor-A level (mean \pm SD)

Group	6 h	24 h
I	183.44 \pm 11.65	185.04 \pm 10.11
II	185.32 \pm 12.06	154.55 \pm 11.77 ^a
III	181.42 \pm 9.97	142.32 \pm 10.30 ^{a,c}
IV	177.93 \pm 10.40	126.44 \pm 16.03 ^{b,c,e}

^a*P* < 0.05 vs group I; ^b*P* < 0.01 vs group I; ^c*P* < 0.05 vs group II; ^e*P* < 0.05 vs group III.

PCNA protein expression appeared brown in the cell nucleus. The cholangiocyte proliferation index was measured as the number of PCNA-positive cholangiocytes per 100 cells under high magnification (\times 400).

Apoptosis of bile duct epithelial cells

Apoptosis of bile duct epithelial cells was identified by detecting DNA fragmentation *in situ* in serial sections at 6 h and 24 h after hepatic arterial reperfusion. DNA fragmentation was detected by terminal -deoxynucleotidyl transferase mediated nick end labeling (TUNEL) staining, which was performed on deparaffinized and dehydrated sections using the In Situ Cell Death Detection kit (Zhongshan Biomedical Technology Co., Beijing, China) according to the manufacturer's instructions. TUNEL-positive cholangiocytes displayed a characteristic morphology of apoptosis, including chromatin condensation, cell fragmentation and apoptotic bodies. Apoptotic cells were examined at original magnification \times 400 in 10 randomly selected fields per section. The apoptotic index was calculated as the percentage of apoptotic cells in the total number of cholangiocytes.

Histological evaluation of bile duct injury

Six liver specimens were collected at 24 h after hepatic arterial reperfusion in each group. The liver specimens for light microscopy were fixed with 10% formalin and embedded in paraffin. The sections were stained with hematoxylin and eosin for histological examination. Bile duct injury in the specimens was semiquantified by calculating a bile duct injury severity score (BDISS)^[12] based on the following three components: bile duct damage (graded as 0, absent; 1, mild; 2, moderate; 3, severe; modified from the Banff criteria for acute rejection); ductular proliferation (graded 0-3, using a similar scale as stated earlier); and cholestasis (graded 0-3, using a similar scale as stated earlier). This resulted in a minimal BDISS of zero and a maximum score of 9 points. All examinations were conducted by an experienced pathologist who was unaware of the other study data.

Statistical analysis

The results were expressed as mean \pm SD. Data were analyzed using the Statistical Analysis System (SAS Institute, Cary, NC, United States). One-way analysis of variance was used for multiple comparisons with Student-Newman-Keulstest. *P* < 0.05 was considered statistically significant.

RESULTS

Assessment of VEGF-A level

There was no significant difference in VEGF-A at 6 h postoperatively between group I and the other three groups, but there was a significant decrease in VEGF-A at 24 h in groups II-IV. The VEGF-A was lower in group IV than in groups II and III, and there were significant differences among these three groups (*P* < 0.05) (Table 1).

Bile ducts and blood vessels in the portal area

The number of bile ducts in each portal area in group I was 6.10 \pm 0.74, and the bile ducts were always accompanied by blood vessels. Compared with group I, there was a significant decrease in the number of bile ducts, blood vessels and bile ducts with blood vessels in the portal area, and a significant increase in bile ducts without blood vessels in the other three groups. No significant difference in the number of bile ducts and blood vessels was found at 6 h postoperatively among groups II, III and IV. The number of bile ducts, blood vessels and bile ducts with blood vessels in portal area at 24 h was significantly lower in group IV than in groups II and III (*P* < 0.05) (Table 2).

Assessment of cholangiocyte proliferation with PCNA immunolabeling

Compared with group I, the number of PCNA-positive cholangiocytes was significantly reduced at 6 h and 24 h postoperatively in groups II and III (*P* < 0.05), and a very significant decrease in cholangiocyte proliferation was observed in group IV (*P* < 0.01). The number of PCNA-positive cholangiocytes in group IV was lower than in groups II and III, and there were significant differences among these three groups at 6 h and 24 h postoperatively (*P* < 0.05). The number of PCNA-positive cholangiocytes at 24 h after hepatic arterial reperfusion was reduced in all groups compared with the result at 6 h, but no significant differences were noted (Table 3, Figure 1).

Apoptosis of bile duct epithelial cells

There were a few apoptotic bodies in the liver sections of group I. Compared with group II, a significant increase in apoptosis index was found at 6 h and 24 h postoperatively in groups III and IV (*P* < 0.05). The apoptosis index in group IV was significantly higher than in group III at 6 h postoperatively (*P* < 0.05). In groups II, III and IV, the apoptosis index at 24 h after hepatic arterial reperfusion was higher than that at 6 h, but no significant differences were noted (Table 3, Figure 2).

Histological evaluation of bile duct injury

The histological findings indicated that the degree of bile duct injury was mild in group I. The main bile duct injuries in group II included cholangiocytes lining in disarray with diversified morphous, edematous, inflammatory cell infiltration, migrated chromatin, and necrotic

Table 2 Effect of different secondary warm ischemia time on the number of bile ducts and blood vessels in portal area (mean \pm SD)

	Time (h)	Group I	Group II	Group III	Group IV
Bile ducts	6	6.10 \pm 0.74	3.40 \pm 1.17 ^a	2.80 \pm 0.79 ^a	2.60 \pm 0.51 ^a
	24		3.00 \pm 1.15 ^a	2.40 \pm 0.74 ^{a,c}	1.85 \pm 0.63 ^{a,c,e}
Blood vessels	6	5.50 \pm 0.94	2.90 \pm 0.74 ^a	2.40 \pm 0.88 ^a	2.00 \pm 0.84 ^a
	24		2.40 \pm 0.93 ^a	1.67 \pm 0.67 ^{a,c}	1.10 \pm 0.83 ^{a,c,e}
Bile ducts with blood vessels	6	5.42 \pm 1.35	2.20 \pm 1.23 ^a	1.60 \pm 0.70 ^a	1.40 \pm 0.81 ^a
	24		1.80 \pm 0.53 ^a	1.10 \pm 0.67 ^{a,c}	0.40 \pm 0.65 ^{a,c,e}
Bile ducts without blood vessels	6	0.65 \pm 0.42	1.0 \pm 0.67 ^a	1.20 \pm 0.70 ^a	1.31 \pm 0.92 ^a
	24		1.12 \pm 0.53 ^a	1.40 \pm 1.03 ^{a,c}	1.58 \pm 0.61 ^{a,c,e}

^a*P* < 0.05 vs group I; ^c*P* < 0.05 vs group II; ^e*P* < 0.05 vs group III.

Table 3 Effect of different secondary warm ischemia times on cholangiocyte proliferation, apoptosis index of bile duct epithelial cells and bile duct injury severity score (mean \pm SD)

Group	Cholangiocyte proliferation		Apoptosis index		Severity score	
	6 h	24 h	6 h	24 h	6 h	24 h
I	25.81 \pm 3.50	26.13 \pm 2.60	0.87 \pm 0.50	0.53 \pm 0.60	0	0
II	17.38 \pm 4.31 ^a	16.91 \pm 5.67 ^a	5.83 \pm 0.51	7.15 \pm 0.62	2.6 \pm 0.3	2.8 \pm 0.2
III	14.36 \pm 3.69 ^{a,c}	12.90 \pm 2.48 ^{a,c}	7.57 \pm 0.32 ^c	8.98 \pm 0.65 ^c	3.7 \pm 0.3 ^c	3.8 \pm 0.4 ^c
IV	10.19 \pm 0.49 ^{c,e}	9.01 \pm 3.65 ^{c,e}	8.96 \pm 0.67 ^{c,e}	9.92 \pm 0.47 ^c	4.8 \pm 0.2 ^{c,e}	5.0 \pm 0.3 ^{c,e}

^a*P* < 0.05 vs group I; ^c*P* < 0.05 vs group II; ^e*P* < 0.05 vs group III.

cell debris in the lumen. The bile duct showed more histological changes in groups III and IV, and more marked injuries in group IV. Microthrombi were found in the microangium around the biliary tract in some sections from groups III and IV.

Compared with group II, a significant increase in BDISS was observed at 6 h and 24 h postoperatively in groups III and IV (*P* < 0.05). The BDISS in group IV was higher than that in group III, and there was significant difference between the two groups (*P* < 0.05). BDISS at 24 h after hepatic arterial reperfusion increased in all groups compared with BDISS at 6 h, but no significant differences were noted (Table 3, Figure 3).

DISCUSSION

Warm ischemia time in DCD is associated with a higher risk of biliary strictures^[15], and the incidence of NAS in DCD ranges from 10% to 30% compared with an incidence of 1%-10% in donation after brain death^[14,15]. We used a model of rat autologous orthotopic liver transplantation to simulate ischemia-reperfusion injury of the biliary tract, which mimics the whole process of clinical liver transplantation. This model decreases the possibility of blood vessel or vascular anastomosis damage compared with the allogeneic orthotopic liver transplantation, and it minimizes the effects of immunologic rejection. It is a simple model used with a high success rate, which better reflects the pathophysiologic process of bile ducts, and affords a useful tool for the investigation of intrahepatic bile duct damage in liver transplantation caused directly by ischemia-reperfusion injury^[16].

Hepatocytes are supplied by both the hepatic artery and the portal vein, but bile ducts entirely rely on arterial blood supply for oxygenation. The terminal branches of the hepatic artery end in the PBP, which is the direct source of blood supply to the intrahepatic bile ducts. Therefore, the changes of PBP often result in alterations of intrahepatic bile duct structure^[17]. Post-transplantational hepatic arterial ischemia induces ischemia and occlusion of PBP, thus aggravating ischemia of intrahepatic bile ducts^[18,19]. The pathomorphologic changes of bile ducts indicated that there was a time-dependent relationship between secondary ischemia time and pathological injury, and in this study, group IV had the most severe bile duct injury among the four groups. Microthrombi were found in the microangium around the biliary tract in some sections from groups III and IV.

The etiopathogenesis of nonanastomotic stenosis of bile ducts after liver transplantation is complicated, and the factors that cause damage to the bile ducts are mediated by either direct or indirect effects of the PBP. Cholangiocytes can express VEGF and its receptors to regulate the adaptive proliferation of the PBP^[20]. In this study, VEGF-A in groups III and IV decreased significantly, and the number of blood vessels and bile ducts with blood vessels in the portal area also decreased significantly.

Cholangiocyte proliferation is regulated by a number of factors including cAMP, gastrointestinal hormones (e.g., gastrin and somatostatin), bile salts, cholinergic, adrenergic and serotonergic neurotransmitters, and vascular growth factors^[21-23]. The number of PCNA-positive cholangiocytes was reduced compared with that

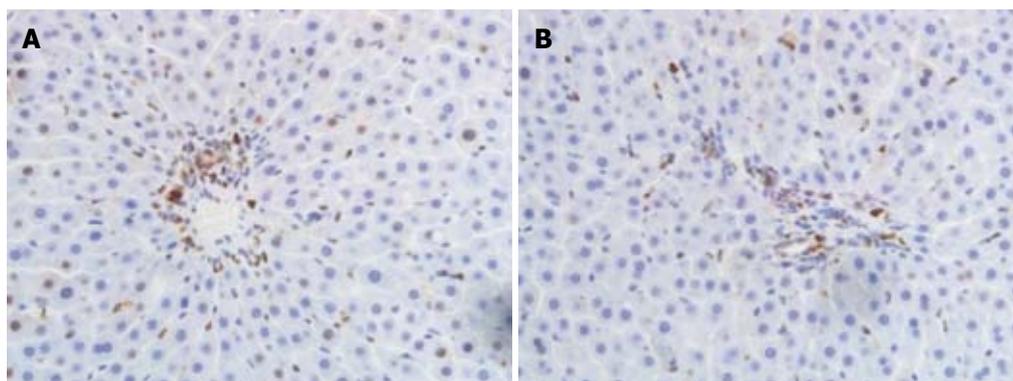


Figure 1 With prolongation of secondary biliary warm ischemia time, the number of proliferating-cell nuclear antigen-positive cholangiocytes was significantly reduced. A: Proliferating-cell nuclear antigen (PCNA)-positive cholangiocytes in group II at 24 h after hepatic artery (HA) reperfusion; B: PCNA-positive cholangiocytes in group IV at 24 h after HA reperfusion. A and B, original magnification $\times 400$.

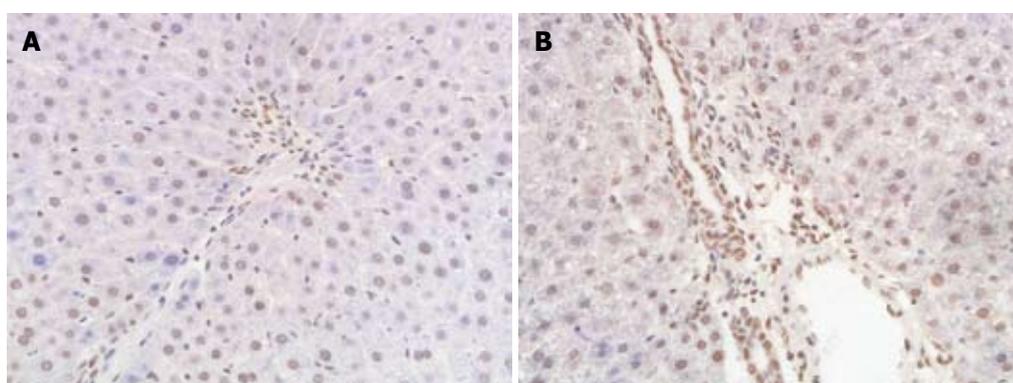


Figure 2 With prolongation of secondary biliary warm ischemia time, more biliary epithelial cells became apoptotic. A: Biliary epithelial cell apoptosis in group II at 24 h after hepatic artery (HA) reperfusion; B: Biliary epithelial cell apoptosis in group IV at 24 h after HA reperfusion. A and B, original magnification $\times 400$.

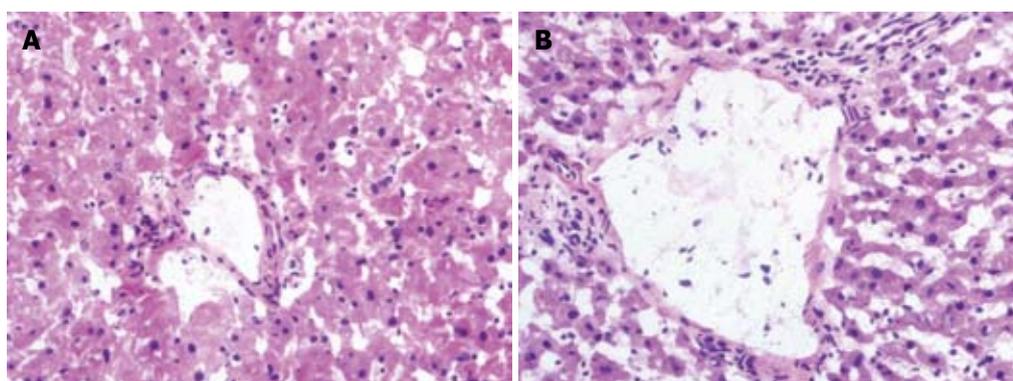


Figure 3 Histological examination of the liver at 24 h after hepatic arterial reperfusion. A: Cholangiocyte injury can be found in group I ; B: More marked injury occurs in group IV. A and B: Hematoxylin-eosin, original magnification $\times 400$.

in normal rats, most obviously in group IV. And the numbers of bile ducts and bile ducts with blood vessels decreased significantly in group IV.

Bile duct epithelia are highly susceptible to reoxygenation after anoxia^[24]. The increased susceptibility to reoxygenation injury by cholangiocytes is associated with increased production of toxic reactive oxygen species by cholangiocytes during reoxygenation, with concomitant low basal levels of the antioxidant glutathione in these

epithelial cells^[25,26]. There are two mechanisms by which cell death occurs: one is apoptosis and the other is the pathological process of necrosis. Accumulating evidence suggests that apoptosis plays an important role in ischemia-reperfusion injury in organ transplantation^[11], and it is widely taken as a reference index to evaluate bile duct epithelial injury. With the prolongation of biliary warm ischemia time, the biliary epithelial cell apoptosis index was significantly elevated.

In conclusion, a prolonged biliary warm ischemia time would result in aggravated injury of the bile duct and the surrounding vascular plexus in autologous orthotopic liver transplantation. The secondary biliary warm ischemia time in liver transplantation should be minimized to reduce the injury of the bile duct and its surrounding blood vascular plexus.

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COMMENTS

Background

With the improvement in surgical techniques, the incidence of anastomotic biliary strictures after liver transplantation decreased markedly, whereas non-anastomotic biliary strictures (NAS) became the major type of biliary complications of liver allografting. Diffuse NAS remain the most challenging type of biliary complication as they are frequently therapy-resistant and are often associated with long-term consequences.

Research frontiers

Warm ischemia time in donors after cardiac death, in addition to subsequent cold ischemia-reperfusion injury, is believed to result in increased damage to biliary epithelial cells. Compared with liver cells, the bile duct epithelial cells experience an extra ischemic process from portal venous recanalization to hepatic arterial recanalization, which is defined as the "secondary warm ischemia time (SWIT) in the biliary tract" or "relative warm ischemia time in the biliary tract". This is a special phase of biliary warm ischemia in the graft; and more and more studies have shifted their focus to the effect of SWIT on bile duct injury in organ transplantation.

Innovations and breakthroughs

The function of the intrahepatic biliary tree is linked with its vascular supply sustained by the intrahepatic peribiliary arterial plexus (PBP). Alterations of intrahepatic bile duct mass are associated with changes in the PBP architecture. In this study, the authors investigated the impact of different SWITs on the bile duct and PBP in a rat autologous liver transplantation model.

Applications

The authors concluded that the prolonged biliary warm ischemia time would result in aggravated injury of the bile duct and the surrounding vascular plexus in autologous orthotopic liver transplantation. Therefore, secondary biliary warm ischemia time in liver transplantation should be minimized to reduce injury to the bile duct and its surrounding blood vascular plexus.

Peer review

This is a concise manuscript, addressing the important problem of non-anastomotic biliary injuries in liver transplantation. The experimental design is clear, methods are valid and the results support the idea that with increasing secondary biliary warm ischemia time, an increase of bile duct injury, mediated by adverse effects of ischemia on the bile duct surrounding blood vascular plexus, could be observed.

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Does antecolic reconstruction for duodenojejunostomy improve delayed gastric emptying after pylorus-preserving pancreaticoduodenectomy? A systematic review and meta-analysis

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Abstract

AIM: To evaluate whether antecolic reconstruction for duodenojejunostomy (DJ) can decrease delayed gastric emptying (DGE) rate after pylorus-preserving pancreaticoduodenectomy (PPPD) through literature review and meta-analysis.

METHODS: Articles published between January 1991 and April 2012 comparing antecolic and retrocolic reconstruction for DJ after PPPD were retrieved from the databases of MEDLINE (PubMed), EMBASE, OVID and Cochrane Library Central. The primary outcome of interest was DGE. Either fixed effects model or random effects model was used to assess the pooled effect based on the heterogeneity.

RESULTS: Five articles were identified for inclusion: two randomized controlled trials and three non-randomized controlled trials. The meta-analysis revealed

that antecolic reconstruction for DJ after PPPD was associated with a statistically significant decrease in the incidence of DGE [odds ratio (OR), 0.06; 95% CI, 0.02-0.17; $P < 0.0001$] and intra-operative blood loss [mean difference (MD), -317.68; 95% CI, -416.67 to -218.70; $P < 0.0001$]. There was no significant difference between the groups of antecolic and retrocolic reconstruction in operative time (MD, 25.23; 95% CI, -14.37 to 64.83; $P = 0.21$), postoperative mortality, overall morbidity (OR, 0.54; 95% CI, 0.20-1.46; $P = 0.22$) and length of postoperative hospital stay (MD, -9.08; 95% CI, -21.28 to 3.11; $P = 0.14$).

CONCLUSION: Antecolic reconstruction for DJ can decrease the DGE rate after PPPD.

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Key words: Pylorus-preserving pancreaticoduodenectomy; Delayed gastric emptying; Antecolic reconstruction; Retrocolic reconstruction; Duodenojejunostomy

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Su AP, Cao SS, Zhang Y, Zhang ZD, Hu WM, Tian BL. Does antecolic reconstruction for duodenojejunostomy improve delayed gastric emptying after pylorus-preserving pancreaticoduodenectomy? A systematic review and meta-analysis. *World J Gastroenterol* 2012; 18(43): 6315-6323 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i43/6315.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i43.6315>

INTRODUCTION

Pylorus-preserving pancreaticoduodenectomy (PPPD), which preserves the whole stomach and 2.5 cm of duodenum^[1], is generally accepted as a standard modality for periampullary malignancies. Compared with the classical pancreaticoduodenectomy (PD), PPPD was reported to have many advantages: (1) easier to perform; (2) less operative time and blood loss; and (3) better improvement of quality of life, nutritional status and weight gain^[2-5]. However, despite the improvements in surgical techniques and postoperative management, PPPD has been associated with a higher delayed gastric emptying (DGE) rate than classical PD^[6,7], although controversies still exist^[8,9]. DGE, with an incidence ranging from 33% to 44%, is reported to be the major complication after PPPD^[10-12]. Although not a lethal complication, DGE is often responsible for prolonged hospital stay and increased associated morbidity and hospital costs. Several studies revealed that DGE was closely related to the reconstruction technique^[13,14]. Therefore, various modifications of reconstruction technique have been advocated to decrease the incidence of DGE.

A recently reported modification in the PPPD procedure is the performance of antecolic duodenojejunostomy (DJ) instead of retrocolic one. It has been found that antecolic reconstruction for DJ could significantly decrease the DGE rate after PPPD^[15-19]. The reported DGE rate was > 30% for retrocolic reconstruction whereas it was < 15% for the antecolic reconstruction^[19]. Nevertheless, two randomized controlled trials (RCTs) demonstrated that antecolic reconstruction was not superior to retrocolic reconstruction for DJ with respect to DGE after PPPD^[20,21]. Up to date, the use of antecolic reconstruction for DJ to decrease the incidence of DGE after PPPD remains a topic of debate.

The primary objective of this study is to analyze the existing evidence regarding the antecolic and retrocolic reconstruction for DJ after PPPD in a systematic review and to perform a meta-analysis of operative outcomes, postoperative mortality, morbidity and length of postoperative hospital stay. The primary outcome of interest was DGE.

MATERIALS AND METHODS

Selection of studies

Multiple databases, including MEDLINE (PubMed), EMBASE, OVID, and Cochrane Library Central, were searched for RCTs or non-RCTs (N-RCTs) that evaluated antecolic *vs* retrocolic reconstruction for DJ after PPPD from January 1991 to April 2012. The following Mesh search headings were used: pylorus-preserving pancreaticoduodenectomy, duodenojejunostomy, delayed gastric emptying, gastrostasis, antecolic reconstruction and retrocolic reconstruction. Citations were limited to those published on humans and in English language. A search was also performed for reference lists of the retrieved relevant articles for additional trials.

Inclusion and exclusion criteria

All included studies should fulfill the following criteria: (1) reporting the indication of PPPD; (2) comparing the results of antecolic and retrocolic reconstruction for DJ after PPPD; (3) reporting the incidence of DGE and other complications; and (4) when two or multiple studies were published by the same institution and/or authors, either one of the higher quality or the most recent article was included in the meta-analysis. Abstracts, case reports, letters, commentary, reviews without original data, studies lacking control groups or appropriate data for extraction and the number of patients less than 35 were excluded.

Study eligibility assessment

Two authors (Cao SS and Zhang Y) independently screened the title and abstract of each publication for potentially eligible studies. Then full articles of eligible trials were obtained for detailed evaluation. Any disagreement in the selection process was resolved through discussion by the two authors. If the two authors could not reach an agreement, a third person (Tian BL) would make a final decision on the eligibility of the study.

Data extraction

Two authors (Cao SS and Zhang Y) independently extracted data from all eligible studies, and then cross-checked the data. Data extracted from each study included: first author, study period, study design, inclusion and exclusion criteria, participant characteristics, interventions used, technique of reconstruction, morbidity and mortality rates, definition of DGE, DGE rate, and length of postoperative hospital stay. Any disagreements were resolved using the same method as mentioned above.

Quality assessment

Jadad scoring system, which evaluates studies based on appropriate randomization, proper blinding, and an adequate description of withdrawals and dropouts, was used to assess the quality of RCTs^[22]. The N-RCTs were scored on the following basis: prospective *vs* retrospective data collection; assignment to antecolic route or retrocolic route by means other than surgeon preference; and an explicit definition of DGE (studies were given a score of 1 for each of these areas; score 1-4)^[23]. The study was considered to be of high quality if the quality score is ≥ 3 .

Statistical analysis

Meta-analysis was performed in accordance with the recommendations of the Cochrane Collaboration. The effect outcomes estimated were odds ratio (OR) for dichotomous variables and mean difference (MD) for continuous variables, both reported with 95% CI. OR was defined as the odds of an adverse event occurring in the antecolic group (AG) *vs* the retrocolic group (RG) and it was considered statistically significant at $P < 0.05$.

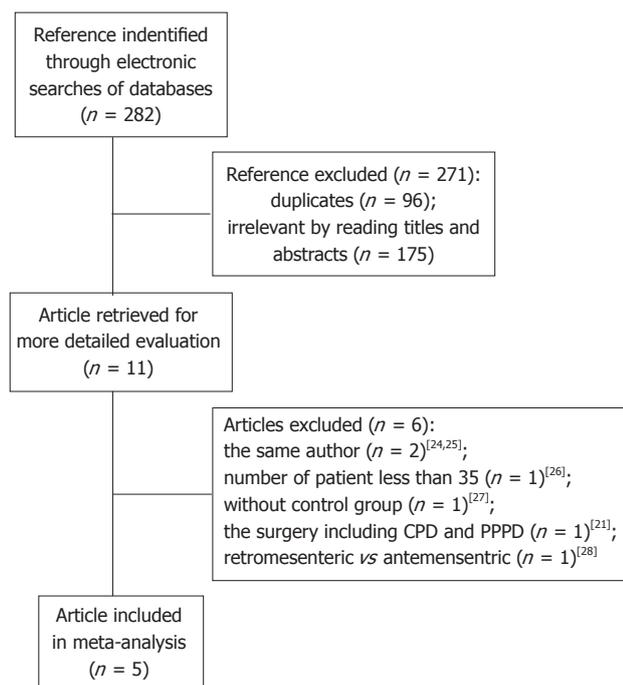


Figure 1 Flow chart showing the search strategy used to identify studies. CPD: Classic pancreaticoduodenectomy; PPPD: Pylorus-preserving pancreaticoduodenectomy.

if the 95% CI did not cross the value 1. MD represented the difference between the two groups in the continuous variables and it was considered statistically significant at $P < 0.05$ if the 95% CI did not cross the value 0. Heterogeneity between studies was measured using χ^2 and I^2 , and $I^2 > 50\%$ was considered statistically significant. Either fixed effects model or random effects model was applied to calculate the pooled effect based on the heterogeneity. But random effects model was used first to assess the heterogeneity. Subgroups were used for sensitivity analysis and a funnel plot was used to identify publication bias.

RESULTS

Eligible studies

The literature searching strategy identified five articles^[16-20] that met the inclusion criteria: two RCTs and three N-RCTs (Figure 1). The five studies involved a total of 451 patients: 240 in the AG and 211 in the RG. The details of the included studies are summarized in Table 1. The sample size ranged from 35 to 200 patients. The mean age of the patients varied between 61 and 70 years. The mean proportion of males varied between 41% and 67% and the proportion of malignancy varied between 63% and 100%. There were no significant differences between the two groups in age (MD, 1.50; 95% CI, -1.67 to 4.66; $P = 0.35$), sex (OR, 0.91; 95% CI, 0.62-1.32; $P = 0.61$) and the proportion of malignancy (OR, 0.75; 95% CI, 0.49-1.16; $P = 0.20$). Of the five studies, only one reported the length of follow-up^[19]. Surgical reconstruction and the definition of DGE are

described in Table 2. In three studies^[18-20], the description of reconstruction method revealed adequate consistency. There was some variation in the postoperative management, including the indication for nasogastric tube (NGT) removal, and administration of somatostatin analogues (SSA), antacid and prokinetic agents (PA).

Meta-analysis of operative outcomes

Operation time (min): Four studies^[16,17,19,20] provided information regarding operation time. The random effects model was used because of significant heterogeneity ($I^2 = 83\%$) between studies, and the result of pooled analysis showed no statistically significant difference between the two groups (MD, 25.23; 95% CI, -14.37 to 64.83; $P = 0.21$) (Figure 2A).

Intra-operative blood loss (mL): Four studies^[16,17,19,20] reported on intra-operative blood loss. It was significantly lower in the AG than in the RG (MD, -317.68; 95% CI, -416.67 to -218.70; $P < 0.0001$) (Figure 2B).

Meta-analysis of postoperative outcomes

Mortality: All the five studies reported on hospital mortality. Among the 451 patients involved, only one patient reported by Tani *et al.*^[19] died from acute hemorrhagic shock because of a Dieulafoy's type ulcer in the RG. Therefore, there was no difference in mortality between the two groups.

Morbidity: Three studies^[17,18,20], including 367 patients, were analyzed for the overall postoperative morbidity. No statistically significant difference was found between the two groups: 17.9% (AG) vs 32.0% (RG) (OR, 0.54; 95% CI, 0.20-1.46; $P = 0.22$). But there was statistically significant heterogeneity between the groups in the three studies ($I^2 = 71\%$) (Figure 2C). All studies provided data on DGE rate and pancreatic fistula (PF) rate. The summarized effect of DGE with random effects model ($I^2 = 52\%$) revealed a statistically significant result favoring AG with a DGE incidence of 7.1% (17/240) compared with a DGE rate of 45.5% (96/211) in the RG (OR, 0.06; 95% CI, 0.02-0.17; $P < 0.0001$) (Figure 2D). However, the difference of the occurrence of PF between the two groups was not statistically significant. Concerning other postoperative complications, there was no significant difference between AG and RG in hemorrhage, intra-abdominal abscesses, bile leakage, the anastomotic leakage, wound infection and reoperation (Table 3).

Postoperative time to remove NGT (d): Time for postoperative removal of NGT was reported in four studies^[16,17,19,20], and three^[17,19,20] studies reported the data using mean \pm SD. No SD was reported by Kurosaki *et al.*^[16] (3 vs 14, $P < 0.0001$). The random effects model was used due to significant heterogeneity ($I^2 = 98\%$) between studies, and the overall effect indicated no difference between the AG and RG (MD, -7.38; 95% CI, -17.39 to 2.63; $P = 0.15$) (Figure 2E).

Table 1 General characteristics of the included studies

Ref.	Country	Study period	Design	Group	Patients	M/F	Mean age (yr)	Etiology of malignancy	Quality score
Kurosaki <i>et al</i> ^[16]	Japan	1996-2002	N-RCT	AG	25	13/12	65 ¹	25 (100)	1
				RG	19	10/9	61 ¹	17 (89.5)	
Hartel <i>et al</i> ^[18]	Germany	1996-2003	N-RCT	AG	100	41/59	61 (53-71) ²	70 (70)	2
				RG	100	46/54	65 (53-74) ²	75 (75)	
Murakami <i>et al</i> ^[17]	Japan	1994-2006	N-RCT	AG	78	46/32	67 ± 11	49 (62.8)	2
				RG	20	10/10	66.7 ± 12.2	16 (80)	
Tani <i>et al</i> ^[19]	Japan	2002-2004	RCT	AG	20	11/9	63.1 ± 9.21	16 (80)	3
				RG	54	36/18	64 ± 12	39 (72.2)	
Chijiwa <i>et al</i> ^[20]	Japan	2005-2007	RCT	AG	17	11/6	69.7 ± 11.0	12 (70.6)	2
				RG	18	9/9	66.9 ± 12.9	16 (88.9)	

¹Medians; ²medians with ranges in parentheses. RCT: Randomized controlled trial; N-RCT: Non-randomized controlled trial; AG: Antecolic group; RG: Retrocolic group; M/F: Male/female.

Table 2 Surgical reconstruction, definition of delayed gastric emptying and postoperative management

Ref.	Group	Reconstruction	Definition of DGE	Indication for removing NGT	SSA	Antacid	PA	
Kurosaki <i>et al</i> ^[16]	AG	II	E-T-S PJ	E-T-S DJ	(1) NGT ≥ POD 10;	Aspiration	NM	NM
	RG	I	E-T-S PJ or PG	E-T-E DJ	(2) reinsertion of NGT	< 200 mL/d	No	NM
Hartel <i>et al</i> ^[18]	AG	II	E-T-S PG	E-T-S DJ	(1) NGT ≥ POD 10;	Aspiration	No	H2 blocker
	RG	I	E-T-S PG	E-T-E DJ	(2) inability to tolerate a solid diet ≤ POD 14	< 500 mL/d	Yes	PPI
Murakami <i>et al</i> ^[17]	AG	II	E-T-S PJ	E-T-S DJ	(1) NGT ≥ POD 10;	(1) After tracheal extubation;	Yes	PPI
	RG				(2) inability to tolerate regular diet ≤ POD 10;	(2) Aspiration of reintubation		Yes
Tani <i>et al</i> ^[19]	AG	II	E-T-S PJ	E-T-S DJ	(1) aspiration > 500 mL/d from NGT left ≥ POD 10;	Aspiration	No	H2 blocker
	RG				(2) reinsertion of NGT;	< 500 mL/d		No
Chijiwa <i>et al</i> ^[20]	AG	II	E-T-S PJ	E-T-S DJ	(3) failure of unlimited oral intake by POD 14	NM	NM	H2 blocker
	RG				(1) NGT ≥ POD 10;			No
					(2) reinsertion of NGT;			
					(3) inability to tolerate an appropriate amount solid food ≤ POD 14			

AG: Antecolic group; RG: Retrocolic group; I, II: Billroth I and Billroth II; E-T-S: End-to-side; E-T-E: End-to-end; PJ: Pancreaticojejunostomy; PG: Pancreaticogastrostomy; DJ: Duodenojejunosotomy; POD: Postoperative day; DGE: Delayed gastric emptying; NGT: Nasogastric tube; NM: Not mentioned; SSA: Somatostatin analogues; PPI: Proton pump inhibitors; PA: Prokinetic agents.

Table 3 Postoperative complications and antecolic vs retrocolic reconstruction for duodenojejunosotomy

Complications	Number of studies	Number of patients		OR	95% CI	P value	Heterogeneity (I ²)
		AG	RG				
Pancreatic fistula	5 ^[14-17,19]	10/240	8/211	1.00	0.40, 2.50	0.99	0%
Hemorrhage	4 ^[14,16,17,19]	3/162	5/157	0.63	0.18, 2.29	0.49	0%
Intra-abdominal abscesses	4 ^[14,16,17,19]	11/162	14/157	0.72	0.30, 1.72	0.46	0%
Bile leakage	3 ^[14,17,19]	0/62	2/57	0.28	0.03, 2.77	0.27	0%
The anastomotic leakage	3 ^[16,17,19]	0/137	2/138	0.2	0.01, 4.14	0.29	—
Wound infection	3 ^[14,17,19]	5/62	4/57	1.21	0.31, 4.72	0.78	0%
Reoperation	3 ^[14,16,17]	2/145	6/139	0.33	0.07, 1.48	0.15	0%

AG: Antecolic group; RG: Retrocolic group; OR: Odds ratio.

Postoperative time to start liquid meal (d): Three studies^[16,17,20] evaluated postoperative time to start liquid meal, but one of them did not provide detailed information (8 vs 22, $P < 0.0001$)^[16]. Meta-analysis of the remaining two studies with random effects model ($I^2 = 98%$) showed no significant difference in the postoperative time to start

liquid meal (MD, -5.59; 95% CI, -15.98 to 4.80; $P = 0.29$) (Figure 2F).

Postoperative time to start solid food (d): Four studies^[16,17,19,20] reported the postoperative time to start solid food, but one of them did not provide sufficient infor-

Table 4 Sensitivity analysis performed for studies comparing antecolic and retrocolic reconstruction for duodenojejunostomy

Outcome	Number of studies	Number of patients		OR/MD	95% CI	P value	Heterogeneity (I^2)
		AG	RG				
Randomized controlled trials							
Delayed gastric emptying	2 ^[17,19]	2/37	14/38	0.1	0.02, 0.47	0.004	0%
Mortality	2 ^[17,19]	0/37	1/38	0.32	0.01, 8.26	0.49	–
Postoperative hospital stay (d)	2 ^[17,19]	37	38	-7.4	-27.2, 12.40	0.46	79%
Non-randomized controlled trials							
Morbidity	2 ^[15,16]	26/178	49/154	0.33	0.19, 0.57	< 0.00 001	0%
Delayed gastric emptying	3 ^[14,15,16]	15/203	82/173	0.05	0.01, 0.20	< 0.00 001	72%
Reconstruction with Billroth II in the two groups							
Morbidity	2 ^[16,19]	20/117	30/118	0.85	0.16, 4.69	0.86	79%
Delayed gastric emptying	3 ^[16,17,19]	7/137	38/138	0.15	0.06, 0.34	< 0.00 001	0%
Mortality	3 ^[16,17,19]	0/137	1/138	0.32	0.01, 8.26	0.49	–
Postoperative hospital stay (d)	2 ^[17,19]	37	38	-7.4	-27.2, 12.40	0.46	79%
Reconstruction with Billroth II in AG and Billroth I in RG							
Delayed gastric emptying	2 ^[14,15]	10/103	58/73	0.03	0.01, 0.06	< 0.00 001	0%

AG: Antecolic group; RG: Retrocolic group; OR: Odds ratio; MD: Mean difference.

mation (14 *vs* 28, $P < 0.0001$)^[16]. The summarized effect with random effects model ($I^2 = 95\%$) revealed no difference between the two groups (MD, -8.32; 95% CI, -17.89 to 1.26; $P = 0.09$) (Figure 2G).

Length of postoperative hospital stay (d): Data of length of postoperative hospital stay was available in four studies^[17-20], but Hartel *et al*^[18] did not report the SD (11.5 *vs* 17.5, $P < 0.001$). The other three studies showed no statistically significant difference between the two groups (MD, -9.08; 95% CI, -21.28 to 3.11; $P = 0.14$), which was associated with significant heterogeneity between the groups in all available studies for pooled analysis ($I^2 = 77\%$) (Figure 2H).

Sensitivity analysis

The following four subgroups were used for the sensitivity analysis: RCTs, N-RCTs, reconstruction with Billroth II in the AG and RG and reconstruction with Billroth II in AG and Billroth I in RG. The results of the analysis (Table 4), were the same as those when all studies were selected.

Publication bias

A funnel plot of all the studies reporting on DGE used in this meta-analysis is shown in Figure 3. There was no strong evidence of publication bias because all the studies were equally distributed around the vertical axis.

DISCUSSION

This meta-analysis found that antecolic reconstruction for DJ during PPPD was associated with a statistically significant decrease in the incidence of DGE and intra-operative blood loss. But antecolic reconstruction was not superior to retrocolic reconstruction with respect to operation time, postoperative mortality, overall morbidity, postoperative time to remove NGT and start liquid

meal and solid food, and length of postoperative hospital stay.

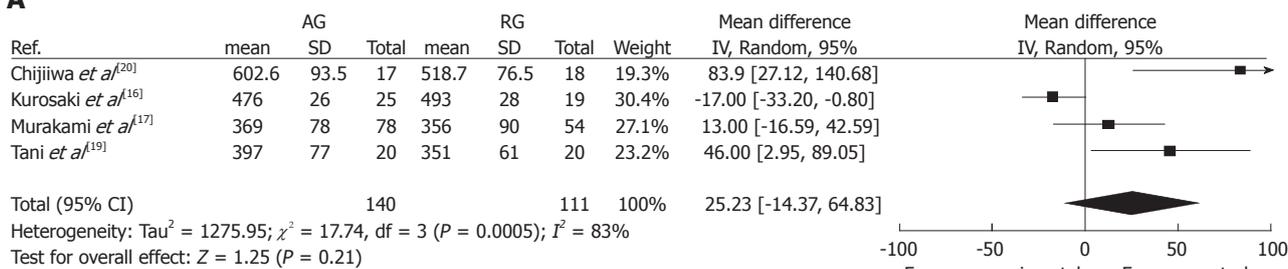
Although DGE is the most frequent postoperative complication after PPPD, the true mechanism has not been fully clarified. A number of theories, including local ischemia of the antrum, low plasma motilin concentrations, gastric atony, transient pancreatitis, and gastric dysrhythmias, have been postulated to explain the occurrence of DGE after PPPD^[29]. Moreover, DGE is always associated with angulation or torsion of the DJ in the early postoperative period^[30].

Compared with retrocolic reconstruction, antecolic reconstruction may have several theoretical advantages. Antecolic reconstruction is believed to be less prone to torsion or angulation, causing DGE by mechanical obstruction^[18,31]. In the antecolic reconstruction, the DJ anastomosis is located further away from pancreaticojejunostomy compared with the retrocolic reconstruction, which reduces the negative effect on antroduodenal motility by a small pancreatic anastomotic leak or a transient mild postoperative pancreatitis^[18]. Furthermore, the descending jejunal loop is more mobile after antecolic reconstruction than after retrocolic reconstruction because of a minor degree of venous congestion and bowel edema^[28].

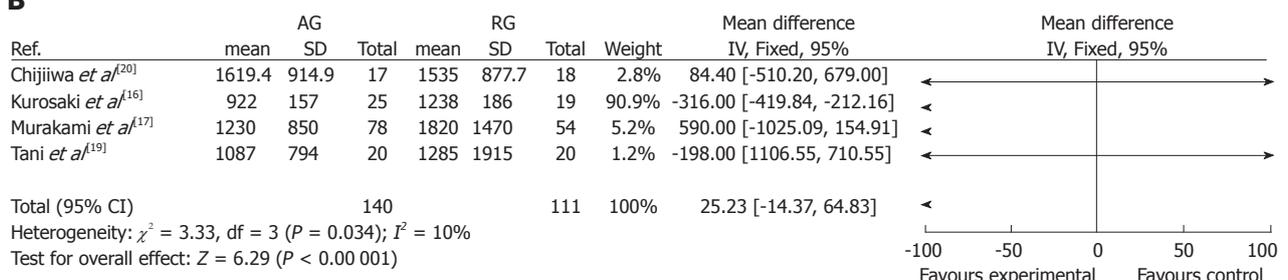
DGE due to postoperative complications has been an accepted concept in the literature^[31,32]. However, in the current study, the postoperative complications, including PF, hemorrhage, intra-abdominal abscesses, bile leakage, the anastomotic leakage, wound infection and reoperation, were similar in both groups. The lack of generally accepted definitions of postoperative complications may influence the results. Perhaps the significant higher intro-abdominal blood loss during surgery in the retrocolic reconstruction group may contribute to a risk for DGE.

DGE not only leads to repeated episodes of nausea and vomiting which prolongs NGT intubation and delays

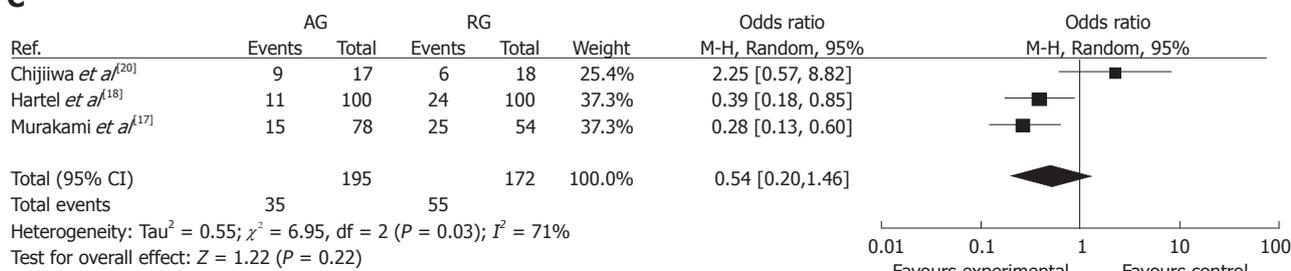
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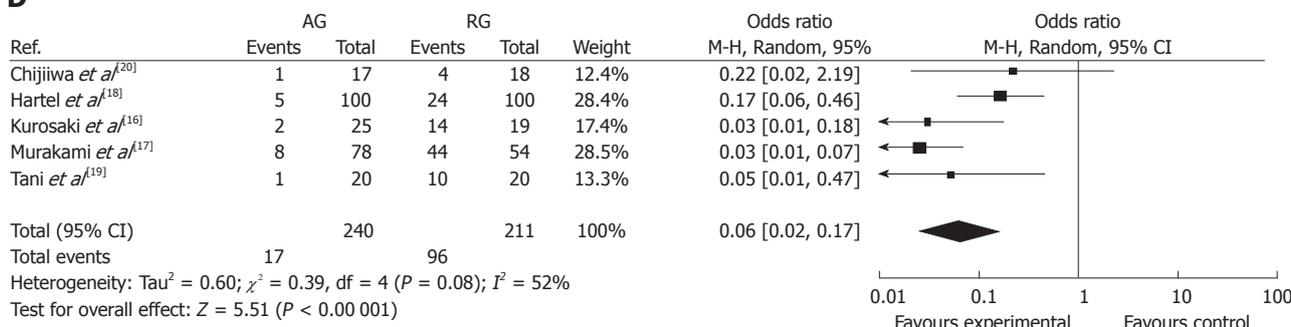
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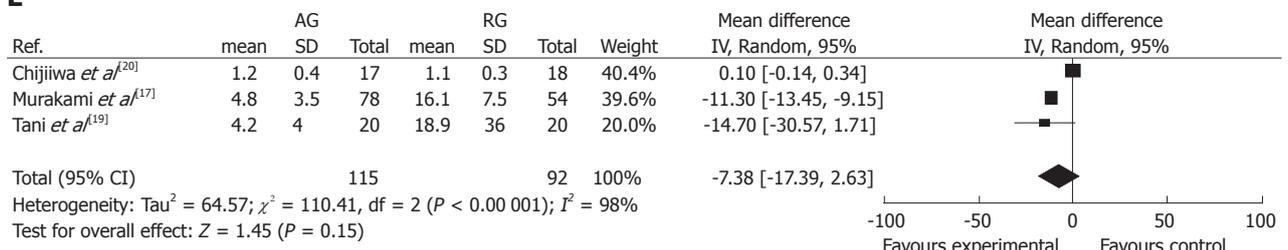
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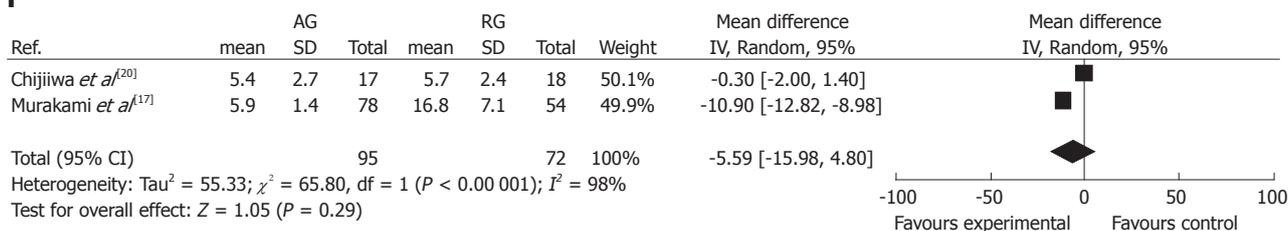
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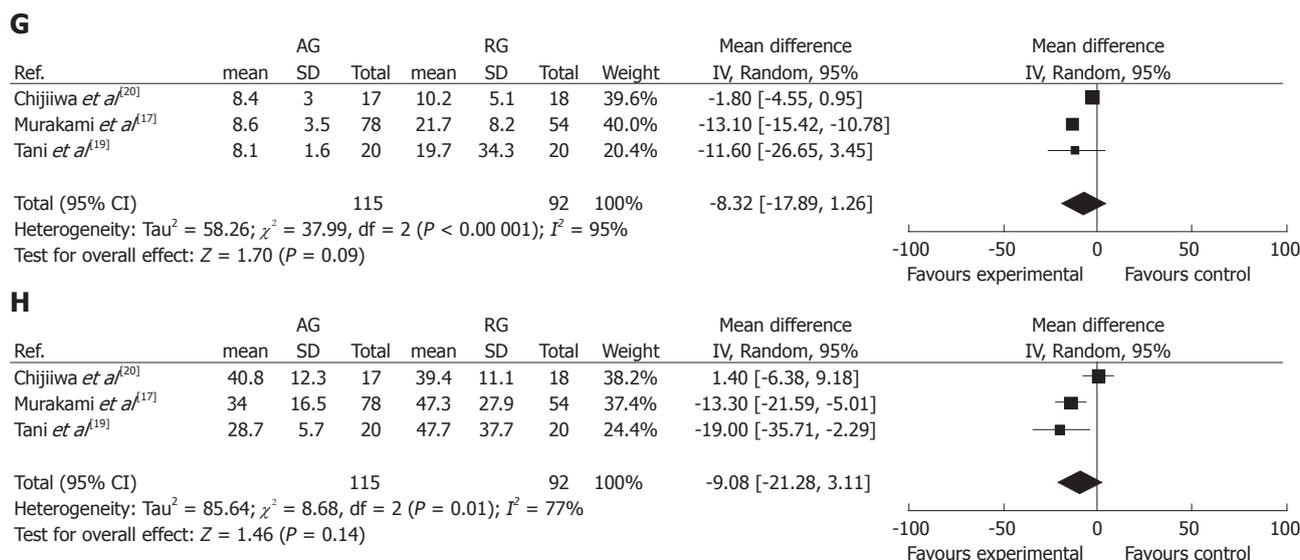


Figure 2 Meta-analysis of all available data. A: In operative time with random effect model; B: In intra-operative blood loss with fixed effect model; C: In overall morbidity with random effect model; D: In delayed gastric emptying with random effect model; E: In postoperative time to remove nasogastric tube with random effect model; F: In postoperative time to start liquid meal with random effect model; G: In postoperative time to start solid food with random effect model; H: In length of postoperative hospital stay with random effect model. AG: Antecolic group; RG: Retrocolic group.

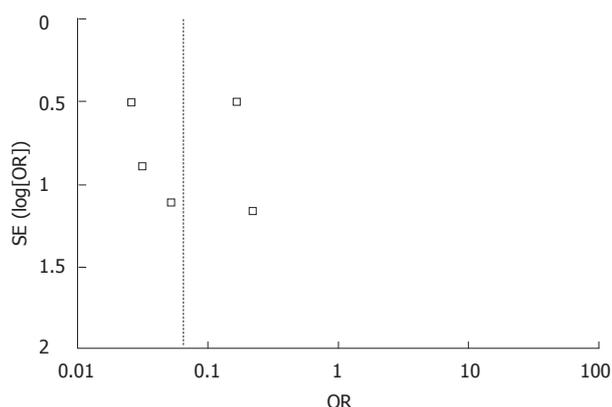


Figure 3 Funnel plot of comparison of antecolic vs retrocolic reconstruction for duodenojejunostomy in delayed gastric emptying. OR: Odds ratio.

food intake, but also has an impact on duration of hospitalization^[33]. Nevertheless, this meta-analysis demonstrated that antecolic reconstruction did not seem to offer an advantage with respect to postoperative time to remove NGT and start liquid meal and solid food, and length of postoperative hospital stay. This may result from a small number of studies providing insufficient information for analysis. Kurosaki *et al*^[16] reported that postoperative time to remove NGT and start liquid meal and solid food were significantly shortened in antecolic reconstruction group. Hartel *et al*^[18] also found that the median postoperative stay was significantly shorter in the antecolic reconstruction group than in the retrocolic reconstruction group. But neither of the studies reported the SD, which would greatly influence these pooled results.

The three types of reconstructions, including Billroth I, Billroth II and Roux-en Y, are frequently performed for digestive tract reconstruction after PPPD.

Of the five studies included in the current meta-analysis, three studies applied Billroth II reconstruction for both groups^[18-20] and pooled analysis showed a significantly decreased DGE rate in the antecolic reconstruction group. One study showed that Billroth II reconstruction with antecolic DJ achieved a significantly lower incidence of DGE than Billroth I reconstruction with retrocolic DJ^[16]. Another research showed that DGE rate was significantly lowered with antecolic Roux-en Y reconstruction. However, according to the description and schematic illustration of the reconstruction method, the reconstruction method used should be the antecolic Billroth II reconstruction, but not the Roux-en Y reconstruction^[17]. These data suggest that antecolic Billroth II reconstruction for DJ could be a useful method after PPPD to decrease the occurrence of DGE.

The present study has some limitations and the results should be interpreted with caution. First, this meta-analysis included a small number of studies and patients. Second, some low-quality studies were incorporated, and 60% of the data came from N-RCTs. Third, a test for heterogeneity was significant for most outcomes analyzed. The differences between the studies have led to heterogeneity, including differences in the type of digestive tract reconstruction, definition of DGE and postoperative management. In order to reduce the heterogeneity, subgroup analysis was performed and the results were the same as those when all studies were selected, which further confirmed the conclusion drawn above.

In conclusion, the current study suggests that antecolic reconstruction for DJ can decrease DGE rate after PPPD. However, further standardized RCTs with general type of digestive tract reconstruction and definition of DGE are urgently needed to draw a definitive conclusion.

COMMENTS

Background

Various modifications of reconstruction technique have been advocated to decrease delayed gastric emptying (DGE) rate after pylorus-preserving pancreaticoduodenectomy (PPPD). A recently reported modification is the performance of antecolic duodenojejunostomy (DJ) instead of retrocolic approach. Up to now, however, the selection of antecolic or retrocolic reconstruction for DJ remains an issue of debate. In this paper, therefore, a systematic review and meta-analysis were performed to evaluate whether antecolic reconstruction for DJ can decrease DGE rate after PPPD.

Research frontiers

DGE is reported to be the leading complication after PPPD. Although not a lethal complication, DGE is often responsible for prolonged hospital stay and increased associated morbidity and hospital costs. In the area of decreasing DGE rate with different reconstructions for DJ, the research hotspot is to evaluate the effect of antecolic and retrocolic reconstruction for DJ on the incidence of DGE after PPPD.

Innovations and breakthroughs

This review suggests that antecolic reconstruction for DJ can decrease DGE rate after PPPD. According to the authors, this is the first systematic review using the meta-analysis to study the benefit of antecolic reconstruction for DJ in decreasing the DGE rate after PPPD.

Applications

The study result that antecolic reconstruction for DJ can decrease DGE rate after PPPD could guide the selection of the reconstruction route for DJ.

Peer review

This is a technically good study of antecolic vs retrocolic reconstruction for DJ after PPPD. The results are interesting and suggest that antecolic reconstruction for DJ can decrease DGE rate after PPPD.

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Granular cell tumor of the common bile duct: A Japanese case

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Abstract

Granular cell tumor (GCT) of the biliary system is rare. It is reported that it occurs more commonly in young black women. We report here our seldom experience of a Japanese case in whom icterus was found as a first symptom just after a caesarean operation. A 36-year-old Japanese woman developed icterus after delivery by the Caesarean operation. A surgical operation was performed without can deny that there was a tumor-related change in a bile duct as a result of examination for various images. As a result of pathological evaluation, GCT was diagnosed. By the preoperative organization biomicroscopy result, it was not able to be attached a right diagnosis. It was thought that this tumor, although rare, should be considered as one of the causes of biliary stenosis in the younger population.

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INTRODUCTION

Granular cell tumor was initially described by Abrikossoff in 1926; it was found in the skeletal muscle of the tongue^[1], and first reported in the biliary system in 1952 by Coggins^[2]. A lot of researchers favor the theory that such tumors are generated from Schwann cells. The theory of Schwann cell origin is based on histological, electron microscopic, and immunohistochemical findings^[3,4]. The tumors generally arise in the oral cavity, skin, and subcutaneous tissues, and less than 1% of granular cell tumor (GCT) occurs in the biliary tract^[5,6]. To date, there have been no reports on malignant GCT in bile duct. Most patients suffering from GCT in the bile duct are young, female, and black. Benign tumors in bile duct are rare (4%) and GCT in bile duct is very rare^[5].

This is a Japanese case report of bile duct GCT. This case was first suspected of being adenocarcinoma by the result of tumor biopsy on endoscopic retrograde cholangiopancreatography (ERCP) before the operation, as this was the result in other previous cases.

CASE REPORT

A 36-year-old Japanese woman was hospitalized for toxemia during pregnancy. She had hypertension and renal disorder at gestational age 30 wk and 2 d. Her blood pressure was 160-170/100 mmHg with albuminuria. Because her condition was resistant to treatment, she gave birth to twins by preparative Caesarean section at gestational age 32 wk and 2 d. During surgical procedures, her and her babies' general conditions were good. The Caesarean section was completed without noticeable complications.

After delivery, her systolic arterial pressure decreased to 120 mmHg and the proteinuria disappeared in several weeks. Three days after the delivery, liver enzymes and bile tract enzymes suddenly increased. In addition, six days after the delivery, icterus was recognized (Table 1). Serum bilirubin increased to 5.0 mg/dL (direct bilirubin 4.0 mg/dL) in 3 d. At first, the liver damage was regarded as a side effect of the various medicines. For example, Cephalosporin antibiotic was administered on the day and the day after the Caesarean operation and Ca blocker was prescribed for the purpose of lowering the blood pressure. Abdominal ultrasonography showed a swollen gallbladder, as well as debris associated with it. However, for the common bile duct (CBD), no expansion was observed given its diameter of 0.7 cm in the measurement (Figure 1). Tumor shadows or stenosis of CBD were also recognized. Contrast-enhanced abdominal computed tomography and magnetic resonance cholangiopancreatography also showed about 10 mm long stenosis and wall thickening in the middle part of the bile duct. Tumor marker Carcinoembryonic antigen was 2.0 ng/mL (normal 0-5 ng/dL) and carbohydrate antigen 19-9 was 32.0 U/mL (0-37 U/mL).

ERCP suggested there was a 6.1 mm-long stenosis at the middle part of the bile duct. The shape of the tumor was nodular (Figure 2). Mucosal layer (m) and fibromucosal layer (fm) thickening was identified by intraductal ultrasonography. Endoscopic brush cytology of the bile duct resulted in a status of "quantity not sufficient" and findings of forceps biopsy were atypical but inconclusive. In spite of these investigations, bile duct carcinoma could not be ruled out, so pancreaticoduodenectomy was performed 37 d after the Caesarean section.

At the operation, the tumor felt hard to touch in liver-duodenum ligament. Lymph nodes around the aorta and the lymph node on the posterior surface of the pancreatic head were slightly swollen, but they were free of malignancy on frozen section. The tumor was a poorly circumscribed nodule, measuring 9.0 mm × 5.5 mm, located at the lower portion of the CBD at the level of the upper margin of the pancreas and involved all layers of the bile duct wall (Figure 3A). **Microscopically**, tumors were rounded or polygonal with eosinophilic granular cytoplasm, arranged in nests and cords separated by fibrous tissue septa (Figure 3B). **The GCT cells** partly infiltrated a peripheral nerve fibrous tissue bunch in the bile duct wall. In addition, they infiltrated the vein



Figure 1 Upon abdominal ultrasound, the gallbladder was swollen. Dilatation of the bile duct was not apparent upon measurement, however, a segment of the common bile duct seemed to be obstructed, warranting further investigation.

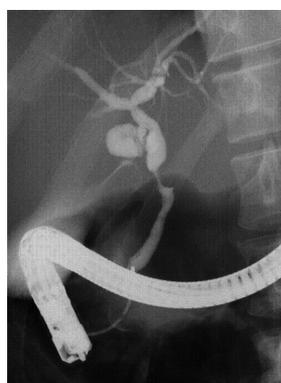


Figure 2 Endoscopic retrograde cholangiopancreatography shows stricture at the middle part of the bile duct.

Table 1 Laboratory data on the day of first appearance of icterus

Complete blood count	Blood chemistry
WBC 6000 / μ L (3500-9800/ μ L)	AST 88 IU/L (6-40 IU/L)
Hb 12.2 g/dL (11.0-15.3 g/dL)	ALT 132 IU/L (0-35 IU/L)
PLT 31.8×10^4 / μ L (13.0-37.0 $\times 10^4$ / μ L)	LDH 580 IU/L (160-420 IU/L)
Blood coagulation test	ALP 3016 IU/L (115-360 IU/L)
PT-INR 0.93 (0.8-1.3)	γ GTP 189 IU/L (5-45 IU/L)
Tumor markers	T-bil 5.0 mg/dL (0.2-1.0 mg/dL)
CEA 2.0 ng/mL (0-5 ng/mL)	D-bil 4.0 mg/dL (0.0-0.4 mg/dL)
CA19-9 32.0 U/mL (0-37 U/mL)	Amy 81 IU/L (50-220 IU/L)
	CRP 1.09 mg/dL (0.01-0.4 mg/dL)

WBC: White blood cells; Hb: Hemoglobin; PLT: Platelets; PT-INR: Prothrombin time international normalized ratio; CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LDH: Lactate dehydrogenase; ALP: Alkaline phosphatase; γ GTP: γ -glutamyl transferase; T-bil: Total bilirubin; D-bil: Direct bilirubin; Amy: Amylase; CRP: C reactive protein.

in the bile duct wall and caused some vein lumina to occlude (Figure 4). Tumor cells are diastase-resistant periodic acid-Schiff stain positive, and immunoreactive for S-100 protein and neuron-specific enolase. The cellular atypia and mitosis were inconspicuous. These findings were compatible with benign GCT. In addition, regional

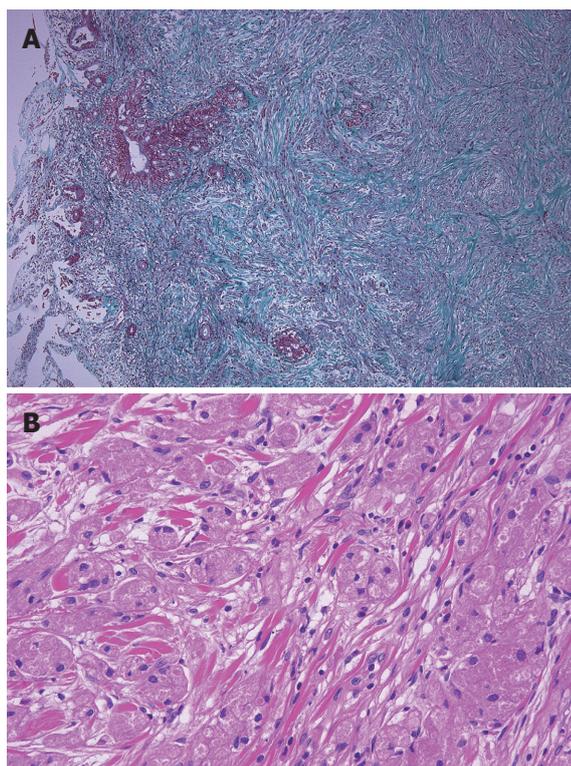


Figure 3 The pathological result of the tumor. A: Neoplastic cells of granular cell tumor infiltrates the mucous membrane epithelium of the bile duct (Elastica-Masson stain, $\times 1.25$); B: Granular cells (HE stain, $\times 10$).

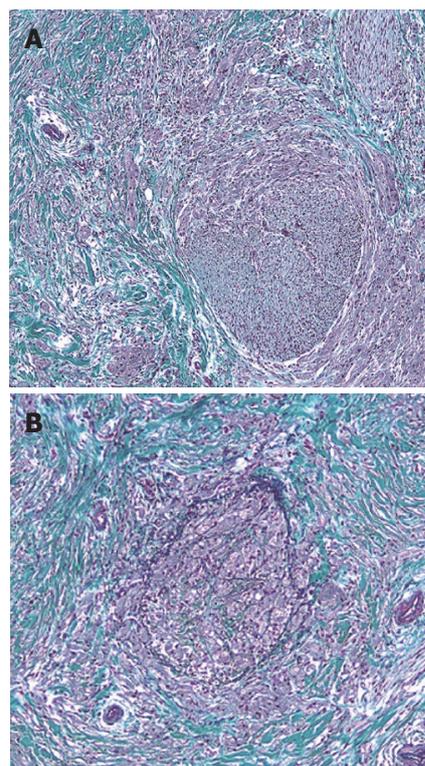


Figure 4 The granular cell tumor cells partially infiltrated a peripheral nerve fibrous tissue bunch in the bile duct wall. In addition, they infiltrated the vein in the bile duct wall and occluded some of the vein lumina. A: Granular cell tumor involving a small nerve (Elastica-Masson stain, $\times 4$); B: A vein is replaced by a neoplastic cell (Elastica-Masson stain, $\times 4$).

lymph nodes were free of tumor. Five years have passed since the surgery and the patient is well.

DISCUSSION

Almost all bile duct tumors or stenosis are suspected as being malignant because benign tumors are comparatively rare in the bile duct. In 2003, Principe *et al*^[5] reviewed 179 (90 men and 89 women) patients who were diagnosed with malignant stricture of bile duct and judged to be operable from 1982 to 2001. In 153 cases, an operation was carried out. Of these 147 (96.1%) cases were found to have a malignant cause as gall bladder carcinoma or cholangiocarcinoma. Cases in which radical operation was possible constituted only 24.8% of these. It was judged not to be possible to remove the tumor surgically in the other cases, and only an operation for reducing jaundice was carried out. The remaining 6 cases (4%) were benign strictures and only one of them was GCT (0.7% of the total). Benign tumor in bile duct is rare so almost all cases of stenosis in it tend to be considered as malignant stricture. We searched for “granular cell tumor” and “bile duct” in the Pub-Med database and obtained reports on 47 previous cases of GCT of the bile duct. We verified 48 cases including our case.

Most of the patients were young. The average age at diagnosis was 33.9 years old (range 11 years to 56 years). Almost all of the patients (81.0%) were female and many were black. In terms of ethnic origin, 60.5% of them were black (including Jamaican and Ethiopian), 31.6%

were Caucasoid (including Algerian), and 7.9% of them were Mongoloid (including Japanese and Thai). The chief complaints were jaundice, pain, and a pruritus feeling, which are symptoms caused by obstructive jaundice. There is no specific symptom of this tumor, as forecast. The range of tumor size was 0.5 cm^[7] to 4.0 cm^[8] and the average was 1.6 cm. As for the site of the tumors, 58.1% were in CBD, 23.3% in common hepatic duct (CHD), 14.0% in cystic duct (CD), and 2.3% in gallbladder and ampulla of Vater. They might tend to be located at confluences with 41.9% of them near the confluence of CBD (CHD and CD and CBD), and 11.6% of them near the confluence of CHD (right and left hepatic ducts). However, the divergence position of the CD was not mentioned in all reports, and some contained vague descriptions, for example, “CBD” or “CD”.

It is generally difficult to make diagnoses as GCT histologically before an operation. A correct diagnosis might be made from several repeated biopsies, as in our case. Because almost all tumors of bile duct are malignant, the diagnosis of “suggested adenocarcinoma” is difficult to the default setting doubt.

Histologically, GCT involves large granular-like eosinophilic cells. It generally occurs in skin and the oral cavity, and two-thirds of cases occur in cutis and submucosa^[9]. It is rare in infants, and occurs in adults of 30-40 years old. As for the sex ratio, its prevalence is four times higher in women than in men. Many cases of

GCT occurring in bile duct in women, the young, and in black people have been reported. In the literature, GCT is described as being not often diagnosed with histology before an operation. Even if it is diagnosed as benign tumor, surgical operation may be chosen for various reasons. One is that it is not easy to rule out malignant tumor completely because of the frequency of malignant tumor in bile duct. Another reason is that some symptoms like jaundice or pain can be caused, no matter how small it is. The average size was 1.6 cm, which is small. The smallest size was 0.5 cm. Generally, it is difficult to discover small tumors without symptoms. These tumors tend to cause small symptoms, at an early stage.

It occurs at a rate under 2%, but there is malignant GCT^[9-11]. There are some cases that relapsed after surgical excision and spread, finally resulting in death. These malignant GCTs are usually larger than 5 cm^[12]. However, no one has reported malignant GCT in bile duct, for example, a case of it spreading to other organs, recurring several years later, or invading surrounding tissue. Although only one case resulted in mortality^[13], this was not due to primary disease but to postoperative severe adult respiratory distress syndrome and disseminated intravascular coagulopathy with fulminant sepsis, 3 wk after Roux-en-Y hepaticojejunostomy.

Benign tumors in the biliary system are so rare that it is difficult to rule out malignant tumor. If intraoperative frozen section could establish an exact diagnosis, we would be able to select reduction operation depending on the location of GCT in bile duct.

GCT is a rare disease among Asian population. According to past case reports, it is a tumor that tends to occur in the bile duct in young black women. A benign tumor developing in the bile duct is very rare and it is unusual in Asians.

In conclusion, we experienced a case of GCT that occurred in a young Japanese woman. It was thought that this tumor, although rare, should be considered as one of the causes of biliary stenosis in the younger population.

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Lymphangioma of the small bowel mesentery: A case report and review of the literature

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Abstract

Lymphangioma is a rare benign condition characterized by proliferation of lymphatic spaces. It is usually found in the head and neck of affected children. Lymphangioma of the small-bowel mesentery is rare, having been reported for less than 1% of all lymphangiomas. Importantly, it can cause fatal complications such as volvulus or involvement of the main branch of the mesenteric arteries, requiring emergency surgery. Moreover, the gross and histopathologic findings may resemble benign multicystic mesothelioma and lymphangiomyoma. Immunohistochemical study for factor VIII-related antigen, D2-40, calretinin and human melanoma black-45 (HMB-45) are essential for diagnosis. Factor VIII-related antigen and D2-40 are positive in lymphangioma but negative in benign multicystic mesothelioma. HMB-45 shows positive study in

the smooth-muscle cells around the lymphatic spaces of the lymphangiomyoma. We report a case of small-bowel volvulus induced by mesenteric lymphangioma in a 2-year-and-9-month-old boy who presented with rapid abdominal distension and vomiting. The abdominal computed tomography scan showed a multiseptated mass at the right lower quadrant with a whirl-like small-bowel dilatation, suggestive of a mesenteric cyst with midgut volvulus. The intraoperative findings revealed a huge, lobulated, yellowish pink, cystic mass measuring 20 cm × 20 cm × 10 cm, that was originated from the small bowel mesentery with small-bowel volvulus and small-bowel dilatation. Cut surface of the mass revealed multicystic spaces containing a milky white fluid. The patient underwent tumor removal with small-bowel resection and end-to-end anastomosis. Microscopic examination revealed that the cystic walls were lined with flat endothelial cells and comprised of smooth muscle in the walls. The flat endothelial cells were positive for factor VIII-related antigen and D2-40 but negative for calretinin. HMB-45 showed negative study in the smooth-muscle cells around the lymphatic spaces. Thus, the diagnosis was lymphangioma of the small bowel mesentery with associated small bowel volvulus.

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Key words: Lymphangioma; Mesentery; Small bowel; Volvulus; Factor VIII-related antigen; D2-40

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INTRODUCTION

Lymphangioma is a benign condition characterized by proliferation of the thin-walled lymphatic spaces^[1]. It is believed to result from congenital lymphatic malformation rather than a true lymphatic neoplasm^[2,3]. It is usually found in the head and neck regions during the first few years of life^[1]. Lymphangioma of the small-bowel mesentery is rare, representing less than 1% of all lymphangiomas^[2]. Volvulus is the most common manifestation of mesenteric lymphangioma^[3].

CASE REPORT

We report a case of a 2-year-and-9-mo-old boy who suffered from unexplained intermittent abdominal pain with vomiting since he was 6 mo old. These symptoms were temporarily relieved by treatment with anti-flatulence and anti-vomiting agents. However, the frequency and severity seemed to gradually increased. Fifteen days prior to admission to the hospital, he developed mucous bloody diarrhea with low-grade fever. He was treated by oral and intramuscular antibacterial agents and oral rehydration. Three days later, the diarrhea had diminished, but the low-grade fever persisted. When he was first brought to the emergency room, his vital signs were not remarkable except the pulse rate, 130 beats/min. Physical examination revealed abdominal distension. Other features were unremarkable. Stool exam was not performed. The initial diagnosis was acute infectious diarrhea. Intravenous fluid was administered, but the patient was not hospitalized. He was treated by oral antibacterial agents and oral rehydration. Five days later, the child was brought to the hospital for the second visit with a 1-d history of intermittent abdominal pain, rapid abdominal distension, and vomiting. However, there was no mucous bloody diarrhea or fever. The vomited content was food material admixed with greenish watery fluid. The patient's vital signs were unremarkable except for the pulse rate, 110 beats/min. Physical examination revealed abdominal distension with diffuse tenderness and hyperactive bowel sounds but no abdominal rigidity. Rectal digital examination revealed yellow feces. Other features were unremarkable. Plain abdominal radiography revealed dilatation of the small-bowel loops at the upper and mid abdomen with multiple air-fluid levels, suggestive of small-bowel obstruction (Figure 1A and B). Abdominal CT scan revealed a thin-walled, fluid-filled, multiseptated mass, about 7.8 cm × 7 cm × 6.9 cm, at the right lower quadrant with compression of the adjacent bowel and generalized dilatation of the small-bowel loops in a whirl-like pattern, suggestive of a chylous mesenteric cyst associated with midgut volvulus (Figure 1C and D). Laparotomy was performed. During the operation, small-bowel volvulus with small-bowel dilatation and a mesenteric mass were found (Figure 2A and B). The mesenteric mass was lobulated, yellowish pink, cystic, and huge, measuring approximately 20 cm × 20 cm × 10 cm. The mass content was milky white, approximately 100 mL. The mesenteric mass with adjacent

small-bowel segment were resected. Small-bowel anastomosis and decompression were performed. After formalin fixation, the mesenteric mass collapsed and shrank to 5.5 cm × 4.5 cm × 2.4 cm (Figure 2C). It was lobulated, cystic, semitranslucent, and pale tan with an adjacent small-bowel segment 5 cm long and 2 cm across. Cut surfaces of the mass revealed multicystic spaces of varying size (Figure 2D). The cystic walls were generally thin, but some walls were relatively thick. There was no fluid in the mass because it had been previously drained. However, there was residual milky white fluid in the specimen container representing the drained lymphatic fluid (inset of Figure 2D). The small-bowel mucosa was grossly unremarkable without invagination into the mass. Microscopic examination revealed that the cystic walls comprised of smooth muscle were lined with flat endothelial cells (Figure 3A and B). The stroma showed various sizes of small lymphatic spaces lined by a flat endothelium and containing small lymphoid cells (Figure 3C). The stroma also contained smooth-muscle bands and scattered lymphoid infiltrates (Figure 3C). Few subendothelial lymphoid follicles were observed, supporting the diagnosis of cystic lymphangioma (Figure 3D). The cell lining of the cystic walls was immunoreactive for factor VIII-related antigen (Figure 3E) and D2-40 (focal) (Figure 3F) but not for calretinin (Figure 3G) and human melanoma black-45 (HMB-45) (Figure 3H).

In conclusion, the diagnosis was cystic lymphangioma as indicated by proliferation of cystically dilated lymphatic spaces, immunoreactivity with factor VIII-related antigen and D2-40, and milky white content. Negative study of calretinin and HMB-45 excluded benign multicystic mesothelioma and lymphangiomyoma, respectively.

DISCUSSION

Lymphangioma is a mass-forming lesion characterized by numerous thin-walled lymphatic spaces and usually manifests in the first few years of life^[1]. The common sites are the head, neck, and axillary regions. Other locations such as the abdominal or mediastinal cavity are rare, accounting for approximately 5% of lymphangiomas^[2]. Among these, lymphangioma of the small-bowel mesentery has been described in less than 1% of lymphangiomas^[1]. Lymphangioma appears to result from congenital malformation of lymphatic vessels rather than a true lymphatic tumor^[2,3]. The former causes sequestration of lymphatic vessels during the embryonic period^[1]. However, some data suggest that inflammation, abdominal trauma, abdominal surgery, radiation, or lymphatic obstruction may play a role in the genesis as a tumor^[4,5]. Of note, the patient described in this case study had a history of intermittent abdominal pain without an identifiable cause from the time he was 6 mo old. This supports the theory of lymphangioma resulting from congenital malformation of lymphatic vessels rather than a tumor. Lymphangiomas are traditionally classified into three histologic types: capillary (simple), cavernous, and cystic^[1]. The capillary (simple) type usually originates in the skin and con-

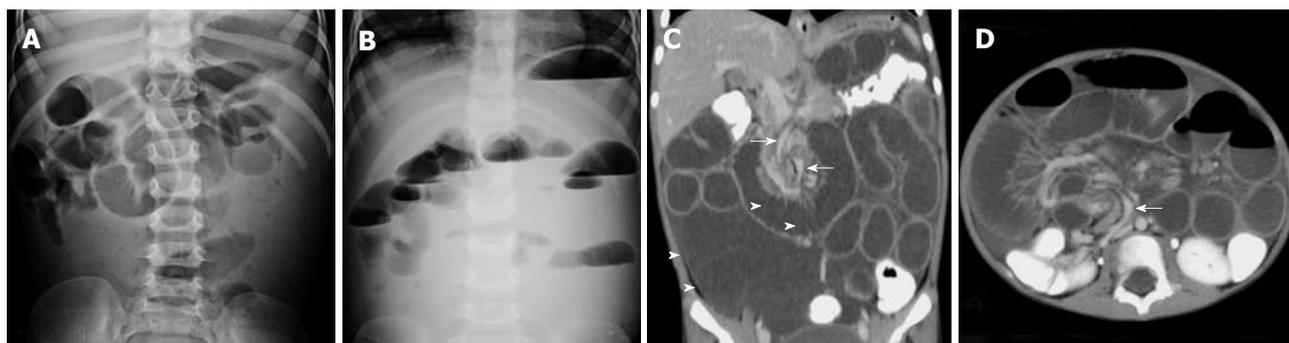


Figure 1 Radiologic study. A, B: Plain abdominal radiography in supine (A) and upright (B) positions showed small-bowel dilatation with multiple air-fluid levels in the upright position (B); C: Coronal view of the abdominal computed tomography (CT) scan revealed twist of the mesentery and its vessels (arrows) with a fluid-filled multiloculated mass in the right lower quadrant (between arrowheads); D: Axial view of the abdominal CT scan showed a twist of the mesenteric fat and its vessels (arrow) with small-bowel dilatation.

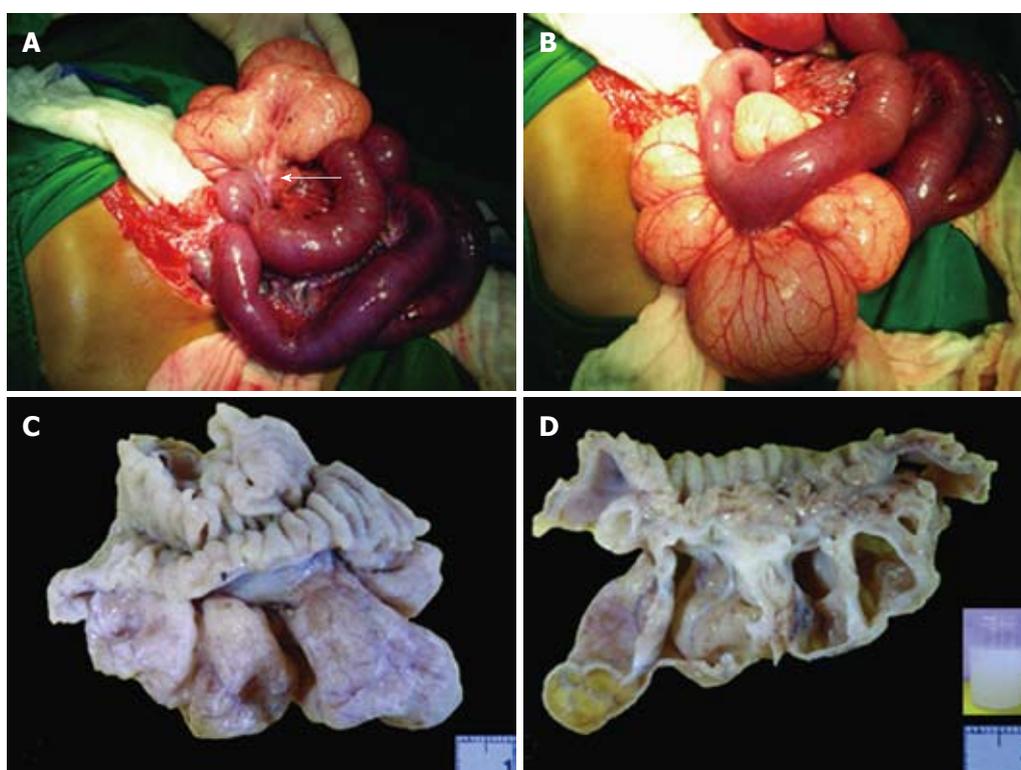


Figure 2 Gross pathology. A, B: Intraoperative photographs show a lobulated cystic yellowish pink mass that originated from the small-bowel mesentery (arrow in A) with small bowel dilatation. The mass shows vascular streaks (B); C, D: The formalin-fixed specimen reveals a partially collapsed, cystic, lobulated, pale tan mesenteric mass with vascular streaks (C). The cut surface reveals thin-wall multicystic spaces with thickened white appearance in some cystic walls (D). The inset shows milky white fluid. The small-bowel mucosa is not remarkable.

sists of uniform small thin-walled lymphatic spaces. The cavernous type is composed of various sizes of dilated lymphatic spaces associated with lymphoid stroma and shows a connection with the adjacent normal lymphatic spaces. The cystic type consists of dilated lymphatic spaces of various sizes associated with collagen and smooth-muscle bundles in the stroma but lacks connection to the adjacent normal lymphatic spaces. Cystic lymphangioma findings are similar to cavernous lymphangioma findings in that dilated lymphatic spaces of variable size are seen for both^[6].

In this case study, the mass was composed of cysti-

cally dilated lymphatic spaces of varying size associated with smooth-muscle cells, characteristic of cystic lymphangioma. An immunohistochemical study for D2-40, a lymphatic endothelial marker, showed focal positivity in the cystic wall endothelium, supporting the diagnosis^[7]. Lymphoid follicles and lymphoid infiltrates in the stroma supported the diagnosis^[8]. The milky white fluid content supported lymphangioma, in which the white color results from accumulated lymphoid cells. The differential diagnoses were lymphangiomyoma and benign multicystic mesothelioma. The former shows smooth-muscle cell proliferation around the lymphatic spaces with pericytic

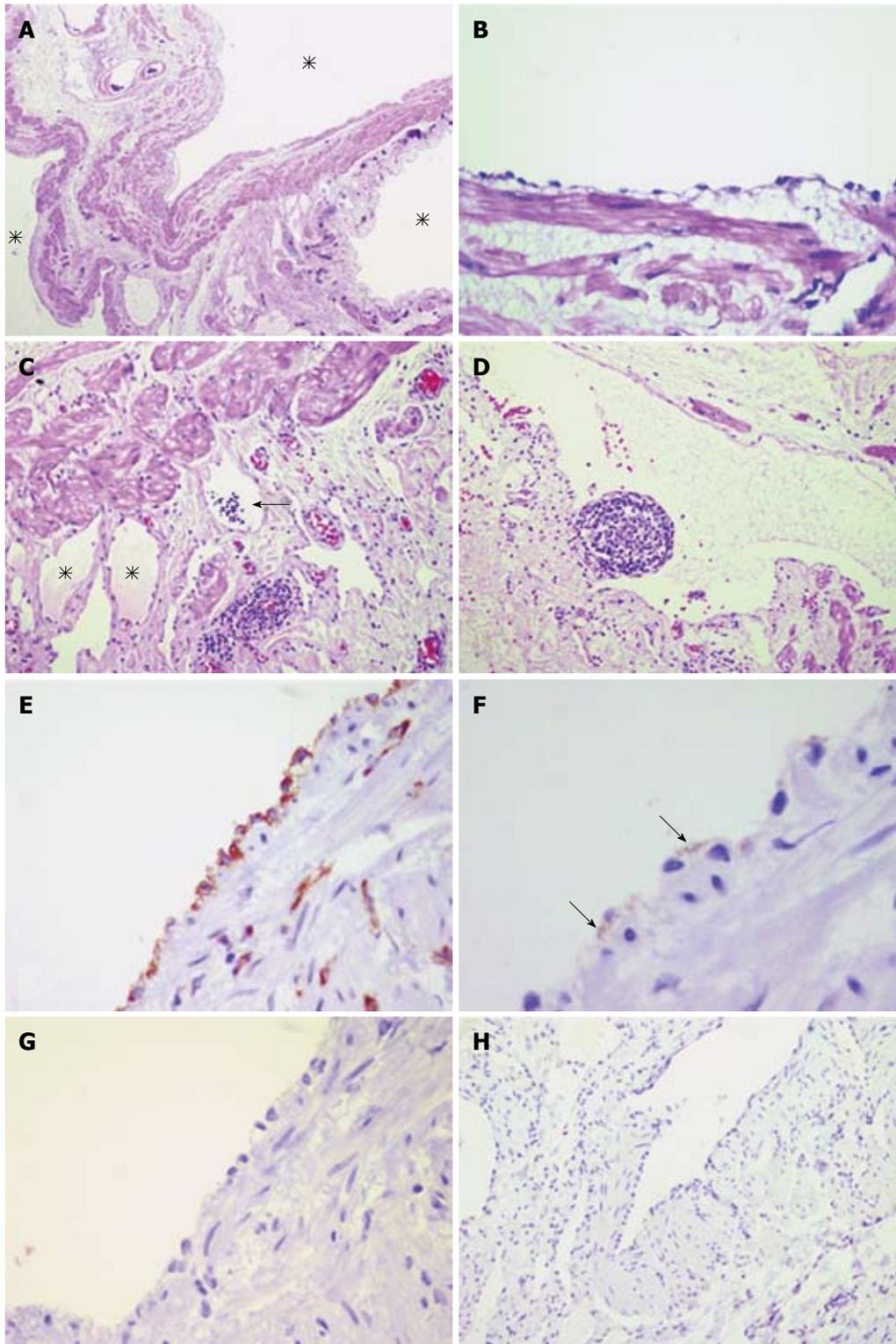


Figure 3 Histopathology. A: Cystic spaces (asterisks) with smooth muscle in their walls (hematoxylin and eosin, 40 ×); B: Flat lining cells of the cystic spaces with smooth muscle below them (hematoxylin and eosin, 400 ×); C: Lymphatic spaces in the stroma containing lymphoid cells (arrow) and pale eosinophilic fluid, suggestive of lymph (asterisks) (hematoxylin and eosin, 200 ×); D: Subendothelial lymphoid follicle (hematoxylin and eosin, 200 ×); E-G: Immunohistochemical study of the lining cells of the cystic wall shows strong cytoplasmic immunoreactivity for factor VIII-related antigen (E, 600 ×) and focal reactivity with D2-40 (F, 1000 ×, arrows) but not for calretinin (G, 600 ×); H: Immunohistochemical study for human melanoma black-45 shows negative result in the smooth muscle of the lymphatic wall (100 ×).

differentiation, demonstrated by HMB-45 immunoreactivity^[8]. Benign multicystic mesothelioma consists of variably sized spaces lined by cuboidal or flattened mesothelial cells, with positive staining for mesothelial marker such

as calretinin^[9]. In this case study, the smooth-muscle cells around the lymphatic spaces were non-immunoreactive for HMB-45, and the lining cells of the cystic walls were non-immunoreactive for calretinin. Therefore, lymph-

angiomyoma and benign multicystic mesothelioma were excluded.

Intra-abdominal lymphangioma usually presents as abdominal distension, a palpable abdominal mass, or acute intestinal obstruction^[2]. The latter is the most common presentation of mesenteric lymphangioma in the form of small-bowel volvulus^[3]. Small-bowel volvulus is the rotation of the small bowel and its mesentery, usually complicated by acute intestinal obstruction. The precipitating factors to volvulus include postoperative adhesion bands, congenital bands, colostomy, ileostomy, fistula, tumors, omental defect, and Meckel's diverticulum^[10]. Mesenteric lymphangioma induces rotation of the small bowel, resulting in small-bowel volvulus with subsequent closed-loop small-bowel obstruction. Partial small-bowel obstruction induced by volvulus was responsible for the patient's first visit, as manifested by abdominal distension. The patient's fever and mucous bloody diarrhea might have resulted from secondary bowel inflammation related to volvulus, particularly hemorrhagic infarction, or from unrelated infectious diarrhea. Small-bowel volvulus with closed-loop obstruction resulted in the patient's second visit and required emergency surgery. One month after the operation, the patient was brought for follow-up and did not manifest postoperative complications.

In conclusion, lymphangioma of the small-bowel mesentery is rare but can cause fatal complications such as volvulus or involvement of the main branch of the mesenteric arteries that requires emergency surgery^[1]. The gross and microscopic findings may mimic benign multicystic mesothelioma in case of an overwhelming

flattened mesothelium, and these conditions can be distinguished by immunohistochemical study.

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Octreotide in Hennekam syndrome-associated intestinal lymphangiectasia

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Abstract

A number of disorders have been described to cause protein losing enteropathy (PLE) in children. Primary intestinal lymphangiectasia (PIL) is one mechanism leading to PLE. Few syndromes are associated with PIL; Hennekam syndrome (HS) is one of them. The principal treatment for PIL is a high protein, low fat diet with medium chain triglycerides supplementation. Supportive therapy includes albumin infusion. Few publications have supported the use of octreotide to diminish protein loss and minimize hypoalbuminemia seen in PIL. There are no publications on the treatment of PIL with octreotide in patients with HS. We report two children with HS and PLE in which we used octreotide to decrease intestinal protein loss. In one patient, octreotide increased serum albumin to an acceptable level without further need for albumin infusions. The other patient responded more dramatically with near normal serum albumin levels and cessation of albumin infusions. In achieving a good response to octreotide in both patients, we add to the publications supporting the use of octreotide in PIL and suggest that octreotide should be tried in patients with PIL

secondary to HS. To the best of our knowledge, this is the first case report on the use of octreotide in HS-associated PIL.

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Key words: Hennekam syndrome; Lymphangiectasia; Octreotide; Protein losing enteropathy

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INTRODUCTION

Protein losing enteropathy (PLE) occurs in a variety of intestinal disorders leading to excessive loss of proteins into the gastrointestinal (GI) tract^[1]. There are two different mechanisms through which intestinal protein loss can occur: lymphatic system abnormalities and mucosal injury. Primary intestinal lymphangiectasia (PIL) is a congenital disorder of the lymphatic system resulting in impaired lymphatic drainage. In the intestines, impaired lymphatic drainage leads to excessive protein-rich chyle loss, hypoalbuminemia and peripheral edema. Mucosal edema leads to malabsorption and steatorrhea. The diagnosis of PIL is based on typical endoscopic and/or histological findings plus exclusion of a secondary cause of intestinal lymphangiectasia such as cardiac disease, malignancy or post-abdominal surgical complications^[2,3]. A

number of disorders have been described to cause PLE in children. Few syndromes are associated with PIL; Hennekam syndrome (HS) is one of them^[4]. HS (OMIM 235510) is an autosomal recessive disorder comprising intestinal lymphangiectasia, severe lymphedema of the limbs, genitalia and face, facial dysmorphism and mental retardation^[4]. Several authors have reported additional symptoms of HS. The frequency of HS is uncertain with widespread occurrence of the gene. There is inter-familial variability in the phenotype. HS is characterized by generalized maldevelopment of the lymphatic system. PIL and PLE are reported in most patients^[4-6]. Phenotypic abnormalities are due to impaired lymphatic flow resulting from insufficient *CCBE1* gene function during lymphangiogenesis. The *CCBE1* protein plays a direct role in the formation of lymphatic vessels and venous sprouting^[7]. Mutations in the *CCBE1* gene have been identified as a cause of HS^[5,7,8]. The principal treatment for PIL is a high protein, low fat diet associated with medium chain triglycerides (MCT) supplementation. Supportive therapy includes albumin infusion and paracentesis, when required. In patients not responding to such therapy, other options, such as octreotide, antiplasmin, tranexemic acid and surgical resection of segmental or localized disease may be a therapeutic option^[9,10]. Somatostatin and its synthetic analogue, octreotide, have been used to treat secretory diarrhea and other GI and pancreatic disorders^[11]. The mechanism of action of octreotide in diminishing protein loss through the GI tract is still unclear. Both somatostatin and octreotide decrease splanchnic blood flow *via* splanchnic vasoconstriction. They also decrease intestinal motility, gastric emptying, gallbladder contraction and pancreatic secretion. Octreotide inhibits GI hormone secretion and results in decreased chloride secretion, increased chloride and sodium absorption and decreased water loss. Therefore, octreotide has been used in the treatment of secretory diarrhea associated with Zollinger-Ellison syndrome, acquired immunodeficiency syndrome, graft versus host disease, carcinoid syndrome, and multiple endocrine neoplasia-2A^[11]. Octreotide inhibits triglycerides absorption^[12]. Little is known about its action on chyle production and pressure regulation in the lymphatic system. Somatostatin inhibits thoracic lymph flow in dogs^[13]. Reports of somatostatin and its analogues on reducing lymphatic fluid outflow in both pediatric and adult patients were described in surgically created thoracic duct injuries^[14,15]. The most commonly reported adverse reactions in clinical trials following octreotide administration were diarrhea, abdominal pain, nausea, flatulence, headache, cholelithiasis, hyperglycemia and constipation. Other commonly reported adverse reactions were pruritus, rash, alopecia, dizziness, localized pain, biliary sludge, thyroid dysfunction, loose stools, vomiting, asthenia, and hypoglycemia. In very rare instances, acute pancreatitis has been reported within the first hours or days of sandostatin treatment. Cardiac adverse effects include bradycardia and less commonly tachycardia^[16].

CASE REPORT

Case 1

A male baby was born at term with a normal birth weight. There were no antenatal or postnatal complications. The infant was born with generalized anasarca and was found to have low serum total protein and serum albumin. There was a history of consanguinity and a family history of a 15-year-old maternal cousin with generalized edema diagnosed to have HS and managed at a different center. The infant was referred to our hospital at the age of 1 mo for evaluation of generalized edema. He had a history of diarrhea, occasional vomiting, abdominal distension and poor feeding. He was not thriving well. There were no symptoms of heart failure. He had normal urine output. Physical examination revealed generalized pitting edema of the lower limbs and non-pitting edema of the hands. There were dysmorphic features manifested as down slanting palpebral fissure, broad nasal bridge, flat stubby nose with midface hypoplasia, long philtrum and hypoplastic low set ears. Chest and cardiovascular examinations were unremarkable. The abdomen was distended and non-tender with ascites and no hepatosplenomegaly. He had scrotal edema. Investigations revealed normal full blood count (FBC), renal function, liver enzymes and liver function. He had low serum total protein (23 g/L), low serum albumin (16 g/L), and low serum immunoglobulin levels (immunoglobulin A of 0.09 g/L, IgG of 0.9 g/L and immunoglobulin M of 0.26 g/L). Urine analysis was negative for protein and 24-h urine protein was low (0.2 g/24 h). Human serum albumin scintigraphy revealed loss of protein into the intestinal tract. An upper GI endoscopy study revealed widespread whitish patches with snowflake appearance seen in the second and third parts of the duodenum. The stomach showed slight gastric wall edema. Histological examination of a biopsy specimen from the duodenum showed marked edema in the lamina propria. There were no dilated lymphatics observed on the examined specimens. Gastric biopsy revealed congestion and edema of the lamina propria. The lack of dilated lymphatics on histological examination of the intestinal biopsies was due to the patchy distribution of lymphangiectasia in the intestinal mucosa. The child was diagnosed with PLE due to PIL. Given the family history of HS, clinical features and dysmorphism, and a biochemical diagnosis of PLE, we suspected a diagnosis of HS. We performed a genetic test for mutation in the *CCBE1* gene. He was found to have homozygous mutation c.305G>C (p.Cys102Ser) in the *CCBE1* gene which confirmed the diagnosis of HS. He was started on MCT-based formula, a low fat, high protein diet, fat soluble vitamin supplements and intravenous infusion of albumin every few days due to the occurrence of severe pericardial effusion and recurrent ascites. However, his serum albumin continued to be on the low side. Given the severity of hypoalbuminemia and his requirement for frequent albumin infusions, he was started on octreotide subcutaneous (s/c) injections,

Table 1 Cases of intestinal lymphangiectasia treated with octreotide in the literature

Number of patients	Age of patients	Etiology of PLE	Dose of octreotide	Pre octreotide serum albumin	Post octreotide serum albumin	Ref.
6	0-24 mo	PIL	15-20 µg/kg twice daily	14-25 g/L	Normal in 3/6	[2]
1	38 yr	IL	100 µg twice daily	12 g/L	Above 40 g/L	[17]
1	21 yr	PIL	150 µg twice daily	22 g/L	39 g/L	[18]
1	25 yr	IL	Slow release octreotide 20 mg every 4 wk	20 g/L	35 g/L	[19]
1	27 yr	Type I IL	200 µg twice daily then SR octreotide 30 mg every 4 wk	19 g/L	NA (graph indicates 30-40 g/L)	[20]
1	47 yr	Cirrhosis induced IL	0.1 mg three times daily	22 g/L	28 g/L	[21]
1	17 yr	PIL	200 µg twice daily	15 g/L	22-26 g/L	[24]
2	2-12 mo	HS associated IL	100 µg twice daily	16 g/L	28-36 g/L	Current report

IL: Intestinal lymphangiectasia; PLE: Protein losing enteropathy; PIL: Primary intestinal lymphangiectasia; SR: Slow release; NA: Not available; HS: Hennekam syndrome.

which gradually reached 100 mg twice daily. A few weeks later, the patient was discharged home on s/c octreotide injections. Albumin infusion requirement was reduced to once monthly 2 mo after starting octreotide therapy, and subsequently stopped. Total serum protein increased to 49 g/L and serum albumin to 28 g/L. He is now 3 years old, tolerating octreotide with no complications.

Case 2

A term male baby was born to a primi mother with a normal birth weight. There were no antenatal or postnatal complications. The infant was noted to have generalized edema from day 1 of life with low serum total protein and serum albumin. There was a history of consanguinity and 2 maternal cousins were diagnosed with HS (patient in case 1 and his older cousin). He was referred to our hospital at the age of 6 wk. There was no weight gain. There was no history of diarrhea, vomiting or jaundice. He was passing urine normally. There were no signs of heart failure or ascites. Physical examination revealed dysmorphic features in the form of hypertelorism, broad flat nasal bridge and a long hypoplastic philtrum. There was generalized edema, periorbital edema, bilateral non-pitting edema of the upper limbs and bilateral pitting edema of the lower limbs with scrotal edema. The remainder of the systemic examination was unremarkable.

Investigations revealed normal FBC, renal function, liver enzymes and liver function. He had low serum total protein (26 g/L) and low serum albumin (16 g/L). Urine analysis was negative for proteinuria. Twenty-four hour urine protein was normal. His bone profile revealed rickets with low 1, 25 hydroxyvitamin D and 25 hydroxyvitamin D. Stool alpha 1 antitrypsin was high at 2.34 g/L. Given the family history of HS, clinical features and dysmorphism, and a biochemical diagnosis of PLE, we suspected a diagnosis of HS and performed a genetic test for familial mutation in the *CCBE1* gene. He was found to have homozygous mutation c.305G>C (p.Cys102Ser) in the *CCBE1* gene using sequence analysis. This confirmed the clinical diagnosis of HS. The patient was managed conservatively with MCT-based formula, fat soluble vitamins, and rickets treatment with frequent

albumin infusions and diuretics. Following albumin infusions, his generalized edema improved. The infant was left with genital, periorbital and lower limb non-pitting edema. Serum albumin decreased to very low levels of 12-16 g/L within a few days of albumin infusions. At the age of 8 wk, we started him on octreotide subcutaneous injections of 80 mg twice daily and increased this to 100 mg twice daily at the age of four months. His serum albumin levels increased to 36 g/L a few months later with no further albumin infusions required. He is now 11 mo old with a good response to octreotide in terms of reducing hypoalbuminemia and cessation of albumin infusions with no noted complications to octreotide.

DISCUSSION

To date, no publications have supported the use of octreotide to diminish protein loss and minimize hypoalbuminemia seen in PIL. These publications are summarized in Table 1. Most clinical research on the use of octreotide in humans has been limited to adults. One of the first experiences of using octreotide in PIL was by Bac *et al*^[17] in 1995. They reported a patient with PLE due to intestinal lymphangiectasia. The patient was treated with and responded to octreotide with normalization of serum albumin and a decrease in fecal protein loss. Few subsequent case reports have supported this experience^[18-21]. The suggested dose of octreotide ranges from 100 µg two or three times a day to 200 µg two times a day or the slow release formulation which can lead to clinical, biochemical and histological improvement^[10,18,20]. The first use of somatostatin in pediatric patients was for non-GI conditions such as excessive growth hormone release, hyperinsulinism and others^[22,23]. Almost all the experience in pediatric patients with PIL is in the form of case reports^[11]. In 1998, Ballinger *et al*^[24] reported the use of octreotide in an adolescent with PIL. Enteric protein loss was decreased and serum albumin levels were stabilized with resolution of peripheral edema and cessation of re-accumulation of recurrent pleural effusions. A recent study of a series of 6 pediatric patients^[2] suggested that octreotide might be useful in controlling findings and maintaining serum albumin

at normal levels. Serum albumin level was maintained at normal levels in 3 patients. The requirement for albumin infusions decreased in all 6 patients. This study concluded that octreotide should be considered in the long-term treatment of PIL when other options are ineffective. They also suggested weighing the benefits of octreotide against the risk of adverse effects. Pediatric case reports have shown that octreotide decreases stool output in a variety of cases^[25-27]. In these case reports, there was no standardization of dose or duration of octreotide use to achieve the required effect. The specific use of octreotide in diarrhea secondary to chemotherapy^[28-30], GVHD^[31,32] and immunodeficiency^[33] has been reported in adults. Despite this, there are reports on the failure of octreotide to diminish stool output in a variety of cases^[34,35]. In one case report, octreotide failed to induce a clinical response and a study performed on an established guinea pig showed that octreotide did not alter lymphatic function^[36].

We report 2 children who were genetically diagnosed as having HS and associated PIL with PLE, who suffered from generalized mixed edema secondary to hypoalbuminemia and lymphangiectasia. By using octreotide in both patients, we eliminated the need for albumin infusions with resolution of pericardial effusion and ascites in one patient. The other younger patient's serum albumin increased dramatically to a normal level with resolution of pitting edema. In both patients, scrotal edema and non-pitting edema were still present. This is likely to be related to generalized maldevelopment of the lymphatic system seen in HS and the possibility that lymphatic function is not altered by octreotide.

In conclusion, our 2 children with genetically proven HS and PIL leading to PLE responded well to octreotide. In both patients, non-pitting edema was still present. This was attributed to lymphangiectasia and a probable reduced effect on the lymphatics in HS compared with other reports in the literature. Octreotide has been used in various GI disorders associated with PIL and PLE. To the best of our knowledge, this is the first case report on the use of octreotide to diminish PLE in HS-associated PIL. The lack of clinical data based on randomized trials in the pediatric population makes it difficult to confirm a positive effect of octreotide on PIL and PLE from the literature. There is a need for multicenter pediatric trials in this regard.

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Total colorectal and terminal ileal duplication presenting as intussusception and intestinal obstruction

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Abstract

Colonic intussusception and gastrointestinal duplication are diseases that arise in young children. The clinical presentation of adult cases of intussusception and enteric duplication is non-specific and thus poses a diagnostic challenge. A computed tomography (CT) scan is recommended in adult cases as the most sensitive diagnostic tool and the pathognomonic finding of outer intussusceptans and central intussusceptum is diagnostic. A septum of a duplicated colon in a non-intussuscepted segment has been rarely reported in the literature. With advancements in radiological imaging technology and the increased availability of CT scanners, the capacity for a correct pre-operative diagnosis has been significantly enhanced. Our current case report illustrates the importance of considering an uncommon etiology for enteric intussusception and duplication as a differential diagnosis of acute abdomen in an adult patient. Our analyses of this patient also highlight the successful use of CT scanning to make this diagnosis.

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Key words: Ileal duplication; Colonic duplication; Intussusception; Intestinal obstruction

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Ho YC. Total colorectal and terminal ileal duplication presenting as intussusception and intestinal obstruction. *World J Gastroenterol* 2012; 18(43): 6338-6340 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i43/6338.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i43.6338>

INTRODUCTION

Colonic intussusception and gastrointestinal duplication are diseases of young children. Symptomatic colonic duplication is very rare in adults. We report a case of a young adult patient with a duplication of the terminal ileum, appendix, colon and rectum who presented in our hospital with symptoms of acute intestinal obstruction and intussusception.

CASE REPORT

A 25-year-old man was admitted to our hospital for central abdominal pain for three days. He had a history of situs inversus and a watery bowel opening with blood streaks was noted on the day of admission. A physical examination revealed a distended abdomen, and tenderness and rebound over the lower abdomen.

Abdominal radiographs further revealed some dilated bowel loops at the right abdomen, which was a relatively non-specific finding (Figure 1). Fresh blood was found in the rectum during a sigmoidoscopic examination. Advancement beyond 30 cm could not be achieved during this examination due to an acute kink. A computed tomography (CT) scan was then performed for further assessment and an intestinal obstruction was confirmed. Intussusception at the region of the sigmoid and descending colon was detected at the right lower abdomen (since the patient had situs inversus) (Figure 2A). The duplicate appearance of the large bowel at the right lower quadrant of abdomen was also noted (Figure 2B).

Emergency surgery was performed and revealed a



Figure 1 Abdominal radiographs taken as an initial evaluation of the abdominal pain in the patient. A: Supine radiograph showing non-specific dilated bowel loops at the right abdomen; B: Erect radiograph revealing a right-sided stomach bubble in this patient with situs inversus.

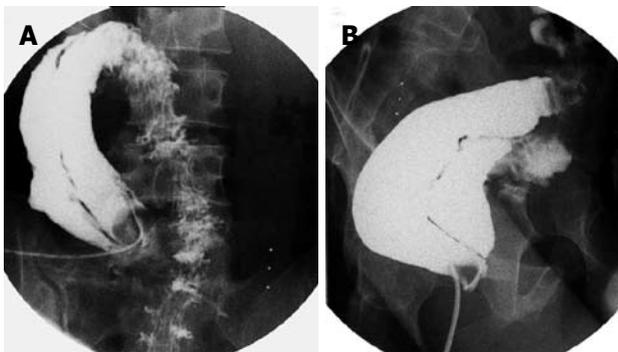


Figure 3 Gastrografin enema. A: Enema performed via an enterostomy showing a duplicated terminal ileum; B: Enema performed via the anus showing a duplicated rectum.

grossly dilated sigmoid and descending colon. Intussusception into the distal sigmoid colon with a large gangrenous diverticulum formed by the septum of a duplicated colon was also observed. A partial small bowel resection, decompression and a colostomy were subsequently performed.

A post-operative gastrografin enema *via* the enterostomy and anus further confirmed a duplication of large bowel and terminal ileum in this patient (Figure 3).

DISCUSSION

Colonic intussusception and gastrointestinal duplication are diseases of young children that usually manifest before two years of age (male:female ratio, 3:1)^[1]. Intestinal intussusception in adults is very rare. In a previously reported surgical series of 20 adults with intussusception, no single diagnosis of gastrointestinal duplication was identified as the underlying pathology^[2]. Colonic duplication represents 4%-18% of gastrointestinal duplication cases^[3]. Duplications are classified according to location or shape; cystic (more than 80%) or tubular^[4]. The classification of this disorder in accordance with the vascular pattern had also been proposed^[5].

The clinical presentation of an adult case with intussusception and enteric duplication is typically non-

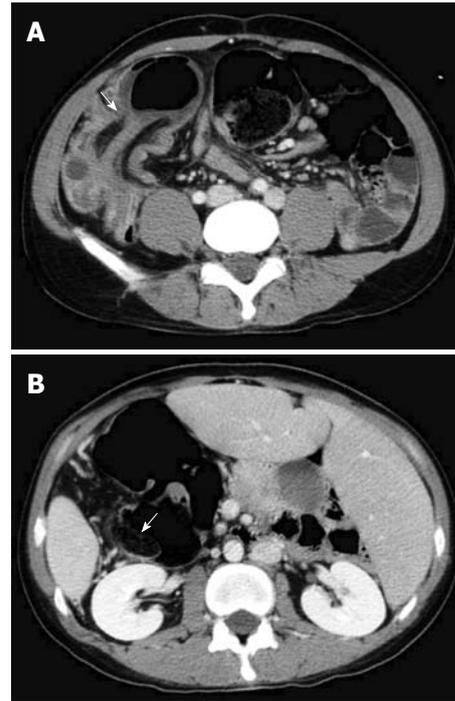


Figure 2 Axial computed tomographic scans of the lower abdomen. A: Intussusception in the region of the sigmoid and descending colon at right lower quadrant of the abdomen (arrow); B: Scan at a more cranial level revealing a distended descending colon with a thin enhancing rim of a septum (arrow).

specific, i.e., abdominal pain, nausea and constipation. The mode of presentation can also be acute, subacute or chronic^[1].

CT scans are recommended as the most sensitive diagnostic tool in adult cases of intestinal intussusception^[1]. The pathognomonic finding of outer intussusceptans and central intussusceptum confirms the diagnosis of these cases. The complex mass is "sausage-shaped" or "target" like when the CT beam is oriented parallel or perpendicular to its axis, respectively^[6]. It can in rare cases present as a cystic mass at the left upper abdomen, thus causing diagnostic confusion as a pancreatic tumor^[7]. The diagnosis of enteric duplication in the absence of intussusception or intestinal obstruction is challenging and it is often difficult to do before surgery^[1,3]. CT scanning can reveal a cystic mass attached to the colon^[3]. The demonstration of a septum of a duplicated colon in a non-intussuscepted segment is a rarity in the current literature.

In summary, our present case illustrates the importance of considering an uncommon etiology involving enteric intussusception and duplication as a potential differential diagnosis for acute abdominal pain in an adult patient. Furthermore, our present findings demonstrate the applicability of a CT scan in making such a challenging diagnosis.

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Gastric metastasis from ovarian carcinoma: A case report and literature review

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Abstract

An isolated parenchymal gastric metastasis from ovarian carcinoma without any other sites of recurrence is extremely rare. Only two cases have been reported, both of which were symptomatic. We herein report such a case without any symptoms. A 61-year-old woman presented with a high cancer antigen-125 level without any other clinical manifestation. A subsequent ¹⁸F-fluorodeoxyglucose (¹⁸F-FDG) positron emission tomography/computed tomography scan revealed a submucosal mass with hypermetabolism of ¹⁸F-FDG (standardized uptake value: 5.36) in the gastric antrum. The final pathology after gastric antrectomy showed a metastatic gastric tumor from a primary ovarian carcinoma. We also performed an extensive literature review about gastric metastasis from ovarian carcinoma published until recently, and this is the first case of an isolated parenchymal gastric metastasis from ovarian carcinoma without any symptoms.

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Key words: Ovarian carcinoma; Gastric antrum; Metastasis; Submucosal tumor; Parenchymal tumor

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INTRODUCTION

Ovarian carcinoma usually metastasizes along the peritoneum throughout the pelvic and abdominal cavity, such as pelvic wall, omentum and mesentery. Gastrointestinal involvement is not common. Even it happens, gastrointestinal tract metastasis of ovarian carcinoma is merely limited to serosa. Solitary parenchymal gastric metastasis from ovarian carcinoma is extremely rare, and only two cases have been reported in English up till now^[1,2]. We herein present a case of gastric metastasis from ovarian carcinoma without any symptoms and other sites of recurrence.

CASE REPORT

In December 2011, a 61-year-old woman was admitted to our hospital because of a high cancer antigen (CA)-125 level of up to 116.5 U/mL (normal, < 35U/mL), and she had no epigastric pain and fullness, hematemesis, melena, weight loss and any other clinical manifestations. In 1999, she underwent optimal debulking cytoreductive surgery in our hospital for ovarian adenocarcinoma, followed by ten cycles of adjuvant chemotherapy with cisplatin and cyclophosphamide. In May 2006, when her CA-125 level increased to 57.9 U/mL, she received

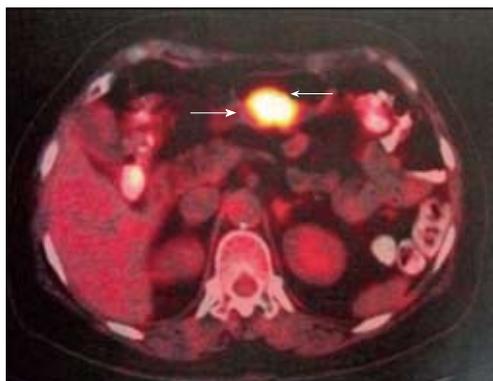


Figure 1 ¹⁸F-fluorodeoxyglucose positron emission tomography/computed tomography shows a hypermetabolic lesion (standardized uptake value: 5.36) in the gastric antrum (arrows).

another ten cycles of adjuvant chemotherapy with taxol, cyclophosphamide, carboplatin and bleomycin. CA-125 level was tested every two months and it exceeded the normal range again in December 2011.

¹⁸F-fluorodeoxyglucose positron emission tomography/computed tomography (¹⁸F-FDG PET/CT) scanning for ruling out the recurrent ovarian carcinoma that was suspected due to the CA-125 level. ¹⁸F-FDG PET/CT revealed a mass located in gastric antrum with high ¹⁸F-FDG uptake (standardized uptake value: 5.36) (Figure 1), and there were no any other lesions with high ¹⁸F-FDG uptake in the abdominopelvic region. A subsequent non-contrast-enhanced CT displayed a 2.4 cm × 3.0 cm submucosal mass in the gastric antrum (Figure 2), which had not been found in the CT scanning done on April 4, 2010. The patient could not tolerate and refuse to take endoscopic examination, so we performed gastroenterography instead. Upper gastroenterography also showed clearly a lesion with a tiny ulceration on the surface of gastric mucosa (Figure 3).

The patient then underwent local gastrectomy. During the operation, we found that both mucosa and serosa were involved, but there was no intumescent lymph node around the gastric antrum. The incision of gastric antrum was fixed with a double-layer hand-sewn suture transversely.

On cut section, a gray-white tumor of 3.2 cm × 2.8 cm × 3.5 cm was situated in the muscularis propria and bulged into the serosa. Microscopically (Figure 4A), serous papillary adenocarcinoma cells infiltrated into normal gastric tissues with cancer embolus in the vessels. There was a deep ulceration on the overlying mucosa. A non-metastatic lymph node was found in the specimen. Values of the immunohistochemical detection of the tumor cells (Figure 4B) were: CA-125 (+++), Wilms' tumor-1 (+++), estrogen receptor (++), cytokeratin 7(+), cytokeratin 20(-), progesterone receptor (-) and CDX-2 (-). The immunohistochemical staining result supported the final diagnosis of gastric metastasis from ovarian serous adenocarcinoma.

CA-125 level was decreased to 53.1 U/mL on the

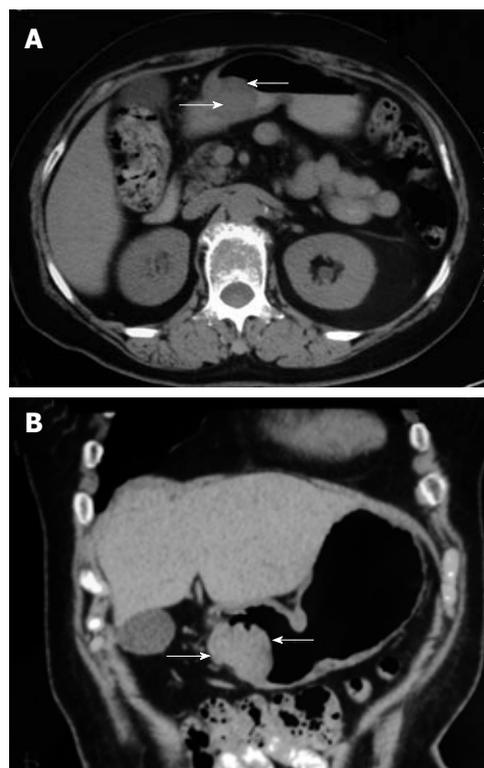


Figure 2 An abdominal computed tomography shows a low density, 2.4 cm × 3.0 cm intramural mass of gastric antrum with tiny ulceration (arrows), which is suggestive of a gastric submucosal tumor, such as gastrointestinal stromal tumor. A: Cross section; B: Coronal section.

7th postoperative day. Her postoperative course was unremarkable and she was discharged on the 9th day after operation. When this manuscript was submitted, she had no experience of recurrent disease.

DISCUSSION

Metastatic disease involving stomach is unusual. A study found 17 metastases to the stomach among 1010 patients with malignant tumors, giving a frequency of 1.7%^[3]. Another series of autopsies discovered 92 gastric metastases among 7165 cases, with a rate of 1.28%^[4]. Most gastric metastases arise from primary breast cancer, followed by melanoma and lung cancer. The incidence of gastric metastases was 3.6% (25/694) in patients with breast cancer and 1.3% (10/747) in patients with lung cancer. No study had analyzed the incidence of gastric metastasis from ovarian carcinoma due to the extremely rare occurrence. According to our review of the literature, there has been no report of gastric metastases from ovarian carcinoma in Chinese.

We performed a very comprehensive review of all case reports of gastric metastasis from ovarian carcinoma. Until this April, ten other reports (Table 1) in English could be searched in PubMed. Patient age ranged from 42 years to 70 years. Two cases^[10,12] were diagnosed with primary ovarian carcinoma simultaneously, the longest time from diagnosis of primary tumor to discovery

Table 1 Literature review

Author	Age	Histology	Recurrence sites	Recurrence time	Symptoms	Survival
Sangha <i>et al</i> ^[1]	55	NR	Stomach	7 yr	Belching reflux, epigastric discomfort	NED NR
Pernice <i>et al</i> ^[2]	42	Adenocarcinoma G3	Stomach + perigastric area	18 yr	Asymptomatic	12 mo NED
Taylor <i>et al</i> ^[5]	62	Serous adenocarcinoma G3	Lung + liver + stomach	10 mo	Haemorrhagy	6 mo DOD
Kobayashi <i>et al</i> ^[6]	48	NR	Spleen + pancreas+ sigmoid colon	21 yr	Hemorrhage, partial bowel obstruction	NR
Dupuychaffay <i>et al</i> ^[7]	65	Adenocarcinoma G3	Stomach + diaphragm + pancreas + peritoneal nodes	16 yr	Fever, aasthenia, anorexia, epigastric pain	NR
Bechade <i>et al</i> ^[8]	51	Adenocarcinoma G3	Stomach + peritoneal nodes + ovaries	NR	Hemorrhage	ED NR
Jung <i>et al</i> ^[9]	49	Serous ovarian adenocarcinoma	Gastric antrum + presacral area	52 mo	Asymptomatic	18 mo NED
Carrara <i>et al</i> ^[10]	70	Adenocarcinoma	Gastric body	Simultaneously	Mild anemia, dyspepsia	NR
Majeurs <i>et al</i> ^[11]	61	Serous adenocarcinoma G3	Stomach + sigmoid colon	7 mo	Epigastric discomfort, vomit	18 mo DOD
Kang <i>et al</i> ^[12]	55	Adenocarcinoma	Gastric antrum + pelvic cavity	Simultaneously	Epigastric pain, abdominal distention	12 mo NED
Present case	61	Adenocarcinoma G3	Gastric antrum	12 yr	Asymptomatic	5 mo NED

DOD: Death of disease; NR: Not report; ED: Evident disease; NED: Not evident disease.



Figure 3 Upper gastroenterography shows a lesion in the gastric antrum (arrows). The antrum was partially obstructed by the mass.

of gastric metastasis being 18 years^[2]. Clinical manifestations were diversified and nonspecific, and three cases were asymptomatic (3/11, 27.27%).

Due to the extremely low incidence, it is hard to make a correct diagnosis of gastric metastasis from ovarian carcinoma. According to our literature review, some cases^[2,9] only presented with CA-125 levels beyond normal range but without any symptoms. Since CT scanning, gastroenterography and gastroscopy all showed a submucosal tumor of stomach, a wrong diagnosis of gastrointestinal stromal tumor^[12] would be easily made^[12]. So, when a patient has a history of ovarian carcinoma, especially when her CA-125 level is high, metastasis from ovarian carcinoma should be considered. ¹⁸F-FDG PET/CT can be useful. In our case, ¹⁸F-FDG PET/CT scanning revealed a high metabolic uptake lesion of gastric antrum, which is similar to the findings as described by other authors^[2,12].

Ovarian carcinoma is more likely to metastasize along the peritoneal surface, but the mechanism of gastric metastasis remains unclear, it may be because of the rich

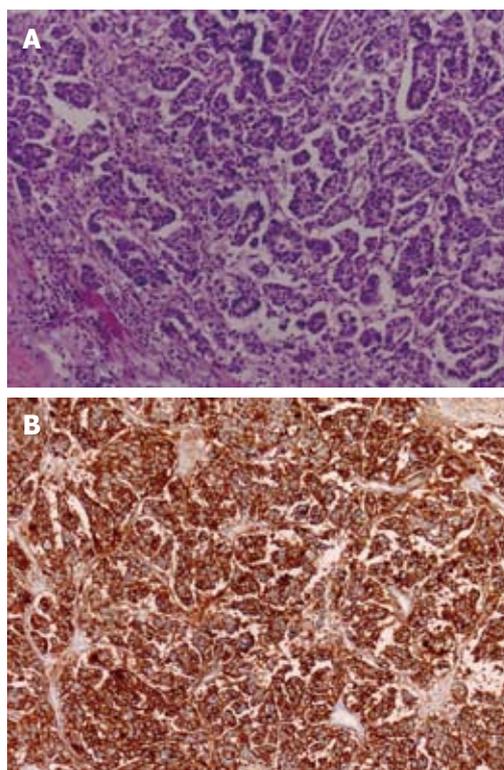


Figure 4 Pathological manifestation of the neoplasm. A: Microscopically, the tumor is composed of irregular sheets of cells with a high-grade nuclear atypia (HE stain, $\times 100$); B: Immunohistochemically, the tumor cells are immunoreactive for cancer antigen 125 ($\times 100$).

blood supply of stomach. Local excision without radical lymphadenectomy following adjuvant chemotherapy is effective and recommended for metastases of ovarian carcinoma. The prognosis of gastric metastases of ovarian carcinoma remains unknown, according to our literature review, a one-year survival rate can be expected optimistically (5/6, 83.33%).

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Is NEDD4-1 a negative regulator of phosphatase and tensin homolog in gastric carcinogenesis?

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Abstract

The expression of phosphatase and tensin homolog (*PTEN*), a tumor suppressor gene, is frequently down-regulated in gastric carcinomas due to mutation, loss of heterozygosity, and promoter hypermethylation. However, it is unknown if additional mechanisms may account for the down-regulation of *PTEN* expression. While neuronal precursor cell-expressed developmentally down-regulated 4-1 (*NEDD4-1*) is believed to be a potential dual regulator of *PTEN*, there are conflicting reports regarding their interaction. To gain further insight into the role of *NEDD4-1* and its association with *PTEN* in gastric carcinoma development, we measured the protein expression of *NEDD4-1* and *PTEN* in gastric mucosae with various pathological lesions and found that *NEDD4-1* increased from normal gastric mucosa to intestinal metaplasia and decreased from dysplasia to gastric carcinoma. These changes did not correlate with *PTEN* expression changes during gastric carcinogenesis. Moreover, we found similar results in protein levels in the primary tumors and adjacent non-tumor-

ous tissues. These results differ from a previous report showing that expression of *NEDD4-1* is up-regulated in gastric carcinomas, and show a more complex pattern of *NEDD4-1* gene expression during gastric carcinogenesis.

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Key words: Neuronal precursor cell-expressed developmentally down-regulated 4-1; Phosphatase and tensin homolog; Gastric carcinogenesis; Immunohistochemistry

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TO THE EDITOR

One of the aims of cancer research is to identify mechanisms controlling the loss of tumor suppressors that promote cancer development. Expression of phosphatase and tensin homolog (*PTEN*), a tumor suppressor gene^[1-3], is frequently down-regulated in gastric carcinomas (GC) due to mutation, loss of heterozygosity, and promoter hypermethylation^[4-8]. In an effort to determine if neuronal precursor cell-expressed developmentally down-regulated 4-1 (*NEDD4-1*), a potential dual regulator of *PTEN*^[9,10], plays a role in the development of gastric carcinoma, Kim *et al*^[11] investigated *NEDD4-1* protein expression in 60 specimens of gastric carcinoma tissues using immunohistochemistry. They found that 75% of GC were immunopositive for *NEDD4-1*, whereas the normal gastric mucosal cells displayed a weak or

Table 1 Differential expression of neuronal precursor cell-expressed developmentally down-regulated 4-1 and phosphatase and tensin homolog at different histological gastric tissue specimens

Group	n	Age (yr)	Sex (M/F)	NEDD4-1				Mean rank	Group	P value	PTEN				Mean rank	Group	P value				
				-	+	++	+++				-	+	++	+++							
Normal gastric mucosa	21	50.0 ± 14.1	13/8	2	6	11	2	87.64	1 vs 2	0.567	3	10	4	4	108.26	1 vs 2	0.607				
																		1 vs 3	0.127	1 vs 3	0.211
																		1 vs 4	0.519	1 vs 4	0.448
																		1 vs 5	0.225	1 vs 5	0.001
																		2 vs 3	0.399	2 vs 3	0.062
Chronic gastritis	21	54.2 ± 14.7	10/11	0	8	9	4	96.26	2 vs 4	0.983	1	10	8	2	116.21	2 vs 4	0.185				
Intestinal metaplasia	41	54.5 ± 12.4	21/20	3	6	23	9	106.16	2 vs 5	0.059	13	15	9	4	91.55	2 vs 5	0.000				
Dysplasia	48	57.4 ± 12.5	26/22	4	14	19	11	95.83	3 vs 4	0.364	13	16	15	4	98.08	3 vs 4	0.535				
Gastric carcinoma	50	54.0 ± 12.3	31/19	7	21	18	4	73.13	3 vs 5	0.001	30	10	9	1	65.9	3 vs 5	0.012				
Overall	181	54.6 ± 13.8	101/80	16	55	80	30		4 vs 5	0.029	60	61	45	15		4 vs 5	0.001				

NEDD4-1: Neuronal precursor cell-expressed developmentally down-regulated 4-1; PTEN: Phosphatase and tensin homolog.

no immunoreactivity. NEDD4-1, an E3 ubiquitin-protein ligase, is believed to play two opposite roles in regulation of PTEN^[9,10]. On one hand, it exercises an oncogenic function by catalyzing PTEN polyubiquitination, resulting in degradation of PTEN protein^[9]. On the other hand, it exerts a tumor suppressive function by catalyzing PTEN monoubiquitination and regulating PTEN nuclear transport^[10]. Together, these data suggest that NEDD4-1 may play a role in gastric carcinoma development and that it may function as a negative regulator of PTEN.

To gain further insight into the role of NEDD4-1, and more importantly, its association with PTEN in gastric carcinoma development, we measured expression of NEDD4-1 and PTEN by immunohistochemistry in biopsies or surgical specimens of 181 patients with various developmental stages of gastric carcinoma collected from January 2007 to September 2009 at the First Affiliated Hospital of Nanchang University. The specimens included 21 cases of normal gastric mucosa (NGM), 21 cases of chronic gastritis (CG), 41 cases of intestinal metaplasia (IM), 48 cases of dysplasia (Dys), and 50 cases of GC (Table 1). There were no significant differences in the age or gender distributions among these groups. These tissue specimens were sectioned and immunostained using the PV-9000 Polymer Detection System (Zhongshan Goldenbridge, Beijing, China), then reviewed and scored semi-quantitatively, as described previously^[12]. An additional 15 pairs of primary tumors and adjacent non-tumorous tissues were subjected to total cellular protein isolation and Western blot analysis of NEDD4-1 and PTEN expression. This work was approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University. Primary antibodies used in this study were: rabbit polyclonal anti-human NEDD4-1 (Millipore, Billerica, MA, United States), PTEN (Abcam, Cambridge, United Kingdom), and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, United States).

The expression of NEDD4-1 was found to increase from NGM to IM, but to decrease from Dys to GC ($P < 0.05$). NEDD4-1 expression level was significantly decreased in GC compared with IM or Dys (Figure 1

and Table 1, $P < 0.05$). There were no significant differences in NEDD4-1 expression between GC and NGM or CG (Figure 1 and Table 1). The expression of PTEN was found to increase from NGM to CG, but to decrease from IM to GC ($P < 0.05$). The PTEN expression level was significantly decreased in GC compared with NGM, CG, IM and Dys (Figure 1 and Table 1, $P < 0.05$). Analysis of the data from cases of GC did not show an obvious association between NEDD4-1 or PTEN expression levels and clinicopathological grades. In addition, we did not find any correlations between NEDD4-1 expression and PTEN expression in different stages. In primary GC, 13 (86.7%) of 15 cases showed a reduction in PTEN expression in tumor tissues compared with the corresponding non-tumorous mucosa. The mean level of PTEN expression was significantly lower in tumor samples than in the non-cancerous counterparts (Figure 2, $P < 0.05$). However, there was no significant difference in the expression of NEDD4-1 between tumor samples and the non-cancerous counterparts (Figure 2, $P < 0.05$).

Since Kim *et al.*^[11] showed an increased expression of NEDD4-1 in GC, we expected to detect increased NEDD4-1 expression in GC. However, we found that NEDD4-1 was increased in the early stages of gastric carcinogenesis, but was then decreased in GC. In addition, we did not find increased NEDD4-1 expression in tumor tissues compared with the corresponding non-tumorous mucosa. More importantly, there was no correlation between NEDD4-1 expression and PTEN expression.

There are several factors that may explain the inconsistencies between the results from our study and those from others. First, although NEDD4-1 is believed to be a potential dual regulator of PTEN, there are discrepancies regarding the nature of the relationship between NEDD4-1 and PTEN. Fouladkou *et al.* reported that NEDD4-1 is not the E3 ligase regulating PTEN stability and subcellular localization^[9,10,13]. We did not see any correlation between NEDD4-1 and PTEN expressions, suggesting that NEDD4-1 may not be a regulator of PTEN in gastric carcinogenesis. Second, there was dif-

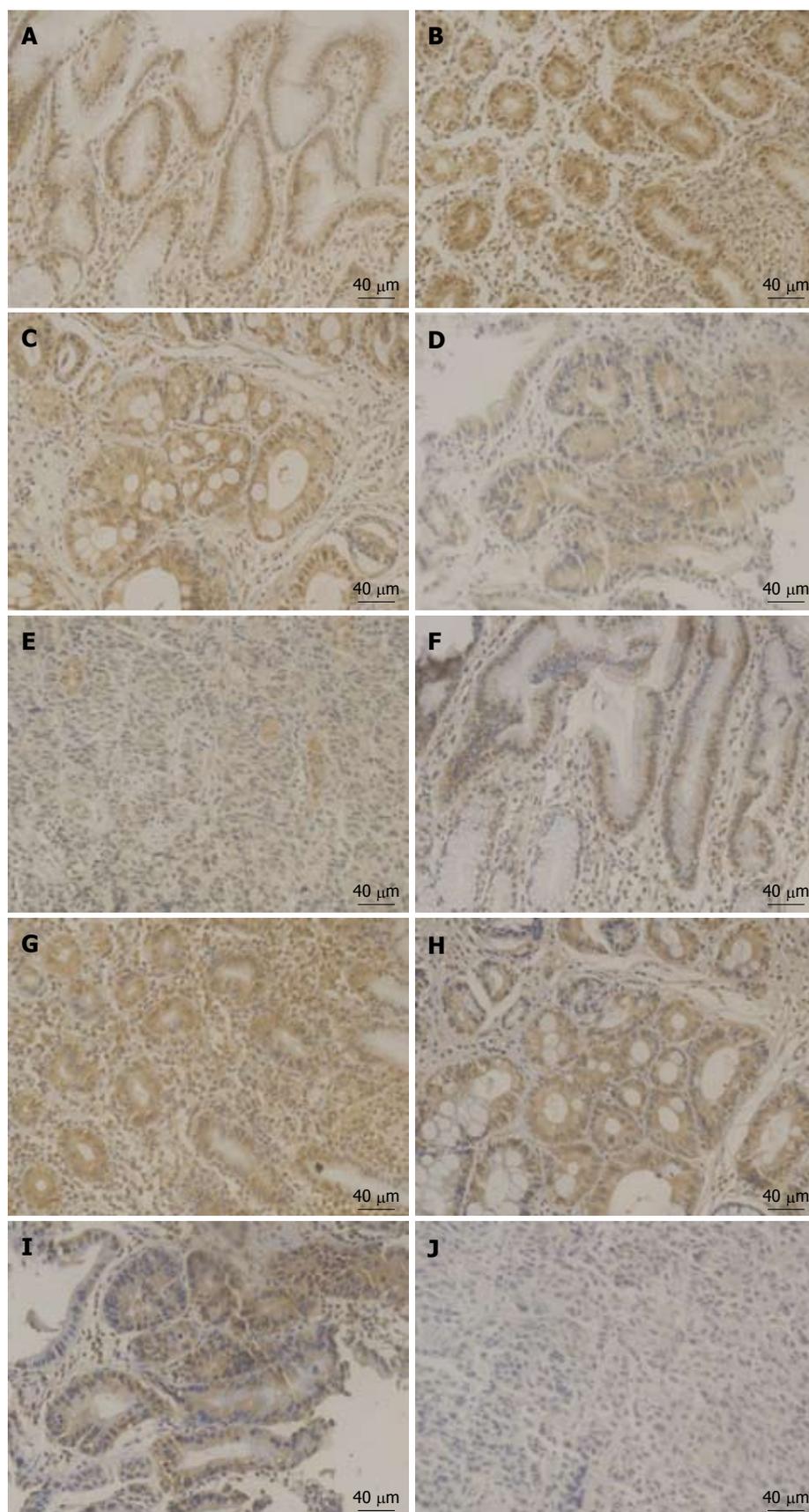


Figure 1 Immunohistochemical staining of neuronal precursor cell-expressed developmentally down-regulated 4-1 and phosphatase and tensin homolog protein in different histological samples of gastric tissues. A: The expression of neuronal precursor cell-expressed developmentally down-regulated 4-1 (NEDD4-1) in normal gastric mucosa (NGM); B: The expression of NEDD4-1 in chronic gastritis (CG); C: The expression of NEDD4-1 in intestinal metaplasia (IM); D: The expression of NEDD4-1 in dysplasia (Dys); E: The expression of NEDD4-1 in gastric carcinoma (GC); F: The expression of phosphatase and tensin homolog (PTEN) in NGM; G: The expression of PTEN in CG; H: The expression of PTEN in IM; I: The expression of PTEN in Dys; J: The expression of PTEN in GC. Magnification: $\times 200$.

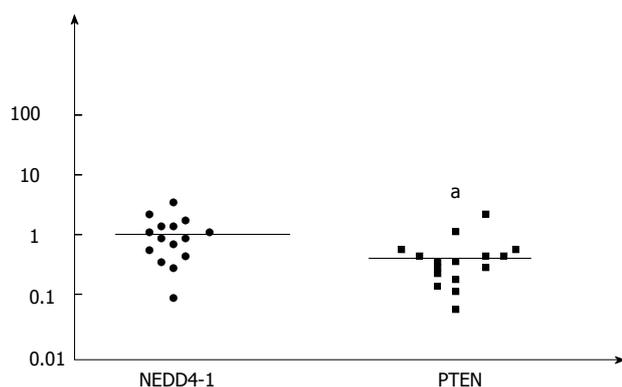


Figure 2 Relative neuronal precursor cell-expressed developmentally down-regulated 4-1 and phosphatase and tensin homolog protein expression level in gastric carcinoma compared to adjacent non-tumorous mucosa. Immunoblots of neuronal precursor cell-expressed developmentally down-regulated 4-1 (NEDD4-1), phosphatase and tensin homolog (PTEN) were scanned and the relative protein expression level of tumor samples vs their non-cancerous counterparts is expressed as % of β -actin. ^a $P < 0.05$.

ference in the genetic background of the cohort from our study and a previous study. In our study, patients were from southern China, whereas in the study by Kim *et al*^[11], patients were from South Korea. In addition, the scoring systems of the two studies were different. In conclusion, we show in the present study that NEDD4-1 and PTEN are distinctly expressed in the various stages of gastric carcinogenesis. In addition, PTEN expression was significantly lower in primary tumors than in adjacent non-tumorous tissues, while there was no difference in NEDD4-1 expression between primary tumors and adjacent non-tumorous tissues. Therefore, NEDD4-1 may not be a regulator of PTEN in gastric carcinogenesis. To elucidate the roles of NEDD4-1 in gastric carcinogenesis, especially the role in regulation of PTEN function, further studies with larger samples in the various stages of gastric carcinoma are needed.

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- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

In press

- 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

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

- 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]

No author given

- 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]

Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

- 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]

No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS A Careaction* 2002; 1-6 [PMID: 12154804]

Books*Personal author(s)*

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 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

Author(s) and editor(s)

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiecezorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

- 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

Electronic journal (list all authors)

- 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>

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean \pm SD or mean \pm SE.

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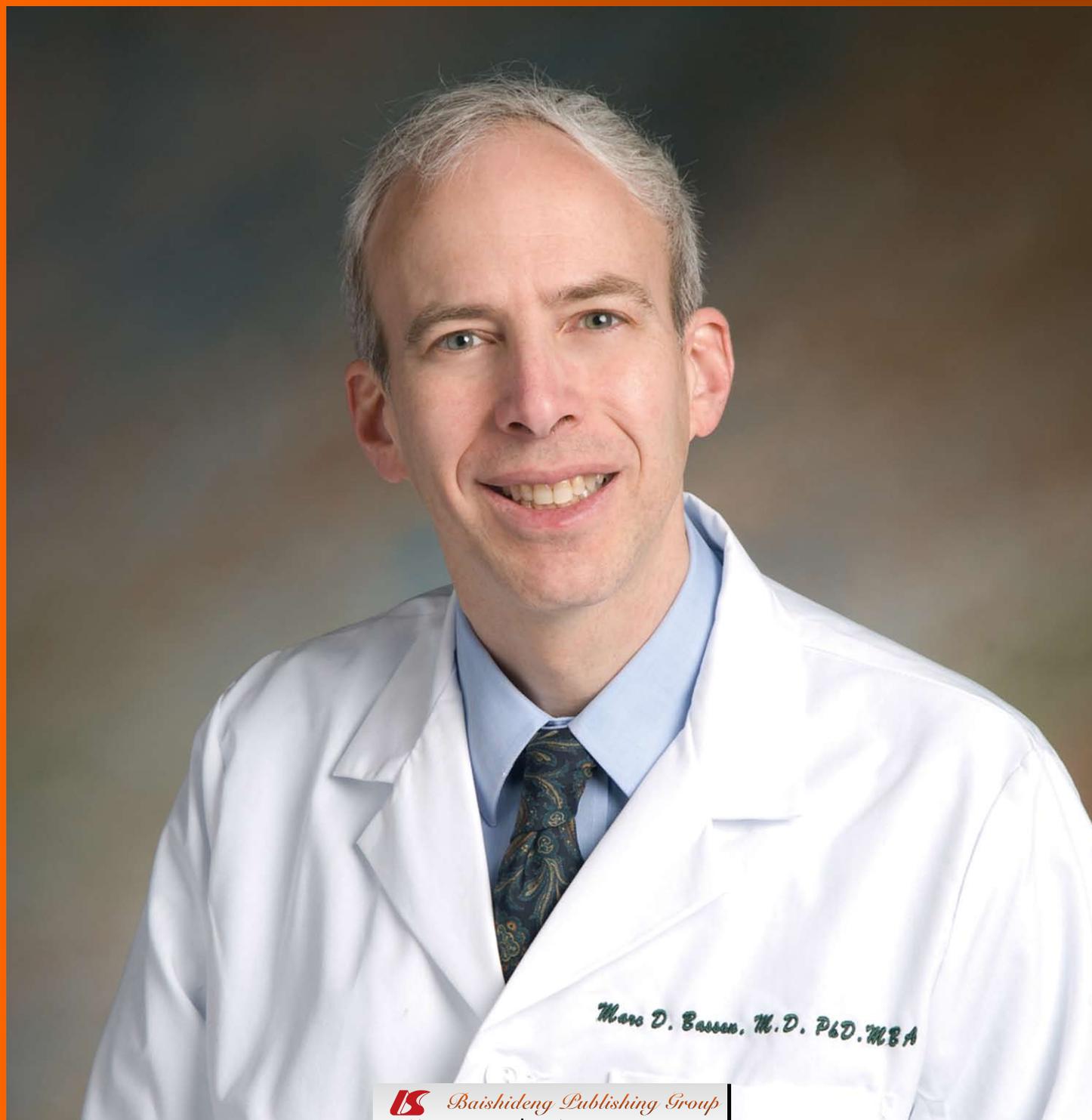
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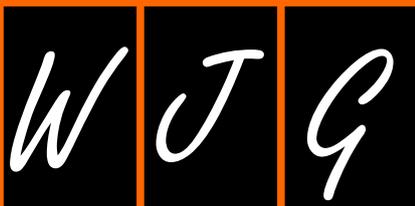
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New approach to anal cancer: Individualized therapy based on sentinel lymph node biopsy

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Abstract

Oncological treatment is currently directed toward a tailored therapy concept. Squamous cell carcinoma of the anal canal could be considered a suitable platform to test new therapeutic strategies to minimize treatment morbidity. Standard of care for patients with anal canal cancer consists of a combination of radiotherapy and chemotherapy. This treatment has led to a high rate of local control and a 60% cure rate with preservation of the anal sphincter, thus replacing surgical abdominoperineal resection. Lymph node metastases represent a critical independent prognostic factor for local recurrence and survival. Mesorectal and iliac lymph nodes are usually included in the radiation field, whereas the inclusion of inguinal regions still remains controversial because of the subsequent adverse side effects. Sentinel lymph node biopsies could clearly identify inguinal node-positive patients eligible for therapeutic groin irradiation. A sentinel lymph node navigation procedure is reported here to be a feasible and effective method for establishing the true inguinal node status in patients suffering from anal canal cancer. Based on the results of sentinel node biopsies, a selective approach could be proposed where node-positive patients could be selected for inguinal node irradiation while node-negative patients could take advantage of inguinal sparing irra-

diation, thus avoiding toxic side effects.

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Key words: Anal carcinoma; Lymphnode metastasis; Sentinel lymphnode; Tumor staging

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INTRODUCTION

In the past two decades, tangible efforts have been made to understand the natural course and behavior of anal canal carcinoma, and, especially, to improve the efficacy of multimodality chemo-radiation treatment. Currently, the main issues in the treatment of this neoplasm are the recognition of a reliable staging system and strategies to obtain long-term survival while reducing radiation related side effects.

Anal canal squamous cell carcinoma represents 1% to 2% of all gastrointestinal malignancies^[1-3] and is associated with human papilloma virus infection^[4]. Diagnostic procedures include clinical and rectal examination, endorectal ultrasound, magnetic resonance imaging (MRI), computed tomography (CT) scan, and positron emission tomography (PET) scan^[1]. Clinical and pathological classification is based on tumor-node-metastasis

staging developed by the American Joint Committee on Cancer^[5].

The two most significant prognostic factors are tumor size and nodal status^[6,7]. Inguinal lymph node involvement is considered a major independent prognostic factor for local recurrence and overall survival^[2] with survival rates dropping from 70% in node-negative to 40% in node-positive patients. Synchronous inguinal metastases are strictly related to the tumor size and occur in 10% to 25% of patients^[8,9]. Metachronous metastases are found, usually during follow-up, in 5% to 25% of patients who were groin lymph node-negative at the time of diagnosis^[9].

Identification of the anal canal lymphatic drainage pattern is essential to predict secondary nodal involvement. Lymphatic drainage of anal canal cancer mostly depends on the tumor location. Based on anatomic and physiopathological studies, tumors located under the dentate line are more likely to drain to groin chains while tumors located above the dentate line are prone to drain to the internal iliac lymph node system, although the two drain systems are not separated from each other (Figure 1)^[10-12]. A tumor located laterally in the anal canal is more likely to drain to the homolateral side. Moreover, tumors located in the midline have the tendency to drain bilaterally in the inguinal regions^[12].

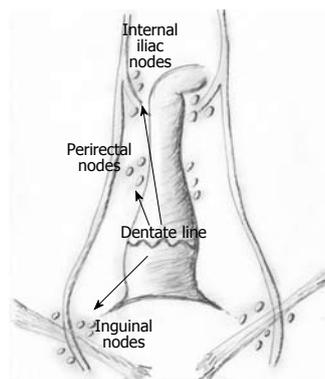


Figure 1 Anal canal lymphatic drainage pattern.

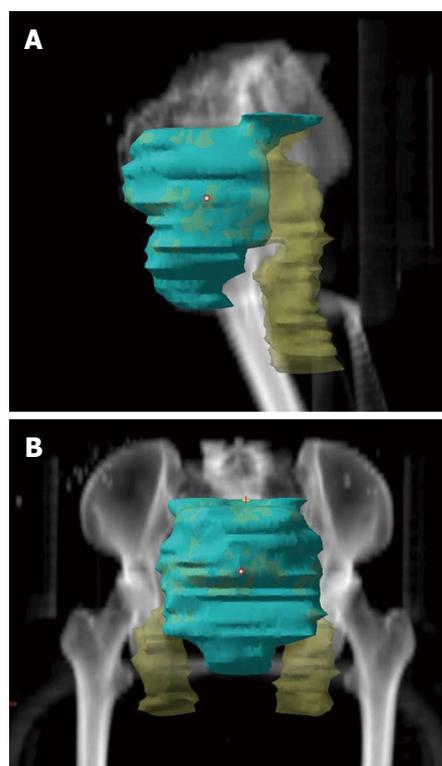


Figure 2 Radiation field of anal carcinoma with exclusion (green field) or inclusion of inguinal regions. A: Lateral view; B: Anterior view.

TREATMENT OPTIONS AND PROGNOSIS

Local excision with neoplasm-free margins is recommended for T1 well-differentiated tumors^[13]. Abdominoperineal resection and permanent colostomy has been traditionally performed for anal canal cancer achieving 40% to 70% survival rate at five years^[2,14]. Chemo-radiation treatment has replaced surgical resection since Nigro *et al*^[15] introduced his protocol in 1974 and has raised survival and eradication of tumors from 70% to 90% in selected patients^[16-19]. Predictably, prognosis is worsened by 50% with nodal involvement and tumor size larger than 5 cm^[2]. Local recurrence shown by pathology or incomplete pathological response after multimodality chemo-radiotherapy treatment is an indication of subsequent abdominoperineal resection^[13,20,21]. However, dose-related radiation side effects, such as anal ulcers, stenosis, and necrosis, can necessitate a subsequent colostomy in 6% to 12% of patients^[18,22-25]. Irradiation fields involving the groin lymph nodes chain can cause inguinal fibrosis, external genitalia edema, epidermolysis with superinfection of skin, necrosis of the femoral head, femoral head fracture, and stenosis of iliac artery^[18,22,24,26-30]. Death from radiation toxicity is reported in 2.0% to 2.7% of patients^[9]. Moreover, risk of radiation side effects do not decrease over time and might pose a lifelong risk of developing late complications^[24,29]. For these reasons, strategies to reduce the radiation field are advisable.

Metastatic involvement of inguinal nodes is a crucial point in the correct assessment of these patients.

In fact, involvement of mesorectal nodes does not affect the therapeutic approach because they are generally included in the radiation fields, while routine inclusion of groin lymph node stations remains controversial. Inguinal lymph node involvement is difficult to establish. The diagnostic accuracy of clinical evaluation and imaging tools remains low. In the case of clinically palpable nodes, a biopsy could be performed. However, as in the majority of cases, a clinically negative groin does not imply absence of metastatic disease. A “wait and see” position is recommended by some institutions^[31], while prophylactic groin irradiation is ordinarily practiced by other groups^[32]. Less frequently, the decision to irradiate the inguinal region is based on primary anal tumor size^[33]

(Figure 2).

Prophylactic groin irradiation has been proposed by several authors with a reduction of inguinal metastatic recurrence as low as 2.5% to 3%^[22,34-40]. Otherwise, Papillon *et al*^[41] and Gerard *et al*^[9] examined a large series of clinically node-negative patients after groin sparing irradiation and observed inguinal metachronous recurrence in 7.4% and 7.8% of patients, respectively.

Given this premise, it is clear that the majority of patients are over treated because effective nodal staging is not achieved. On the other hand, early T stage neoplasms with underrated nodal status may not receive the proper treatment by inguinal sparing.

RATIONALE AND IMPLICATIONS OF SENTINEL LYMPH NODE MAPPING

Because the standard treatment does not provide any specimens for pathological evaluation, the effective node status of these patients is not determined. Moreover, the wide lymphatic drainage pattern that characterizes the inguinal and the pelvic lymph node basin makes it difficult to predict synchronous and metachronous metastatic involvement^[42]. Furthermore, it has been observed that the size of the nodes is not a predictable parameter for nodal involvement because 44% of positive inguinal lymph nodes at pathological examination are smaller than 5 mm^[27]. Conversely, 50% of larger nodes appear to be inflammatory^[14]. Of note, in our previous case series, smaller lymph nodes (4-7 mm) were more likely to harbor metastases than larger ones^[43].

For these reasons, even more advanced imaging techniques, such as MRI, are not accurate in the detection of metastatic lymph nodes^[44]. PET has been used as an aid to achieve better staging and it has been demonstrated to detect up to 20% of metastases not diagnosed by clinical or radiological examination^[44]. Bannas *et al*^[45] has demonstrated that PET/CT is superior to PET or CT alone for staging anal cancer, particularly in identifying local regional lymph node metastases. Recently, Mistrangelo *et al*^[46] demonstrated that sentinel lymph node biopsy (SLNB) was superior to PET-CT for the staging of inguinal lymph nodes. In our experience, PET has a sensitivity of 33% and a specificity of 84%. Even though sensitivity of SLNB of the anal canal has not been yet addressed, the sensitivity of sentinel node biopsy detection in melanoma is reported to be up to 99%^[47-49].

These findings advocate the need to find a more reliable technique to identify positive nodes. Currently, histological evaluation is the gold standard to assess the presence of metastases in lymph nodes. Standard surgical node dissection for suspicious inguinal nodes has been proposed^[50,51]. However, this approach is also fraught with side effects^[52,53]. In addition, elective inguinal dissection for clinically suspicious nodal involvement revealed metastasis only in 50% of cases at histology^[14].

In this scenario, SLNB could help to accurately iden-

tify patients with inguinal metastatic spread and to avoid irradiation morbidity in node-negative subjects or, conversely, to enroll node-positive patients for inguinal irradiation. SLNB should not be performed in patients with clear evidence of clinically positive or suspicious inguinal nodes. Some reports have also suggested the exclusion of patients with locally advanced T4 cancer. Metastatic or even reactive nodes may alter the lymphatic drainage pattern, thus making the SLNB unreliable. For the same reason, patients who underwent prior surgical manipulation in the anal region should be excluded from the procedure^[12,54,55].

PATIENT SELECTION AND FEATURES

Patients with histologically confirmed squamous cell carcinoma of the anal canal were eligible for the SLNB procedure. Previous reports have also included tumors of the anal margin, which should be considered separately due to different clinical behavior. For the reasons explained above, patients with clear or suspicious inguinal nodes were generally not enrolled^[54].

From 2007 to 2012, 23 patients with proven squamous cell carcinoma of the anal canal and clinically negative inguinal nodes were enrolled in the prospective study. Pre-operative work-up included endoscopy and biopsy, pelvic RMI, endoanal ultrasound, and abdominal and lung CT scans. Tumor stage was classified as follows: T1, 3 pts; T2, 9 pts; T3, 7 pts; and T4, 4 pts.

In 2009, an inguinal sparing irradiation protocol was started. Fifteen patients were observed and three patients were excluded from the SLNB study for positive inguinal lymph nodes confirmed by cytology. Twelve patients with clinically negative inguinal regions were enrolled. After the SLNB procedure, patients with histologically positive inguinal nodes were treated with combined chemotherapy using 5-fluorouracil/mitomycin-C, and the standard radiotherapy field, including inguinal basins. Patients with tumor-free nodes did not undergo inguinal irradiation. Patients were then regularly followed-up every three months.

DESCRIPTION OF TECHNIQUES

Many authors have reported a standardized technique to isolate and retrieve sentinel lymph nodes for metastatic assessment^[12,28,54-64]. The first procedural step was a lymphoscintigraphy to evaluate the main lymphatic drainage and the first node in which the tracer is captured. The radiotracer (0.2 mL 99mTc nanocolloid) injection was performed submucosally, directly around the anal lesion in the four cardinal points with the aid of an anoscope. As the injection can be painful, a needle-free injection system^[56] could also be utilized to minimize patient discomfort. After injection, planar anterior and posterior images were taken with a Philips gamma camera, as previously described^[43], to localize the sentinel lymph

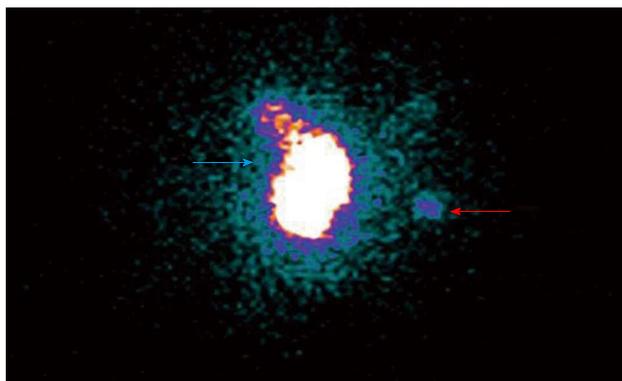


Figure 3 Lymphoscintigraphy of a sentinel lymph node in anal carcinoma. Anterior view showing injection site (blue arrow) and sentinel lymph node (red arrow).

node. Typical drainage patterns displayed radiotracer accumulation in the inguinal area or in the iliac internal lymph node system (Figure 3). If the accumulation was observed in the perirectal or external iliac lymph nodes, then patients did not undergo the sentinel lymph node procedure. Only patients showing radio accumulation in the inguinal area were enrolled for sentinel node surgical retrieval, and the overlying skin was marked by a waterproof pen. Marking the skin assisted in the intraoperative identification of the nodes and minimized surgical incision.

There was no consensus about the time gap between injection, lymphoscintigraphy acquisition, and surgery. Clearance of radioactive colloids by lymphatic drainage is related to the particle size; small particles are cleared first and large particles later^[65]. Moreover, tracers with small particle size are washed out from true sentinel nodes and move to other nodes^[66]. Therefore, a shorter period between injection and surgery is recommended with smaller particles. In our experience, 99mTc-nanocolloid with particles between 80 and 150 nm were employed. By virtue of their delayed wash-out, surgery could be performed even the day after injection. A delay of 12 to 16 h between injection and surgery allowed good intraoperative radio-localization with minimal interference by primary tumor radioactivity.

During surgery a second vital tracer, blue patent, was injected around the site of the anal tumor. In other reports, nearly all groups have used blue dye as a second vital tracer. The addition of intraoperative dye aids in the intraoperative search^[67] and tends to result in higher rates of lymph node detection. There are no data regarding the usefulness of dye with SLNB in anal cancer; however, the importance of dual mapping to reduce false negative results has recently been demonstrated in breast cancer^[68]. When accumulation was shown at lymphoscintigraphy on both groin regions, inguinal dissection was made bilaterally.

Local anesthesia was routinely employed in our institute, although the procedure can be performed under

general or local regional anesthesia. During surgery, a hand-held gamma detection probe was used to identify the radioactive lymph node (NEOPROBE Neo2000 Gamma Detection System). A small incision was made under radio probe guidance and over the marked skin. The sentinel lymph node was retrieved by visualization through the blue dye and radio detection by the hand-held gamma probe. During radio-navigation the gamma probe was directed away from the anus to avoid signal detection from the primary site of injection^[28]. Significant radio-colloid capture, compared to lymphatic basin, should be in a ratio of 5:1. After nodal excision, the surgical site was explored by the gamma probe to identify accessory nodes.

The isolated lymph node was sent for pathological examination and metastatic assessment. Hematoxylin and eosin (HE) staining was used to assess the presence of malignancy. Other reports have suggested using immunohistochemistry with pan-cytokeratin antibody markers for cases of negative HE^[28,54,56]. In our study, the specimen was examined using a particularly accurate technique. Briefly, sentinel lymph nodes were step-sectioned into 50-micron slices and serial sections of three microns thick were cut at each level. This number of sections provided good accuracy without having to resort to the more costly immunohistochemical analysis. Among the other related reports, only Gretschel *et al*^[54] have found micro-metastases or isolated tumor cells in lymph nodes examined after a negative HE.

Common complications related to lymph node mapping include wound infection, hematoma, and lymphorrhea from lymphatic fistula with subsequent seroma in the surgical site. Post-procedural side effects are easily managed and rarely require reintervention.

RESULTS OF SENTINEL LYMPH NODE ASSESSMENT

Since the first report published by Keshtgar *et al*^[56] in 2000, several reports have demonstrated the feasibility and effectiveness of the SLNB procedure in anal cancer^[43,54,57-63,69,70]. The technical aspects and results of the published studies are shown in Table 1. In these studies, patients with T1-T4 anal tumors with clinical or imaging negative inguinal nodes were enrolled. Lymphoscintigraphy accuracy was generally high, rating 90% to 100% in examined reports, and inguinal capture, as explained above, depended on the localization of the primary tumor. Surgical gamma probe detection was nearly 100%^[43,54,57-63,65,69,70]. Almost all groups suggested the double tracer technique to better visualize the sentinel lymph node intraoperatively. Metastatic node rate, among the sentinel nodes, varied between 0% and 33%; however, the case series were very heterogeneous and included advanced tumors (T stage ranging from T1 to T4) even though the majority of patients were clinically node-negative.

Table 1 Technical aspects and results of the published case series

Ref.	Year	T stage	Groin clinical	Tot N cases	Inguinal detection rate (%)	Double tracer	N positive (%)	Complication	Bilateral detection	Surgical retrieval rate
Keshtgar <i>et al</i> ^[56]	2000	NA	Negative	1	1/1	Yes	1/1	NA	0/1	1/1
Perera <i>et al</i> ^[58]	2002	T1-T2	1/12	12	8/12 (66)	Yes	2/18 (11)	NA	0/8	8/8
Péley <i>et al</i> ^[57]	2002	NA	1/8	8	8/8 (100)	Yes	2/8 (25)	No complication	5/8	8/8
Rabbit <i>et al</i> ^[69]	2002	NA	Negative	4	3/4 (75)	Yes	0/3 (0)	NA	2/3	3/3
Bobin <i>et al</i> ^[62]	2003	NA	Negative	33	33/33 (100)	Yes	7/33 (21.2)	NA	NA	33/33
Ulmer <i>et al</i> ^[60]	2004	T2-T4	Negative	17	13/17 (76)	Yes	5/12 (41.6)	Lymphatic fistula (1 pt)	4/13	12/12
Gretschel <i>et al</i> ^[54]	2008	T1-T4	Negative	40	20/40 (50)	Yes	6/20 (30)	Wound infection (2 pts), lymphatic fistula (1 pt), hematoma (1 pt)	NA	20/20
Mistrangelo <i>et al</i> ^[64]	2009	T1-T4	NA	35	35/35 (100)	No	7/35 (20)	Lymphatic fistula (18 pts), lower limb lymphedema (1 pt)	22/35	34/35
Damin <i>et al</i> ^[55]	2010	T1-T3	Negative	15	15/15 (100)	Yes	4/15 (26.6)	Lymphatic fistula (1 pt)	13/15	15/15
de Jong <i>et al</i> ^[70]	2010	T1-T3	4/21	21	21/21 (100)	Yes	7/21 (33)	NA	14/21	21/21
De Nardi <i>et al</i> ^[45]	2011	T1-T3	Negative	11	9/11 (81)	Yes	3/9 (33)	Lymphatic fistula (1 pt)	2/11	9/9

NA: Not applicable.

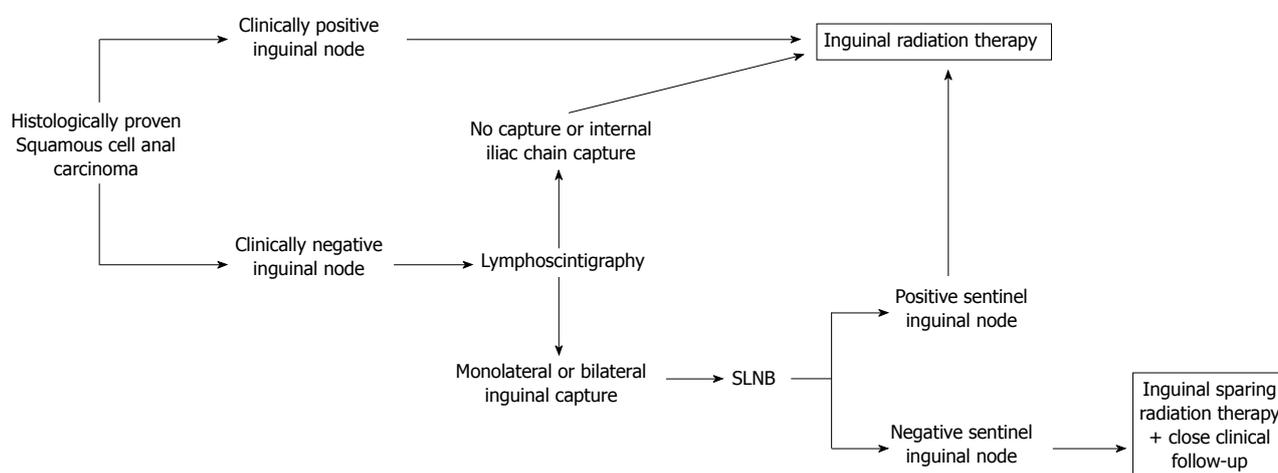


Figure 4 Diagnostic-therapeutic algorithm for squamous cell anal carcinoma. SLNB: Sentinel lymph node biopsy.

Among 23 patients, 19 with inguinal capture at lymphoscintigraphy underwent the SLNB procedure. Inguinal dissection was made bilaterally in two patients where accumulation was shown at lymphoscintigraphy on both groin regions. Sentinel lymph node retrieval by gamma probe was possible in all patients. Histological examination of nodes showed the presence of metastases in five patients (26%).

Among the 12 patients enrolled in the inguinal sparing radiation protocol with a clinically negative inguinal region, 10 patients had negative pathological SLN and received an inguinal sparing irradiation. At a median follow-up of 20 mo, none of these patients had developed inguinal metastases.

Gretschel *et al*^[54] reported that inguinal lymph node assessment was able to change the treatment plan recommended by national guidelines in 50% of patients. In the group of patients with inguinal sparing irradiation, inguinal recurrence was found in two out of 20 patients: one patient suffering from a T4 tumor, associated with

disseminated disease, and one patient with T1 tumor that was previously treated by local excision. Mistrangelo *et al*^[28] did not observe isolated inguinal recurrence in 28 node-negative patients at a median follow-up of 22 mo.

De Jong *et al*^[70] reported that SNLB provided alteration of treatment in at least 11 of 21 patients. However, inguinal recurrence within 12 to 24 mo was observed in two out of 14 node-negative patients undergoing node-sparing irradiation. Figure 4 demonstrates a simple diagnostic-therapeutic algorithm to identify patients eligible for the SLNB procedure to individualize irradiation treatment based on inguinal node status.

CONCLUSION

In spite of the low incidence of anal canal carcinoma, noticeable advances have been achieved in the past 30 years in understanding its etiology, biological behavior and therapy, with the current therapeutic approach be-

ing primary radio-chemotherapy. The identification of lymph nodes metastases, especially in the inguinal area, is still the main issue that needs to be addressed.

The low incidence of metachronous metastases and the considerable side effects after inguinal node dissection and radiotherapy do not justify a prophylactic treatment^[71]. A refined staging system with precise identification of disease extent could allow individualized therapy, ensuring the accurate coverage of disease while sparing disease-free organs.

In this context, SLNB, as a minimally invasive procedure, may improve disease staging and may be useful to select patients for inguinal radiation. Feasibility and efficacy of SLNB has been addressed by several reports and the clinical utility of this procedure in changing the therapeutic plan has also been outlined. However, further larger prospective studies are needed to confirm the clinical impact of this procedure. Continuous and stringent long-term follow-up is necessary to estimate the outcome in node-negative patients who did not undergo groin irradiation.

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Intestinal mucosal atrophy and adaptation

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Abstract

Mucosal adaptation is an essential process in gut homeostasis. The intestinal mucosa adapts to a range of pathological conditions including starvation, short-gut syndrome, obesity, and bariatric surgery. Broadly, these adaptive functions can be grouped into proliferation and differentiation. These are influenced by diverse interactions with hormonal, immune, dietary, nervous, and mechanical stimuli. It seems likely that clinical outcomes can be improved by manipulating the physiology of adaptation. This review will summarize current understanding of the basic science surrounding adaptation, delineate the wide range of potential targets for therapeutic intervention, and discuss how these might be incorporated into an overall treatment plan. Deeper insight into the physiologic basis of adaptation will identify further targets for intervention to improve clinical outcomes.

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Key words: Adaptation; Intestine mucosa; Mucosal differentiation; Short bowel syndrome

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INTRODUCTION

The small intestinal mucosa is adaptable but essential for survival. It has diverse biological roles including nutrient absorption, barrier function, injury response, and immunologic reservoir (Figure 1). Congenital or acquired diseases or medical and surgical interventions can alter intestinal mucosal mass and/or function with profound consequences to which the gut must adapt. The clinical challenges to gut adaptation include massive bowel resection, intestinal atresia, fasting, prolonged ileus, bariatric surgery, and total parenteral nutrition (TPN). The biology that regulates such adaptation represents a critical new frontier for gastroenterology and gastrointestinal (GI) surgery if outcomes are to be improved. This review discusses intestinal mucosal atrophy, hypertrophy, and barrier function, and how they may be influenced. We will begin this review by considering what is known about intestinal mucosal development, as this offers useful parallels to the intestinal mucosal response to pathology. Next, we will explore the biologic phenomena of mucosal atrophy and hypertrophy in more depth. Third, we will discuss the clinical syndrome of intestinal failure in more depth, and consider how our understanding of the biology of intestinal adaptation may guide therapeutic interventions. Finally we will discuss new frontiers in mucosal adaptation, focusing particularly on intersections with GI surgery, including both pro-absorptive procedures like intestinal lengthening procedures and anti-

absorptive procedures like bariatric surgery, and on some lessons that may be learned in the future from recent scientific advances. Many potential approaches are available for facilitating absorption and mucosal homeostasis, but their optimal application may require a better understanding of the biology that regulates these processes.

NATURAL MUCOSAL DEVELOPMENT AND GROWTH

Morphogenesis and cell proliferation

Enterocytes are columnar cells with microvilli at their apices that form the intestinal brush border. The microvilli are covered by a glycocalyx coat that acts as a physical barrier and contains brush border enzymes. Enterocytes are joined by tight junctions to form a relatively impermeable membrane^[1,2].

The small intestinal mucosa is folded to increase its surface area^[1,2]. Submucosal folding forms plicae circularis that each include many crypt-villus units. Villi are mucosal surface modifications, finger-like extensions of lining epithelium formed by projections of lamina propria covered with epithelium. Each villus extends into lamina propria as an intestinal gland or crypt of Lieberkuhn^[1,2]. Crypts have stem cells, paneth cells and enteroendocrine cells. Stem cells proliferate at the crypt base.

Mucosal growth and development are regulated by hormonal, nervous, immune, dietary and mechanical signals^[3]. The small bowel mucosa ultimately develops in stereotypic crypt-villus units containing absorptive, secretory, progenitor and stem cells^[4]. Intestinal stem cells maintained throughout life in the crypts give rise to progenitor cells that undergo a few cell divisions as they move out of the crypts toward the villi before final differentiation^[5,6]. Small bowel ontogeny proceeds in three successive phases: morphogenesis and proliferation, cell differentiation, and functional maturation^[7].

The field is beginning to identify molecular mechanisms that influence intestinal development^[8]. For instance, homeobox (*hox*) genes are early regulators of proximal to distal organ-specific patterning^[9]. Mucosal remodeling and villus formation precedes in a cranial-caudal direction^[10]. A primitive endodermal gut tube surrounded by mesenchyme forms early in gestation^[3]. Later, the endoderm transitions to stratified epithelium which ultimately matures to columnar epithelium starting at the apices of the developing villi. The intervillus epithelium differentiates last, and mitotic activity is restricted to intervillus regions and developing crypts by 16 wk^[8,11]. This correlates with Wnt/ β -catenin pathway activity that appears necessary for stem cell maintenance in fetuses and adults^[12]. Fibroblast growth factor receptor (FGFR)-3 signaling regulates crypt epithelial stem cell expansion and crypt morphogenesis *via* β -catenin/Tcf-4 pathways^[6].

Intestinal villus formation begins at embryonic week 8 in humans^[8]. Influenced by Hedgehog and platelet-derived growth factor (PDGF) signals, mesenchymal cells condense under the epithelium and then grow toward

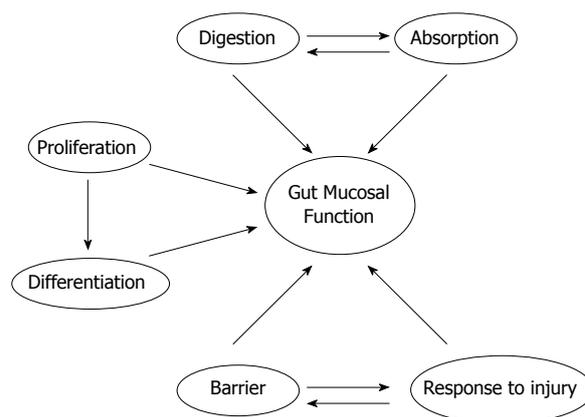


Figure 1 Diversity of gut mucosal function.

the central lumen to form characteristic fingerlike inward projections—the villi^[4,8,13,14]. With increasing age, villus epithelial turn-over, crypt depth, and villus height each increase^[8,15].

Differentiation

Proliferation occurs in the crypts but differentiation occurs as cells migrate up the villus. Thus, differentiated cells populate the villi^[6]. Each villus contains epithelium from the adjacent crypts^[6]. Crypt formation occurs by differential growth of mesenchyme and the crypt-villus junction moves upwards towards lumen^[17]. Crypt-base columnar cells are multipotent cells that differentiate into absorptive enterocytes and secretory mucous secreting goblet cells, entero-endocrine cells and paneth cells^[18]. Notch pathways decide differentiation into absorptive *vs* secretory cells^[19-21]. Crypt stem cells become monoclonal during development^[22]. Critical functional differentiation into distinct apical, lateral and basal cells occurs. Apical cells express digestive enzymes and transporters, while lateral cells chiefly express transporters, and basal cells carry receptors for interaction with basement membrane^[8]. The brush border complex formed by villin and myosin I appears at the apical surface of mature enterocytes^[23,24].

Intestinal growth and differentiation are regulated by an intrinsic program; extrinsic mediators play secondary roles^[25,26]. N-myc is an important regulator of proliferation^[27]. Growth factors like epidermal growth factor (EGF), transforming growth factor (TGF)- α , and TGF- β , insulin-like growth factor (IGF)-2, hepatocyte growth factor (HGF), glucagon like peptide (GLP)-2 and their receptors have been detected in fetal human intestine and likely influence intestinal development^[28]. Luminal and circulating factors are not necessary for fetal intestinal differentiation but may affect growth and maturation^[26,29-31]. Three known triggers are weaning, thyroxine and glucocorticoids^[3]. Regarding intrinsic regulation, it is thought that endoderm has an intrinsic program of regionalization of small intestine and it recruits other cells to complete the formation^[32,33]. Hepatic nuclear fac-

tor (HNF)-3 β , Cdx-1, Cdx-2, GATA transcription factor family and transcription factor CCAAT/enhancer-binding protein α are suggested to have important roles in intestinal development^[8]. Specific regulatory regions of the fatty acid-binding protein (*FABP*) genes are responsible for appropriate temporal, crypt/villus and proximal/distal expression of genes^[34]. Reciprocal permissive and instructive epithelial-mesenchymal interactions and signals direct organ-specific differentiation^[35]. Endoderm can recruit mesenchymal elements^[33]. Sonic hedgehog, *bmp*, *Fkh6*, *H1x* homeobox gene, tyrosine kinase receptors and fibroblast growth factors mediate intestinal growth by epithelial-mesenchymal interaction and direct regional patterning of the gut^[35-40]. The extracellular matrix influences epithelial differentiation by receptor-mediated signaling^[41] and by acting as reservoir for growth factors^[42]. The matrix also drives the enterocyte response to growth factors and physical forces^[43,44]. Matrix has a permissive effect on epithelial cells and is required for maximal differentiation^[45].

Functional development

Brush border membrane enzymes and brush border transport proteins represent the most important functional differentiation of small intestine. These enzymes provide digestive and absorptive functionality for carbohydrates, proteins, fats, minerals and vitamins. Brush border enzymes appear by week eight. Different disaccharidases, alkaline phosphatases, peptidases, and enterokinases mature at different rates in development^[46-48]. Lactase-phlorizin hydrolase (LPH) cleaves lactose into glucose and galactose. Studies of LPH development suggest a proximal to distal gradient in functional maturation of the intestinal epithelium^[46]. Although the human fetus expresses some enzymes and peptidases at levels similar to adults, many of these enzymes have different forms in the fetus^[49-52]. Various transporters for sugar and amino acids appear during gestation in parallel with crypt and villus development^[53,54]. Fetal intestine also starts developing the capacity to secrete lipoprotein fractions, chylomicrons, very-low-density lipoproteins and high-density lipoproteins^[55,56]. Absorptive function is partially detectable at 26 wk^[57].

The intestinal barrier exhibits immune and non-immune protective mechanisms. Although various mucosal defense systems appear early in gestation, immune function remains immature at birth^[58-64]. Tight junctions between enterocytes and goblet cell mucins form a physical barrier by 12 wk^[65].

In summary, the basal differentiation program is encoded in fetal endoderm and mediates spatial and cranio-caudal differentiation of intestinal epithelium *via* a complex intercellular communication network. Epithelial-mesenchymal interaction yields a specialized regional environment that influences gene expression and regulates the growth and differentiation of the intestinal mucosa into crypt-villus units.

MUCOSAL ATROPHY

Mucosal atrophy is characterized by diminished intestinal function as well as morphological changes including decreased villous height, crypt depth, surface area, and epithelial cell numbers^[66]. Atrophy is most common in the absence of enteral nutrition, and is a known long-term consequence of starvation, an effect likely reduced with age^[67]. Animal studies suggest incremental relief of atrophy with progressively greater intake, and that morphologic atrophy is most evident at the villous tip^[68].

Atrophy occurs even if adequate parenteral nutrition is provided. Animal studies first demonstrated the physiology of atrophy during TPN^[69], with atrophy of the proximal small intestinal mucosa with decreased intestinal weight and nitrogen content^[69]. Absence of luminal contents due to either starvation or TPN similarly causes mucosal hypoplasia in rodents, mediated at least in part by an altered tumor necrosis factor (TNF)- α /EGF signaling pathway^[70]. Additionally, rats receiving TPN have fewer Peyer's patches and less total T cells than rats fed enterally, demonstrating an attenuating effect on the gut-associated lymphoid tissue^[71]. Animal studies also demonstrate that TPN evokes enterocyte apoptosis *via* intraepithelial lymphocyte derived interferon-gamma, resulting in a loss of the overall barrier function^[72]. Barrier function is further altered by TPN stimulation of ion secretion, an effect upon intestinal permeability further altered by interferon-gamma^[72]. The effect of TPN on immunologic function (including that in the gut) may have profound clinical consequences. For instance, infectious complications were doubled in pancreaticoduodenectomy patients receiving TPN rather than jejunal feeding^[73].

That the effects of fasting and TPN on the gut mucosa are not just from the TPN has been shown in animals. For instance, when a segment of rat jejunum is defunctionalized by a blind end Roux-en-y anastomosis and the rat is allowed to eat freely, the defunctionalized mucosa undergoes morphologic and biochemical atrophy, while the remainder of the gut mucosa remains intact^[74]. This suggests that direct interaction with luminal chyme is required to sustain the mucosa. Indeed, the effects of the absence of enteral feeding may reflect not only the loss of luminal nutrients themselves, but also aberrations in the physical forces to which the mucosa is subjected during gut interactions with luminal chyme, either directly by peristaltic compression against the non-compressible liquid contents of the bowel or indirectly by villus motility^[75-77]. It has long been known that luminal contents and distension influence postprandial intestinal motor activity^[78] that leads to deformation of the bowel mucosa. In addition, villus motility is markedly stimulated by luminal amino acids and fatty acids (but not glucose)^[79].

At the cellular level, atrophic loss of mucosal mass may reflect both decreased proliferation and increased apoptosis. EGF family cytokines are potent mitogens, but there are others, including GLP-2, while TNF- α and others mediate apoptosis. We found mostly decreased

Table 1 Reported regulators of intestinal mucosal adaptation

Cytokines		Intracellular transducers of physical force effects		Nutrients	
Stimuli	Ref.	Stimuli	Ref.	Stimuli	Ref.
PDGF- α	Sukhotnik <i>et al</i> ^[86]	FAK-Tyr 925	Chaturvedi <i>et al</i> ^[225]	L-arginine	Koppelman <i>et al</i> ^[88]
HGF	Katz <i>et al</i>	Integrin-linked Kinase	Yuan <i>et al</i> ^[243]	Glutamine	Lardy <i>et al</i> ^[252]
Transforming GF- β	Sukhotnik <i>et al</i> ^[151]	RhoA	Chaturvedi <i>et al</i> ^[244]	Ornithine	Lardy <i>et al</i> ^[252]
IGF-1	Lund <i>et al</i> ^[92]	ROCK	Chaturvedi <i>et al</i> ^[244]	Butyrate	Bartholome <i>et al</i> ^[161]
VEGF	Parvadia <i>et al</i>	mDia1	Chaturvedi <i>et al</i> ^[244]	Short-chain fructooligosaccharide	Barnes <i>et al</i> ^[163]
EGF	Warner <i>et al</i> ^[90]	-	-	-	-
GLP-2	Bortvedt <i>et al</i> ^[93]	-	-	-	-

PDGF: Platelet derived growth factor; HGF: Hepatocyte growth factor; EGF: Epidermal growth factor; IGF-1: Insulin-like growth factor-1; GH: Growth hormone; GLP-2: Glucagon-like peptide-2; ROCK: Rho-associated kinases; mDia: The formin homology protein mDia1; VEGF: Vascular endothelial growth factor.

proliferation in defunctionalized rat intestine, perhaps because the incidence of apoptosis is too low to be readily measurable^[74], except in chemotherapy-induced mucosal injury^[80]. Fasting leads to jejunal mucosal atrophy with enhanced apoptosis in a mechanism related to increased nitric oxide^[81]. Feng *et al*^[70] recently explored the interaction between growth factor-stimulated proliferation and cytokine-driven apoptosis in a murine TPN model. BCL-2 expression acts on mitochondria to prevent cytochrome C release, and caspase 3 directed cell death. Bax in contrast, acts on mitochondria to cause caspase 3 release, leading to programmed cell death. The ratio between these determines overall cell survival^[82,83]. Enterocytic differentiation is also impaired in mucosal atrophy, with decreased expression of brush border enzymes and other differentiation markers^[74,84,85], but the mechanism by which this occurs is much less well understood. While translational science strives to find better mitogens to promote enterocytic proliferation, how to promote enterocytic differentiation in patients or animals with mucosal atrophy may represent an important question for basic science in the future.

MUCOSAL ADAPTATION

Mucosal adaptation in many ways opposes atrophy, although that there may be subtle but important differences in the stimuli that prevent atrophy and maintain normal mucosal mass and those that induce adaptation. Teleologically, adaptation may be the attempt of the intestine to compensate for intestinal inadequacy. It has been best described after massive small bowel resection^[86,87]. However, exogenously induced adaptation may reverse chemotherapeutically induced atrophy. For example, profound intestinal injury by methotrexate may be mitigated by supplementation with L-arginine or n-3 fatty acids^[88,89].

Acute absence of nutrition alone cannot trigger full adaptation or fasting would cause intestinal hypertrophy rather than atrophy. The stimuli contributing to adaptation are diverse. Many cytokines facilitate adaptation, including PDGF- α , HGF, and interleukin (IL)-11^[86,90,91]. As detailed later, hormones such as IGF-1 and growth hormone (GH) appear to exert strong adaptive influ-

ences^[92,93]. Within enterocytes, intestinal resection invokes novel signals such as proline-rich protein 2 during wound healing^[94], glutathione reductase during intestinal apoptosis^[95], and basic Kruppel-like factor to activate the IGF-1 promoter^[96].

The anatomic location of the bowel mucosa has an important relationship with adaptive biology. The small and large intestinal mucosa demonstrate many differences in histology, cell phenotype, and transport proteins that reflect their differences in normal function. In addition, the small and large intestinal mucosa respond differently to stimuli of malignant transformation. For example, the APC (Min) mouse is a dominant mutation that leads to multiple intestinal neoplasia^[97]. Crypt cells express a balance of proliferation and differentiation, a process with aberrant regulation in these mutants. In mouse models with this mutation, small bowel neoplasms are much more common than colonic neoplasms, in contrast to the human condition in which colonic neoplasms are more common^[98]. The reason for this regional difference is as yet unknown but further investigation may offer important clues into differentiated intestinal epithelial biology.

Finally, the role of nutrition in adaptation is not yet fully explained. Glutamine has been most studied^[99] (Table 1). There may also be differences between the signals that stimulate the increase in mucosal mass and the signals that augment mucosal functionality during adaptation. Adaptation includes proliferation, augmentation of function and changes in intestinal epithelial phenotype (Figure 2).

Proliferation

Proliferation is one mechanism of adaptation. Proliferation increases villus height, crypt depth, surface area, and intestinal wet weight^[100]. The length of intestine resected correlates with the subsequent change in villus height in humans^[101]. The site of resection also influences adaptation^[102], with a notable increase in jejunal hyperplasia, and to some degree ileal hypertrophy. Animal studies also suggest increased intestinal stem cells after resection; these may contribute to increased crypt formation^[103]. Growth factors and nutritional supplementation stimulate intestinal epithelial proliferation and turnover.

The intestine also adapts from a functional standpoint. Proliferation increases overall function just by

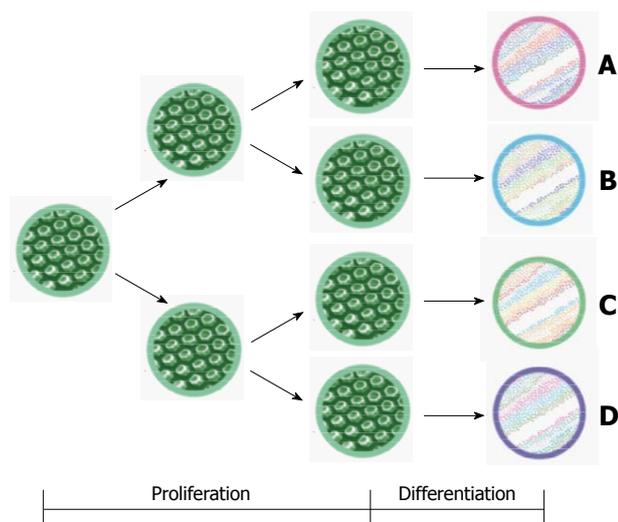


Figure 2 Initial enterocytic stem cell proliferation is supplemented by differentiation to produce the four main intestinal epithelial phenotypes: absorptive enterocytes (A), enteroendocrine cells (B), mucin-secreting cells (C), and Paneth cells (D).

creating more cells that can contribute to amino acid, glucose, and electrolyte uptake. Increased absorption *via* the H⁺/peptide co-transporter 1 after intestinal resection occurs because of hyperplasia and not upregulation of transporters^[104]. Improved glucose uptake after massive small bowel resection is similarly driven by cellular proliferation rather than massive transporter upregulation^[105]. In addition, we can see increase in Na⁺/glucose transporter (Sgt1), Na⁺/H⁺ exchangers (NHE2/3), and some brush border membrane enzymes^[106].

Augmentation of intestinal mucosal function

Although most work has focused on enterocytes, adaptation also increases non-enterocytic mucosal epithelial cells. Goblet and paneth cells exhibit an early and sustained increase after bowel resection; these secretory cells may contribute to juxtacrine signaling that further stimulates intestinal adaptation^[107]. Finally, the role of angiogenesis has been relatively understudied in mucosal adaptation. Bowel resection in rats induces angiogenesis within the adapting intestinal villi^[108]. This may facilitate absorption, protect mucosal integrity and barrier function, and increase nutrients and oxygen delivery to the more rapidly proliferating mucosa.

Cellular differentiation

To maintain and control epithelial cell homeostasis, proliferation and differentiation are transcriptionally regulated in a sequential and spatially defined manner^[109]. The signals that control intestinal development also influence intestinal homeostasis. These include the canonical Wnt/ β -catenin pathway^[110], Notch^[111], Hedgehog^[112], the TGF- β family including bone morphometric proteins (BMP)^[113], PI-3K^[114] and Forkhead Box (FOX) and homeobox (*HOX*) genes^[115]. These pathways use various transcription factors including HNF1 α/β , HNF4 α ,

Table 2 Cellular factors involved in enterocyte differentiation during adaptation

Cellular factors
Wnt/ β -catenin
Notch
Hedgehog
PI3K
HNF1 α/β
GATA
ETS
Cdx2
FGF4
NEUROG3
Schlafen-3
Math1

PI3K: Phosphoinositide 3-kinase; FGF: Fibroblast growth factor; HNF: Hepatocyte nuclear factor.

GATA factors, ETS, and Cdx1/2, alone or in combination^[116-120]. For instance, the combination of HNF1 α , GATA4-6 and Cdx2 regulates sucrase isomaltase transcription^[116] but in differentiated mouse epithelium; HNF4 α regulates expression of genes upregulated during differentiation such as alkaline phosphatase^[121]. GATA transcription factors are required for crypt cell proliferation and absorptive enterocyte gene expression^[119]. HNF3 β is expressed in small intestine and has critical role in foregut and midgut formation^[122-124]. Finally, Cdx2, which is restricted to adult small intestine and colon^[125], is necessary for maintenance of intestinal identity and differentiation of the small intestine epithelium (Table 2)^[126].

A recent landmark study demonstrated the guidance of human pluripotent stem cells into intestinal tissue^[127]. This study demonstrated that the activity of Wnt3a and FGF4 was adequate for hindgut patterning, specification and morphogenesis, with NEUROG3 transcription factor required for enteroendocrine cell development *in vitro*. Similarly, embryonic stem cells have been committed to intestine lineage in medium treated with Wnt3A, in a process that interestingly enough simulated the genes associated with distal gut-associated mesoderm (Foxf2, *hlx*, *Hoxd8*)^[128]. This process was successful in that it allowed engraftment of these cells into murine colonic mucosa.

CLINICAL SETTINGS IN WHICH MUCOSAL ADAPTATION IS IMPORTANT

Atrophy in starvation, fasting or TPN

Intestinal failure occurs when the absorptive surface area falls below a critical level, either because of loss of bowel length or mucosal atrophy with severely flattened epithelium. Intestinal failure presents with diarrhea, dehydration, malabsorption, progressive malnutrition, and electrolyte disturbance^[129].

TPN administration in starving animals or humans does not abrogate the atrophy observed in starvation alone. In addition, TPN may itself impair mucosal barrier function^[130] beyond the effects of starvation on the

epithelium. In adult trauma patients, this loss of barrier function may significantly increase sepsis^[131]. Trauma patients receiving enteral nutrition have fewer pneumonias, intra-abdominal abscesses, and line sepsis and less infections overall compared to patients on TPN^[131]. TPN-associated loss of epithelial barrier function may be related to altered mucosal lymphoid populations with increased interferon gamma and interleukin-10 expression, as well as loss of tight junctions and adherens junction proteins^[72]. Prolonged starvation, as in chronic TPN, pancreatitis, or other medical conditions, can lead to intestinal failure *via* mucosal atrophy. Indeed, poor enteral intake can cause pancreatitis and intestinal mucosal atrophy^[132] which in turn increases enterocyte apoptosis and alters glutamine and arginine transport^[133,134]. Atrophy in turn creates a propensity for bacterial translocation and sepsis^[135].

Short bowel syndrome

Massive small bowel resection can severely test the capacity of the remaining small bowel mucosa to adapt, resulting in short bowel syndrome, a devastating nutritional problem. The most common causes of short bowel syndrome in children include necrotizing enterocolitis, intestinal atresia, and midgut volvulus^[136,137]. In adults, common causes include inflammatory bowel disease, mesenteric ischemia, small bowel obstruction, and radiation enteritis. The loss of mucosal area associated with short bowel syndrome causes substantial malabsorption, with attendant diarrhea, abdominal pain, and weight loss, electrolyte imbalance, and chronic malnutrition. Mucosal adaptation in such patients is a slow and gradual process that may require up to 1-2 years to reach maximum. The simplest and earliest phases of adaptation involve enterocytic proliferation and villous hyperplasia, which may manifest as a reduction in diarrhea and attendant fluid and electrolyte loss. Nutritional adaptation that addresses nutrient absorption and digestion sufficiently to permit weaning from TPN is slower and requires greater complexity.

Current medical management of intestinal failure:

Intestinal failure is initially managed similarly whether due to atrophy or short gut. TPN supplies nutritional requirements while ways to transition to enteral feeding are sought. This is usually successful in mucosal atrophy, although it may be prolonged and difficult in some patients. Such transitions are less frequently successful in the short gut patient. The primary predictor of survival in adults with short gut is small bowel length. Eighty-three percent of adults with less than 50 cm of intestine require lifelong TPN; Twenty-five percent will die within 5 years^[138]. In pediatric short gut patients, cholestasis and age-adjusted small bowel length less than 10% of expected length predict mortality; small bowel length and an intact ileocecal valve predict successful weaning from TPN^[139]. The ileocecal valve slows transit through the small bowel, facilitating absorption and digestion. In

addition, the colon may both absorb water and salvage energy in such patients, perhaps mitigating the need for parenteral nutrition if the small bowel is marginally adequate^[140].

Whether as a bridge to enteral nutrition or as permanent maintenance, TPN is lifesaving in patients who cannot be nourished enterally. However, TPN has significant complications. TPN itself results in mucosal atrophy, impaired mucosal immunity with a proclivity towards intestinal infections, and dysfunction of the gut-associated lymphoid tissue^[141]. It is unclear to what extent these phenomena reflect the lack of enteral feeding and to what extent they are consequences of the infusion of large quantities of hyperosmotic or hyperlipidemic nutrients into the circulation. TPN is also associated with chronic systemic problems including mechanical complications related to the catheter, recurrent infections, liver failure, and death. Randomized, controlled trials have demonstrated the benefits of enteral feeding over parenteral feeding for diverse conditions^[142] with reductions in infection, intraabdominal abscess, anastomotic leak, hospital stay, and all other complications. Many enteral nutrients are essential for intestinal adaptation in both adult and pediatric populations^[143,144]. In both acutely ill hospitalized patients and chronic short gut patients at home, some enteral nutrition is therefore desirable even if parenteral supplementation is required. Such enteral intake both maintains the mucosal barrier and supports the patient psychologically. The central theme of modern management is to provide the gut with at least some nutrients and consequent hormonal stimuli even if parenteral supplementation is required.

“Intestinal rehabilitation” for short bowel syndrome uses chronic home TPN as a bridge to maintain patients while seeking to adapt them to eventual enteral nutrition. Intestinal rehabilitation is a multidisciplinary approach aimed at achieving enteral autonomy, and keeping patients alive while still requiring TPN. Teams of GI and transplant surgeons, gastroenterologists, dieticians, pharmacists, nurses, and social workers collaborate to offer improved nutritional care and dietary manipulation, facilitated discussion about needs for surgical interventions, and formal monitoring and manipulation of essential medications including mucosal mitogens. This approach may improve survival compared to historical controls, although this could also reflect improved treatments over time^[145].

NUTRITION AND INTESTINAL ADAPTATION

In rats with short bowel syndrome, early enteral feeding affects not only cellular proliferation, but also overall gut weight and length^[146]. Even marginal nutrition at the apical or luminal surfaces may improve human intestinal epithelial cell growth, motility, and absorption capacity^[147]. Overall, enteral feeding induces significant intestinal adaptation. Further interest lies in trying to modify the

nature of the diet.

Dietary fats

Dietary lipids encourage intestinal adaptation through several mechanisms. At the simplest level, early feeding of a fatty diet increases lipid absorption in the remnant intestine^[148]. Specific nutrients may be important. For instance, arachidonic acid stimulates intestinal adaptation more than linoleic acid^[149]. Rats on high-fat diet demonstrate increased fat absorptive capacity compared to rats eating standard chow^[150]. However there is some controversy about the role of enteral fatty acids. Other evidence does not demonstrate improved adaptation with enteral omega-3 fatty acids, but only with parenteral supplementation among rats^[151]. Dietary fish oil appears to increase fat absorption without a concurrent increase in bile acid synthesis in rats following ileocecal resection^[152].

Dietary fat intake might also modify gene expression and transport by altering the transcription and activation of signal proteins related to protein synthesis of nutrient transporters, including activation of peroxisome proliferator-activated receptors, HNF-4, and nuclear factor κ -B^[153]. Dietary fat may activate intracellular signals to alter mRNA expression.

Diet also affects gut membrane permeability. Membrane fluidity is altered dramatically by the intrinsic fatty acid saturation and also by cholesterol and ganglioside/glycosphingolipid content, and can inhibit degradation of gut occluding tight junctions in rats^[154]. Specialized parts of the membrane such as lipid rafts and caveolae affect signaling and protein intake in a manner altered by fatty acid intake^[155].

Intestinal lipid transfer is relatively quickly influenced by diet. Rats fed a high-fat diet for only seven days undergo intestinal adaptation, reflected in dramatic increases in the expression of sterol regulatory element-binding protein (SREBP)-1c. The activation of SREBP-1 increases its synthesis and translocation to the nucleus in intestinal cells, altering lipid metabolism^[156]. Further work is needed to identify the signals that influence short term and long term adaptation. This may have even morphological implications. Palmitic acid feeding increases rat bowel and mucosal weight after massive small bowel resection after only 14 d^[157].

Short-chain fatty acids

Interestingly, the colon also contributes to intestinal adaptation in malabsorption. In carbohydrate salvage, short-chain fatty acids (SCFA) produced by fermentation by anaerobic colonic bacteria are absorbed by the colonic mucosa, resulting in net energy absorption. These SCFA consist primarily of acetate, propionate, and butyrate. Luminal butyrate is the primary energy source of the colonocyte, and SCFA are trophic for the colonic mucosa. Adding SCFA to TPN prevents small bowel mucosal atrophy in fasting animals^[158,159]. Adding butyrate to TPN also improves lymphocyte numbers, small intestinal IgA levels, and small intestinal surface area^[159]. This demon-

strates that intravenous nutrition can interact with luminal enterocytes, facilitating their function and altering their structure to promote digestion. Indeed, parenteral butyrate alone increases plasma GLP-2 and directly promotes GLUT2 activity^[160,161]. Parenteral butyrate facilitates small bowel adaptation in piglets after massive resection, improving small intestinal morphology and reducing apoptosis^[161]. Butyrate also must act independently of GLUT2 since it promotes enterocytic differentiation in isolated cells in culture^[162]. Prebiotic supplementation with short-chain fructooligosaccharides may replace butyrate and also promote jejunal adaptation^[163].

Dietary carbohydrates

Traditionally attention has been placed on optimizing carbohydrate/fat/protein ratios to maximize nutrient delivery in short gut syndrome. However, enteral nutrients also influence intestinal adaptation. For example, dietary carbohydrate induces adaptation for monosaccharide absorption by increasing the quantity of carbohydrate transporters^[164]. Dietary fiber may also be helpful in modulating nutrient uptake. In TPN-nourished rats with 85% small bowel resection, supplementation with dietary fiber along with GH synergistically enhanced intestinal adaptation^[165].

Dietary proteins

Increased enteral protein content leads to adaptive amino acid uptake in the small bowel^[166]. Glutamine is a conditionally essential amino acid is also the enterocyte's primary energy source^[141]. Providing parenterally fed rats with glutamine reduces mucosal atrophy^[167], but this effect is less robust in enterally fed animals^[165]. Glutamine supplementation also enhances mucosal immunity in rats with gut-derived sepsis^[168]. However, animal results have been mixed^[169]. Some studies showed glutamine to be effective only when combined with GH as discussed below^[165]. One small uncontrolled study did report that glutamine promoted weaning from TPN, with increased growth and improved nutritional factors^[170]. Human studies for the most part have not demonstrated much efficacy^[171,172]. A recent prospective, randomized human study suggests that human GH may aid adaptation with or without glutamine, but only the patients who received GH along with glutamine maintained the reduction in parenteral nutrition at 3 mo^[173]. This points to the need for multimodality therapy, and suggests caution with regard to glutamine supplementation alone.

Retinoic acid

Adaptation may be facilitated by retinoic acid. Retinoic acid administered intravenously has significant trophic effects in rats undergoing small bowel resection, apparently by inhibiting apoptosis and stimulating crypt cell proliferation^[174]. Retinoic acid may act *via* changes in extracellular matrix^[175], by acting on hedgehog signaling, by increasing Reg1 and Pap1 activity, and by acting on retinoid and peroxisome proliferator-activated receptor pathways. Convincing human data are lacking.

Polyamines

Polyamines can be either synthesized from ornithine or ingested. Diet supplementation with ornithine α -ketoglutarate increases intestinal adaptation after intestinal resection^[176,177]. In one recent study, piglets with 80% small bowel resection were randomized to either parenteral nutrition alone or parenteral nutrition plus enteral feedings beginning on postoperative day 3^[146]. The piglets with additional enteral feedings exhibited greater weight per length of intestine, as well as increased cellular proliferation index and ornithine decarboxylase activity. Response to enteral plus parenteral feedings was greater than the group with sham operation as well. In summary, with only a few days of enteral feeding piglets could undergo exceptional adaptation to extensive surgical resection as marked by polyamine synthesis and crypt cell proliferation. However, it remains unclear to what extent the polyamine synthesis was the critical mechanism for the trophic effects of enteral feedings in this study, and, as for retinoic acid, data suggesting that polyamine supplementation alone will be effective in humans are lacking at this time.

CURRENT AND EMERGING PHARMACOTHERAPIES

Antibiotics

Enteral antibiotics are certainly effective in a very select group of short bowel patients in whom small bowel bacterial overgrowth potentiates malabsorption^[178,179]. The inflammatory response to small bowel resection may also be a potential target for intervention. In massively bowel-resected rats with bowel segment reversal, oral antibiotics were associated with increased IGF-1 and blunted increases in white blood cell count, IL-6, and serum nitric oxide. This demonstrated that antibiotics may attenuate the inflammatory response^[180]. However, more clinical outcomes associated intervention with this have yet to be assessed. This represents an important frontier for future work, but should not justify indiscriminate antibiotic use in patients without demonstrable bacterial overgrowth.

Stimulating cellular proliferation to enhance adaptation

Promoting enterocyte proliferation is an attractive strategy to treat short gut. Many agents have been promising *in vitro* and in animals. We will review several below. As of this writing, only GH and Teduglutide (outside the United States) are in clinical use. It remains unclear how substantial their effects are. In addition, the long term risks of treating the gut mucosa with mitogens over decades are unknown.

GH

GH is an anabolic protein that initiates mitosis. It is released by the anterior pituitary and may act through IGF-1^[181]. GH is perhaps the best studied short bowel

mitogen. Experimental studies suggest that GH might have several beneficial effects on adaptation^[182], including increases in mucosal hyperplasia and absorptive capacity^[165,183], bowel growth, villus height, and crypt depth^[184,185], and even increased length within the remaining intestine after extensive small bowel resection^[92]. In humans, GH alone, or combined with high carbohydrate diets and glutamine supplementation, may increase nutrient absorption^[186]. In children dependent on TPN for more than 50% of their nutritional needs, 12 wk of GH decreased TPN requirements. However, only two children (25%) were definitively weaned from TPN^[187]. This suggests the need for multimodal interventions to achieve clinically meaningful endpoints. Combining GH with dietary modification and glutamine supplementation may permit weaning from TPN in some patients^[183], and another prospective, double-blind randomized placebo-controlled trial demonstrated that a reduction in TPN use can persist for three months if GH is combined with glutamine^[173]. Four randomized, double-blind, placebo-controlled studies have asked whether GH supplementation increases body weight in this setting^[172,188-190]. These have yielded mixed results, although a recent Cochrane review found that glutamine overall increases weight, lean body mass, energy absorption, and nitrogen absorption^[191]. Reported side effects^[172] include myalgia, gynecomastia, insomnia, joint pain, and hyperglycemia. As of this writing, GH is the only FDA-approved agent to treat short bowel syndrome in the United States, but it is certainly not a panacea. To the extent to which GH is effective, it is most likely to benefit patients with 70-100 cm of small bowel remaining and without an intact colon. Side effects are significant.

Glucagon-like peptide-2: Glucagon-like peptide-1 physiologically is a humoral mediator of intestinal adaptation, normally secreted in response to enteral stimulus, especially by foods containing carbohydrates, fatty acids, and fibers^[192,193]. It is a 33 amino acid peptide derived from proteolytic cleavage and modification of proglucagon in the pancreatic α -cells and intestinal L-cells^[194].

GLP-2 production is most robust in the distal small bowel and large intestine^[195]. Effects of GLP-2 are specific to different regions of the bowel and appear to stimulate morphologic adaptation with increase in microvillus height and overall surface area. This was demonstrated in an animal model with 80% small bowel resection, using animals given TPN with or without GLP-2. After only one week intestines were examined for morphology, crypt cell proliferation, apoptosis, SGLT-1 expression and GLUT-5 transport proteins. In addition to the expected finding of morphologic adaptation, GLP-2 increased the jejunal crypt apoptotic index without increasing transport protein expression^[196].

Teduglutide (ALX-0600), a dipeptidyl peptidase IV (dpp-IV) resistant GLP-2 analog, has been reported to promote intestinal growth in short bowel patients, increas-

Table 3 Potential pharmacotherapy targets for intestinal failure

	Dose mg/kg per d	Side effects	Structure	Approval
Growth hormone	0.1	Fluid retention, joint pain, hyperglycemia	191-amino acid protein	FDA
EGF	NA ¹	NA	53-amino acid peptide	Not available commercially
GLP-2	0.1	Abdominal pain/obstructive symptoms	dpp-IV resistant 33-amino acid peptide	Phase III clinical trials available in Europe

¹Dose not specified for human use. FDA: Food and Drug Administration; GLP-2: Glucagon-like peptide-2; EGF: Epidermal growth factor; dpp-IV: Dipeptidyl peptidase IV; NA: Not available.

ing small intestinal villus height, crypt depth, and mitotic index and improving absorption over three weeks^[197]. 11 short bowel syndrome patients with Crohn's disease taking Teguglutide over 2 years demonstrated excellent compliance (93%), safety, and improved quality of life. The major reported side effects appear to be abdominal pain and obstructive symptoms, but it is difficult to determine the extent to which these side effects should be ascribed to Teduglutide or to the patients' underlying Crohn's disease. Whether other short gut patients will report less of these symptoms awaits study^[198]. Teduglutide may also aid weaning from TPN. In a randomized placebo-controlled trial, low-dose Teduglutide promoted weaning from TPN, although puzzlingly high-dose Teduglutide did not have this effect^[199] although secondary endpoints of villus height and body mass were increased by high-dose Teduglutide as well. This puzzling result was attributed to possible baseline differences between groups, although alternative explanations include difference in oral intake and side effects. In addition, a suprapharmacologic effect may limit efficacy. Teduglutide is in clinical use in some countries already, and will likely achieve broader distribution in the near future. Further studies with regard to the ideal dose, time course, and potential for synergy with other interventions would improve our understanding of how Teduglutide should be used (Table 3).

IGF: IGF may also enhance enterocyte proliferation after small bowel resection is^[200,201]. IGF-1 is produced primarily in the liver but it is also synthesized to a lesser extent within the intestine by subepithelial myofibroblasts^[202]. IGF-1 may upregulate digestive enzymes including sucrase, maltase, and leucine aminopeptidases after small bowel resection in animals^[203]. In addition, targeted overexpression of IGF-1 in transgenic mice leads to increased small bowel weight, length, and crypt cell proliferation^[204]. In short bowel syndrome rats on parenteral nutrition, IGF-1 treatment induced jejunal hyperplasia^[205]. In small bowel syndrome rats, IGF-1 increased jejunal mucosal mass by 20% and DNA content by 33%, reflecting increased enterocyte hyperplasia^[206].

EGF: EGF is a 53-amino acid peptide in saliva and pancreaticobiliary secretions. EGF stimulates crypt cell proliferation and suppresses apoptosis^[207]. EGF administration at the time of small bowel resection may facilitate intestinal adaptation, ameliorating weight loss and apoptosis^[208,209]. EGF functions intraluminally as small bowel resection in animals increases salivary EGF without increasing plasma EGF, and either removal of salivary glands^[209,210] or selective oral inhibition of the EGF receptor^[1106] attenuates adaptation after small bowel resection. The EGF receptor is regulated at the level of ligand expression during intestinal epithelial differentiation^[211].

Other hormones of potential interest

Leptin has been studied most regarding appetite and the obesity physiology. It is also a potential target to manipulate adaptation. Parenteral leptin may stimulate structural adaptation in short bowel rats by increasing cell proliferation and decreasing apoptosis. Leptin also increases GLUT-5 levels^[212,213].

Bombesin is also being explored as a therapeutic target of interest. In rats, subcutaneous exposure to bombesin for 2 wk after massive small bowel resection enhanced enterocyte turnover with increased ileal transmural and mucosal weight, DNA and protein, villus height, crypt depth, and proliferation index. These rats also demonstrated increased ileal Bax and Bcl-2 and decreased apoptosis^[214].

Ghrelin is secreted in the stomach and other tissues and influences food intake and nutrition. Plasma ghrelin is decreased in short bowel syndrome^[215]. These changes are unrelated to hyperphagia. It is not yet known whether this decreased ghrelin is only reactive or has an adaptive function^[216].

Glucocorticoids: The stress response may play a critical role in intestinal adaptation. In rats undergoing either 80% small bowel resection or sham operation, dexamethasone infusion reduced weight, DNA content, and mucosal protein content regardless of surgical status. IGF-1 was markedly decreased in the steroid-treated rats, demonstrating a potentially deleterious effect on adaptation^[217]. In contrast, glucocorticoids may impact uptake of sugars by modulating uptake receptors with variable effects among the glucocorticoids^[218]. How to modulate these effects without adversely affecting other physiologic parameters remains unknown.

MULTIMODALITY THERAPY

Integrated multimodality treatment may prove the best strategy. For instance, just as GH may be more effective when combined with glutamine. GH and EGF in combination synergistically increased microvillus height and enhanced nutrient transport in a rabbit short bowel model^[219].

OTHER NEW FRONTIERS IN MUCOSAL ADAPTATION

Surgical interventions for short bowel syndrome

Surgery is more acutely risky than medical intervention but also offers hope to patients who otherwise would be condemned to permanent TPN. Potential interventions include intestinal lengthening procedures such as the Bianchi procedure or the serial transverse enteroplasty (STEP) procedure and small bowel transplantation^[141,220]. Intestinal lengthening procedures are more conservative.

The Bianchi procedure involves splitting a dilated bowel segment longitudinally and reanastomosing it. This could potentially double intestinal length. One institution recently reported a 40% TPN wean rate with Bianchi alone^[221]. There is significant potential, however, for bowel loss in the event of technical misadventure. The STEP is an alternative that involves plicating the small bowel with staple lines alternating on the mesenteric and antimesenteric edges. One series reported a 60% TPN wean rate among adult and pediatric patients with short bowel syndrome using the STEP procedure^[222]. On the horizon is the concept of using slow chronic intestinal distraction to stimulate intestinal mucosal and muscular proliferation and thus lengthen the small bowel slowly over time. This has been successfully performed in animal models with additional benefits including increased mucosal weight, and potentially improved function as given by increased disaccharidase activity^[223]. This makes conceptual sense since pressure^[224] and deformation^[225] stimulate intestinal epithelial proliferation.

Small intestinal transplantation is more aggressive and risky but offers even more potential for weaning from TPN. The indications for small-bowel transplantation according to the American Society of Transplantation include the high risk of death related to the underlying disease as well as intestinal failure with increased morbidity or poor acceptance of parenteral nutrition. The United States Center for Medicare and Medicaid lists home TPN complications including impending liver failure, central venous access thrombosis of 2 or more central veins, recurrent line sepsis, and repeated episodes of dehydration^[226]. We highlight the critical nature of these options to demonstrate the need for less morbid options. Risks specific to small intestine transplant include graft thrombosis, ischemia, infection related to immunosuppression, and graft rejection. Outcomes appear to be improving with time. Graft and patient survival in carefully selected patients have recently been reported as high as 75% and 80%, respectively^[226,227]. Although such surgical interventions are yielding increasingly impressive results, improved medical and nutritional therapy might obviate the need for such risky procedures.

Obesity surgery

Although we generally think about the intersection between mucosal function and surgery with regard to procedures to increase mucosal mass and digestive func-

tion, the biology of intestinal adaptation may also be relevant to morbid obesity surgery. Morbid obesity is a rising epidemic with profound cardiovascular, endocrine, and pulmonary systemic consequences^[228]. Such patients typically do not respond well to conventional instructions to eat less and exercise more. The induction of artificial malabsorption using steatorrheic agents can cause mild weight loss but is associated with poor compliance^[229]. Bariatric surgery has evolved as the most effective treatment for morbid obesity. Although many procedures have been described to induce weight loss, they can generally be categorized as to whether they have restrictive and/or malabsorptive components. Procedures including malabsorptive components, in which some of the small bowel is bypassed, generally achieve superior weight loss to purely restrictive procedures because malabsorption procedures create a functional short gut syndrome.

Such bariatric procedures generate initial weight loss, but many patients gain back weight later. Some failures are attributed to behavioral changes as patients learn to “out-eat” the surgical procedure. However, it seems likely that postoperative intestinal adaptation to the functional short gut also ameliorates weight loss by increasing the absorptive capacity of the intestine that remains in continuity.

Intestinal adaptation does occur after malabsorptive obesity surgery. Humans undergoing classical jejunoileal bypass develop increased small bowel villus height and improved nutritional intake without increases in individual cell height or width, identifying epithelial hyperplasia as part of the adaptive mechanism^[230]. However, the un-bypassed functional segment of small intestine demonstrates not only an adapted morphologic appearance with increased villus height but also increased activity of brush border enzymes^[231]. This suggests that individual intestinal epithelial cells, while not larger, are likely to be more functional, better able to absorb and digest nutrients, consistent with a bimodal model of adaptation in which the adapted intestine has not only more enterocytes but better enterocytes that more fully express characteristics needed for their function. It remains unclear whether similar changes occur in response to decreased nutrient consumption in the bowel of patients who undergo purely restrictive procedures such as laparoscopic gastric banding and gastric sleeve procedures. Human biopsies 11-22 mo after jejuno-ileal bypass reveal marked mucosal villus hypertrophy in the continuous segment of bowel, and atrophy within the bypassed segment^[232]. Obesity surgery also alters gut hormone levels. For instance, 6 mo after Roux-en-Y gastric bypass, fasting leptin and insulin decrease while peptide YY, enteroglucagon, and GLP-1 increase^[233]. This coincides with sustained postprandial satiety that may also be related to intestinal adaptation. These effects may persist for years after surgery^[234].

Mucosal atrophy and adaptation can also be reproduced in rat models for research. Adaptation in the remaining intestinal segment is well described in rats after massive small bowel resection. Conversely, we recently described a novel defunctionalizing Roux-en-Y anasto-

mosis rat model in which the defunctionalized segment (not actually anastomosed proximally but just ligated at its proximal end) displays morphological and biochemical evidence of mucosal atrophy reminiscent of that seen in TPN-nourished animals despite enteral nutrition passing through the remaining gut^[74]. Decreased mucosal mitogenic extracellular signal-regulated kinase (ERK) signaling correlates with decreased proliferation in this bypassed segment. This confirms that mucosal atrophy in the setting of TPN reflects loss of enteral nutrients in direct contact with the gut mucosa rather than loss of indirect neurohumoral effects associated with enteric food consumption.

Interestingly, more classical Roux-en-Y bypass rat models, in which the bypass limb receives continuous biliopancreatic secretions, result in increased villus height and crypt depth in the common limb as compared to the biliopancreatic limb, but decreased glucose transport overall, suggesting the importance of both overall mucosal mass and anatomic rearrangement in determining intestinal function^[235]. Importantly, the biliary limb of this anastomosis exhibits partial adaptation with increased width and increased crypt cell proliferation without further mucosal adaptation. The alimentary and common channel exhibits full adaptation in bowel width, villus height, crypt depth and proliferation. This demonstrates the importance of direct contact and local factors required for full adaptation^[236].

Some endocrine changes after obesity surgery probably contribute to the success of these procedures, beyond their anatomic restrictive and malabsorptive effects. However, some of the neurohumoral consequences of obesity surgery may act synergistically with the decrease in delivery of nutrients to the intestinal mucosa to promote intestinal adaptation which in turn undesirably enhances nutrient absorption and contributes to delayed weight gain. Altering this natural adaptation after obesity surgery could sustain weight loss with less frequent failure. If sufficiently severe mucosal atrophy could be pharmacologically induced, one might even create sufficient malabsorption to obviate the need for any surgical procedure.

Influence of physical forces

The gut mucosa may not only be influenced by chemical interactions with growth factors, cytokines, and nutrients but also by exposure to physical forces such as repetitive deformation and pressure. These forces can originate from peristaltic contractions, villous motility, and physical interactions between the intestinal villi and the relatively non-compressible luminal chyme. *In vitro*, the proliferation of human intestinal epithelial cells is stimulated by rhythmic deformation, and this mitogenic effect is synergistic with the mitogenic effect of L-glutamine supplementation^[237]. The mitogenic effects of strain are amplitude-dependent^[75] as well as frequency-dependent^[237], and occur not only in established cell lines but also in primary

intestinal epithelial cells derived from human surgical specimens^[44].

The pathway governing this mitogenic effect is complex. *In vitro* work suggests it includes a complex web of kinases^[238] (while *in vivo* such signals are activated by repetitive deformation of the intestine in anesthetized animals^[76]). Extracellular pressure may also be mitogenic for intestinal epithelial cells^[224]. Pressure stimulates colon cancer cell proliferation *via* protein kinase C and tyrosine kinase signals. Supraphysiologic extracellular pressure inhibits intestinal epithelial wound healing independently of luminal nutrient flow^[239]. Enterocytic differentiation is influenced by some of these same stimuli^[240,241].

Interestingly, the intestinal epithelial response to repetitive deformation seems regulated by a matrix-dependent switch. Under basal circumstances, repetitive deformation induces proliferation and differentiation consistent with the ideal enterocytic phenotype. However, when fibronectin is added to the matrix substrate or medium *in vitro*^[44] or deposited into the extracellular matrix *in vivo* during inflammation or injury^[239], then deformation promotes a shift to a migratory phenotype and more rapid cell motility to close the resultant mucosal defect and maintain the mucosal barrier. The signal pathways that regulate this mitogenic effect are similar to those by which deformation is mitogenic in the absence of fibronectin, but exhibit subtle but important differences that may permit selective targeting of each effect^[242]. For instance, repetitive deformation promotes epithelial motility across fibronectin *via* a FAK-Tyr 925-phosphorylation that occurs independently of Src, while the FAK-Tyr 925-phosphorylation that occurs in response to strain in the absence of fibronectin requires Src^[225]. We recently demonstrated that integrin-linked kinase, in association with focal adhesion kinase and Src, modifies the downstream response to strain, perhaps implicating ILK as a useful molecular target for intervention^[243].

Animal studies are beginning to validate these *in vitro* observations. The defunctionalized gut, deprived of chemical and physical interactions with luminal nutrients, displays mucosal atrophy throughout its length^[74] and slower mucosal wound healing in wounded areas into which fibronectin has been deposited^[239]. Targeting otherwise deformation-activated signals such as ILK^[243] or small GTP-binding proteins like RhoA, rho-associated kinases and the formin homology protein mDia^[244] may someday maintain the gut mucosa despite fasting or ileus.

TARGETS FOR CELLULAR DIFFERENTIATION IN ADAPTATION

While most therapeutic efforts have emphasized modulating intestinal epithelial proliferation, it may also be useful and important to modulate intestinal epithelial differentiation to achieve a fully and optimally functional intestinal mucosa. Schlafen-3 and Math-1 are potentially interesting targets.

Table 4 Questions for future study

What is the ideal timing for influence of proliferative and differentiation phases in small bowel adaptation with targeted therapy?
What are useful combination regimens of multimodality therapy?
What dietary supplementation is essential for optimization of adaptation?
What is an appropriate algorithm for advanced medical compared to surgical treatment of small bowel syndrome ?
What is the precise role for glutamine supplementation in facilitating adaptation?
What is the ideal dose of Teduglutide?
Can other agents such as polyamines or retinoic acid be useful?
How can surgical procedures best be combined with multimodal therapy?
How can the role of physical force in adaptation be replaced pharmacologically?
Why is neoplasm more common in the large intestine than in the small intestine?
Why do APC (Min) mice have more common neoplasms in the small bowel?

Schlafen-3

Schlafen-3 is a member of the Schlafen superfamily, a poorly understood heterogenous group of proteins first described in 1998 as a family of growth regulatory genes that modulate thymocyte development^[245]. Schlafen-3 is not expressed in humans, but could have a functional human ortholog. We recently demonstrated that the Schlafens could play an important role in modulating intestinal adaptation. Schlafen-3 levels increase in parallel with expression of differentiation markers like dipeptidyl dipeptidase activity and villin expression when non-transformed rat intestinal epithelial IEC-6 cells are induced to differentiate in response to repetitive deformation, TGF- β , or sodium butyrate. More importantly, reducing Schlafen-3 by specific siRNA prevents the differentiating effect of each of these stimuli^[240]. This suggests that Schlafen-3 may represent an important common or convergent node in the differentiating signal pathways invoked by these three very different stimuli. Schlafen-3 is also downregulated in the intestinal mucosa of aging rats^[246], but conversely substantially increases in expression between the fetal state over the first few days after birth when the rat intestine is maturing^[247].

Although Patel *et al*^[246] reported that Schlafen-3 slows proliferation, we observed no effect on basal or EGF-stimulated proliferation when modulating Schlafen-3^[240]. This discrepancy awaits exploration *in vivo*. Tracing and triggering the Schlafen-3-dependent pathway may offer a way to selectively promote intestinal epithelial differentiation, either without affecting proliferation or perhaps even synergistically with agents such as GH or Teduglutide to promote both proliferation and differentiation.

Targeting differentiation is clinically important because altering the function of naive cells may promote intestinal function. A recent piglet study replicated multiple previous findings of increased total villus cell numbers over 6 wk of adaptation to massive small bowel resection. However, this study demonstrated a disconnect between

early proliferation and the absence of increased early weight gain. This suggests that the early proliferation of immature enterocytes alone may not suffice for nutrition, and highlights the need to encourage earlier differentiation to optimize clinical gains^[248].

Math1

Although the Schlafen superfamily may promote differentiation toward an enterocytic phenotype, intestinal stem cells also differentiate into goblet cells, enteroendocrine cells, and paneth cells in addition to enterocytes. Although less abundant in the mucosa, these intestinal epithelial cells are also important for optimal mucosal function. Murine knockout studies have identified Math1 as a transcription factor that influences the differentiation of these secretory cell types^[249]. In fact, deletion of Math1 does not disturb the capacity for self-renewal in intestinal epithelium at the crypt base^[250].

Further terminal differentiation is still under intensive investigation. A series of downstream targets influence differentiation toward the endocrine lineage, including NGN3, BETA2, Pax4, and Pax6^[251]. Manipulating these targets may also be important in the future to promote a fully functional mucosa.

CONCLUSION

Intestinal adaptation is an extraordinary phenomenon, induced by diverse pathological and surgical conditions, but not always successful in recreating adequate mucosal function. The long term consequences of such deficits in intestinal function highlight the need for more effective therapy for short gut syndrome directed by investigation into the physiological basis of adaptation. Despite recent progress, current targets are limited. We propose not only continued efforts to stimulate small bowel mucosal proliferation, but also increased investigation into the role of differentiation in adaptation (Table 4). Addressing both proliferation and differentiation multimodally could greatly improve patient outcomes.

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Advanced gastric cancer: Is there enough evidence to call second-line therapy standard?

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Abstract

Gastric cancer and cancer of the gastro-oesophageal junction (GOJ) are the 4th most common cancer diagnoses worldwide with regional differences in incidence rates. The treatment of gastric and GOJ cancers is complex and requires multimodality treatment including chemotherapy treatment, surgery, and radiotherapy. During the past decade considerable improvements were achieved by advanced surgical techniques, tailored chemotherapies/radiotherapy and technical innovations in clinical diagnostics. In patients with advanced or metastatic gastric/GOJ cancer systemic chemotherapy with fluoropyrimidine/platinum-based regimens (+/-human epidermal growth factor receptor-2 antibody) is the mainstay of treatment. Despite these improvements, the clinical outcome for patients with advanced or metastatic disease is generally poor with 5-year survival rates ranging between 5%-15%. These poor survival rates may to some extent be related that standard therapies beyond first-line therapies have never been defined. Considering that this patient population is often not fit enough to receive further treatments there is an increasing body of evidence from phase-2 studies that in fact second-line therapies may have a positive impact in terms of overall survival.

Moreover two recently published phase-3 studies support the use of second-line chemotherapy. A South Korean study compared either, irinotecan or docetaxel with best supportive care and a German study compared irinotecan with best supportive care-both studies met their primary endpoint overall survival. In this "Field of Vision" article, we review these recently published phase-3 studies and put them into the context of clinical prognostic factors helping to guide treatment decisions in patients who most likely benefit.

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Key words: Gastric cancer; Cancer of the gastro-esophageal junction; Second-line chemotherapy; Best supportive care; Survival

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INVITED COMMENTARY ON HOT ARTICLES

Background

Gastric cancer and cancer of the gastro-esophageal junction are one of the most common cancers in the world with significant impact on health resources^[1]. Extensive

surgery is the therapy of choice in early disease stages and often accompanied by neo-adjuvant and/or adjuvant chemotherapy^[2]. However, a significant number of patients relapse after initial surgery and a large proportion of patients (30%) present with advanced disease. In this setting systemic 5-fluoropyrimidine/platinum based chemotherapy [+/-human epidermal growth factor receptor 2 (HER-2) antibody] has shown to be an effective first-line therapy^[3]. Although first-line therapy is effective in the majority of patients a large proportion has no or limited benefit and may merit further treatment. Over the last decade second-line therapy has been controversially debated as clinical evidence, mostly generated from small, single centre or retrospective studies, was sparse. In this "Field of Vision", we review the results of two recently published trials reporting the benefit of second-line therapy for patients with advanced gastric cancer and put this into the context of current treatment algorithms.

"Field of Vision" commentary

We followed with great interest two recently published papers addressing the use of second-line therapy in patients with advanced gastric cancer.

Briefly, the Arbeitsgemeinschaft Internistische Onkologie (AIO) trial was a prospective, randomised, multicentre, open-label phase-3 study of 40 patients which compared irinotecan ($n = 21$; 250 mg/m² first cycle and 350 mg/m² subsequent cycles, *qw* 3) *vs* best supportive care (BSC; $n = 19$) where crossover into the irinotecan arm was not allowed^[4]. Restaging was performed every 6 wk and toxicity assessed based on the common toxicity criteria version 2.0 (CTCv2.0). Patients were well balanced for performance status (0- \leq 2), pretreatment, primary tumour type, number of metastatic sites, age, however, there was an imbalance in the male:female-ratio in both arms. In total a median number of two cycles was administered (range: 0-9) and 37% of patients in the chemotherapy treatment arm were dose-escalated to 350 mg/m² irinotecan. Irinotecan was generally well tolerated and the main grade 3/4 toxicity was diarrhea (26% of patients)-no treatment related deaths were observed. There was no objective tumour response, however disease stabilisation > 6 wk was documented in 53% of patients and a significant proportion of patients reported improvement of symptoms while on treatment ($n = 9$, 50%). The progression free survival for patients on treatment was 2.5 mo (95%CI 1.6-3.9 mo) with a median overall survival (OS) of 4.0 mo compared to a 2.4 mo OS in the BSC arm [hazard ratio (HR) 0.48, 95%CI 0.25-0.92, $P = 0.012$; one-sided log-rank test]. As a result and supported by evidence from phase-2 studies the German Gastric Cancer national guideline committee approved the use of second-line chemotherapy in patients with advanced gastric cancer.

The second study was recently reported from a group in South Korea where second-line therapy was historically more widely used despite level 3 evidence.

In this prospective phase-3 study, 202 patients with

advanced gastric cancer who received at least one prior therapy were randomised in a 2:1 fashion and received either chemotherapy (irinotecan 150 mg/m², *qw* 2 or docetaxel 60 mg/m², *qw* 3) or best supportive care^[5]. Restaging was performed every 6 wk and toxicity assessed based on the CTCv3.0. Patients were well balanced for performance status (0-1), pretreatment, primary tumour type, number of metastatic sites, age, however, there was an imbalance in the male: female-ratio in both arms. The treatment was generally well tolerated (66 patients, docetaxel; 60 patients, irinotecan; 62 patients BSC). Grade 3/4 toxicities included anemia (30 and 32%), neutropenia (15% and 18%) and fatigue (26% and 10%) in the docetaxel and irinotecan arm, respectively. Anemia, fatigue and anorexia were the most common grade 3/4 toxicities in the BSC arm. After a median follow-up of 20 mo the intention to treat analysis showed an increase in OS from 3.8 mo in the BSC arm (95%CI 3.1-4.5 mo) to 5.3 mo (95%CI 4.1-6.5 mo) with a HR of 0.657 (95%CI 0.485-0.891, $P = 0.007$; one-sided log rank test). There was no difference in the treatment effect of docetaxel and irinotecan; $P = 0.116$. Further exploratory analysis showed that PS (0 *vs* 1), prior chemotherapy (1 *vs* \geq 2) and chemotherapy-free interval (< 3 mo *vs* > 3 mo) were prognostic factors in the uni- and multivariate analyses.

Both phase-3 studies have shed light into a field which has been discussed controversially for the last few years (Table 1). Despite several limitations in design and recruitment there are several factors which we feel are important to highlight.

First, the two trials showed comparable clinical benefit in two different patient populations^[6]-both, the Western World and Asian population, tolerated treatments generally well and had had similar outcomes in terms of survival.

Second, the different choice of chemotherapy, e.g., weekly docetaxel or irinotecan as seen in the South Korean study, did not impact on outcome and therefore offers treatment choices in this setting. These results were recently supported in abstract format by a Japanese phase-3 study (WJOG4007) including 223 patients with advanced gastric cancer. Patients received either weekly docetaxel (80 mg/m²) or irinotecan (150 mg/m², *qw* 2) and were followed up until progression^[7]. In terms of toxicity both treatment regimens were comparable with grade 3/4 toxicities being: neutropenia (39% *vs* 29%), anemia (17% *vs* 7%) and fatigue (13% *vs* 7%), respectively.

Third, the multivariate analysis in the South Korean study identified clinical important prognosticators (performance status, number of prior chemotherapies and chemotherapy-free interval) which could serve physicians to make adequate treatment decisions. In this context, a recent retrospective study by Hasegawa *et al*^[8] identified additional clinical factors which could support treatment decision, namely PS (0-1 *vs* 2), albumin (> 35 mg/dL *vs* < 35 mg/dL) and time to progression on first-line therapy (> 170 d *vs* < 170 d). According to a prognostic model patients with two or more of these factors would not

Table 1 Clinical decision tool for second-line therapy

Prognostic marker	Treatment action	
	Support treatment	Caution-poor outcome
Albumin (mg/dL)	> 35	< 35
Performance status	0-1	2
Chemotherapy free interval (mo)	> 3	< 3
Time to progression on 1st-line therapy (mo)	> 6	< 6

benefit from second-line therapy.

Fourth, improved understanding of gastric/GOJ tumour biology have opened new avenues in combining chemotherapy with novel molecular targeted agents. For example, expression of the HER-2 has been associated with poor prognosis. Targeting this receptor *via* the monoclonal antibody trastuzumab (Herceptin) has shown improved outcome in the first-line setting in terms of response rates, progression free and overall survival^[9]. Other approaches in targeting the human epidermal growth factor family are *via* tyrosine kinase inhibitors such as lapatinib. Although lapatinib in the second-line setting had limited benefits as single agent the combination with taxanes is thought to have synergistic effects and several phase-2 and a randomised phase-3 trial (TYTAN-Study) are ongoing to test this hypothesis^[10]. Other molecular target drugs currently in the clinical arena are directed against the epidermal growth factor receptor (EGFR)-although results of early trials were ambiguous in unselected patient populations there are signs that selected patients with EGFR over-expression may have better outcome. For example, in pretreated patients the combination of irinotecan with cetuximab or nimotuzumab have resulted in trends towards better outcomes in those patients where EGFR was over-expressed^[11]. Other targets of interest are the oncogene c-Met which encodes the hepatic growth factor receptor and the insulin growth factor receptor-1-both receptors are often over-expressed in gastric/GOJ cancer and are thought to play a critical role in chemotherapy resistance^[12]. An increasing field of interest is the study of biomarker and molecular signatures predicting clinical outcome. For example, an increasing body of evidence suggests that downstream mutations in the Kirsten rat sarcoma viral oncogene or loss of the phosphatase and tensin homolog tumour suppressor gene are associated with poor prognosis and moreover predict inferior outcome in patients who are treated EGFR/HER2 targeted therapies^[13]. Other studies suggest that expression of the pro-apoptotic protein Bcl-2-associated protein X is associated with improved clinical outcome for a variety of chemotherapies including irinotecan and others^[14].

In summary, there is now increasing high-level evidence to support the use of second-line therapy in advanced gastric cancer. In addition easily derived clinical prognostic factors in combination with molecular sig-

natures should guide us in our attempt to rationalise the decision-making process to improve patient outcome.

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Dietary supplementation of some antioxidants against hypoxia

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Abstract

The present study aims to clarify the protective effect of supplementation with some antioxidants, such as idebenone (200 mg/kg, *ip*), melatonin (10 mg/kg, *ip*) and arginine (200 mg/kg, *ip*) and their combination, on liver function (T. protein, albumin, alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase), energetic parameters (adenosine triphosphate, adenosine diphosphate, adenosine monophosphate, inorganic phosphate, total adenylate, adenylate energy charge and potential phosphate). The effect on glycolytic and glycogenolytic enzymes (glucose, glycogen, glycogen phosphorylase, pyruvate kinase and phosphofructokinase against hypoxia) was also studied. The drugs were administered 24 and 1 h prior sodium nitrite intoxication. All biochemical parameters were estimated 1 h after sodium nitrite injection. Injection of sodium nitrite (75 mg/kg, *sc*) produced a significant disturbance in

all biochemical parameters of liver function, energetic parameters and glycolytic and glycogenolytic enzymes. Hepatic damage was confirmed by histopathological examination of the liver as compared to controls. The marked changes in hepatic cells induced by sodium nitrite were completely abolished by pretreatment with the drug combination, suggesting potential protection against sodium nitrite-induced hypoxia. It could be concluded that a combination of both idebenone and melatonin or idebenone and arginine provides potential protection against sodium nitrite-induced hypoxia by improving biochemical parameters and preserving liver histology.

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Key words: Hypoxia; Idebenone; Melatonin; Nitrate/nitrite; Adenosine triphosphate

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INTRODUCTION

Hypoxia, or hypoxiation, is a pathological condition in which the body as a whole (generalized hypoxia) or a region of the body (tissue hypoxia) is deprived of adequate oxygen supply. Variations in arterial oxygen concentrations can be part of the normal physiology, hypoxia in which there is complete deprivation of oxygen supply is referred to as anoxia. Hepatic tissue is quite vulnerable to

hypoxic injury. The morphological expression of hypoxic injury seems mediated by changes in the cortical cytoskeleton^[1]. Lemasters *et al*^[2] produced hypoxia in isolated, hemoglobin-free, perfused rat liver by reducing the flow rate of oxygen-carrying fluid entering the organ. This caused anoxia in centrilobular regions. In these anoxic areas, structural derangements developed rapidly, characterized by bleb-like protrusions of hepatocyte plasma membrane through fenestrations in the sinusoidal endothelium. Periportal tissue remained normoxic and was completely spared.

Idebenone (hydroxydecyl benzoquinone), a short chain synthetic analogue of coenzyme-Q10 (CoQ-10), is a vital cell membrane antioxidant and essential constituent of the adenosine triphosphate (ATP) producing mitochondrial electron transport chain. It is a potent antioxidant agent and unlike CoQ-10 has the ability to operate under low oxygen tension situations^[3].

The pineal gland is the main source of melatonin (N-acetyl-5-methoxytryptamine) in the circulation. It is also produced in small amounts in the retina, gastrointestinal system and by leukocytes^[4]. Melatonin (MEL) is tiny^[5] and highly lipophilic, and for this reason, it is found abundantly in all parts of the cell. MEL protects the DNA, lipids and proteins against oxidative damage^[6,7]. The free radical scavenging and antioxidant effects of MEL have been shown in many studies^[8-11].

2-Amino-5-guanidinopentanoic acid (arginine) is an important, versatile and a conditionally essential amino acid. Besides serving as a building block for tissue proteins, arginine plays a critical role in ammonia detoxification, and nitric oxide and creatine production. Arginine supplementation is an essential component for the treatment of urea cycle defects but recently some reservations have been raised with regards to the doses used in the treatment regimens of these disorders^[12].

In the present study, we aimed to compare the protective effects of idebenone, melatonin, arginine, idebenone + melatonin and idebenone + arginine against liver injury through histological (hematoxylin and eosin) and biochemical [T. protein, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP), ATP, adenosine diphosphate (ADP), adenosine monophosphate (AMP), inorganic phosphate (Pi), total adenylate (TA), adenylate energy charge (AEC), *potential phosphate* (PO), glucose, glycogen, glycogen phosphorylase, pyruvate kinase (PK) and phosphofructokinase (PFK) parameters] against hypoxia in ameliorating sodium nitrite induced hypoxia in rat livers.

PRINCIPLES AND TECHNIQUE

Drugs and chemicals

Idebenone, melatonin, arginine and sodium nitrite used in this study were analytically pure products of Sigma-Aldrich Chemical Co., St. Louis, MO, United States. All other chemicals were of the highest analytical grade. Sodium nitrite and melatonin were dissolved in normal

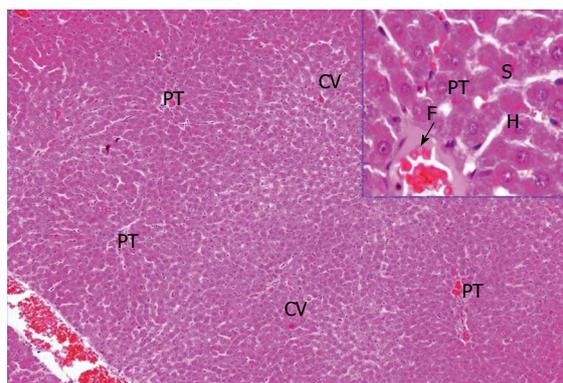


Figure 1 Liver section (hematoxylin and eosin $\times 100$ with lateral magnification $\times 200$) showed the normal structure of liver control.

saline, while, idebenone and arginine were suspended in 1% gum acacia in normal saline.

Animals

Adult male albino rats, weighing 180-200 g obtained from the animal house of King Saud University, were used in this study. They were fed with a standard laboratory diet and tap water ad libitum and housed in cages (ten rats per cage). All animals were kept at standardized laboratory conditions ($25 \pm 5^\circ\text{C}$, $55\% \pm 5\%$ humidity, and a 12 h light/dark cycle). One week after acclimatization in the laboratory where experiments were performed, the animals were fasted for 3 h prior to drugs administration. All experiments were carried out according to recommendations of King Saud University of Experimental Animals Ethics Committee which is matched with international ethics for handling of experimental animals. The doses of sodium nitrite, idebenone, melatonin and arginine used in the current study were chosen according to preliminary studies in our laboratory and were matched with those in the literature^[13-16] respectively.

Experimental design

Animals were divided into seven groups ($n = 10$ rats) and were treated as follows: Group I normal group (Figure 1) treated with saline; group II treated with sodium nitrite only^[13] (75 mg/kg, *sc*); group III was administered idebenone^[14] (200 mg/kg, *ip*); group IV was injected with melatonin^[15] (10 mg/kg, *ip*); group V was treated with idebenone (200 mg/kg, *ip*) followed by melatonin (10 mg/kg, *ip*) one hour later; group VI was treated with arginine^[16] (200 mg/kg, *ip*); group VII was treated with idebenone (200 mg/kg, *ip*) followed by arginine. These drugs were injected 24 h and 1 h before sodium nitrite injection.

Liver tissues homogenate

Liver tissue was homogenized in 0.9 mol/L NaCl (1:10 w/v) for the estimation of glucose, total protein, albumin, ALT, AST and ALP. Liver tissue (0.25 g) was homogenized using 7% trichloroacetic acid for the extraction of adenosine nucleotides according to the method of Wijsman^[17]. For estimation of PFK, 0.25 g liver was

Table 1 Liver function, level of energetic parameters, level of some glycolytic and glycogenolytic enzymes in hypoxica rats at different treated groups

Parameters	Control I	Hypoxia II	Treated-groups					P value
			Idebenone III	Melatonin IV	Id + Melatonin V	Arginine VI	Id + arginine VII	
Liver function parameters								
T.protein (g/dL)	13.4 ± 2.5 ^a	9.2 ± 1.07 ^c	10.26 ± 0.57 ^{bc}	10.63 ± 0.6 ^{bc}	11.28 ± 0.52 ^b	11.03 ± 1.05 ^b	11.54 ± 0.81 ^b	< 0.001
Albumin (g/L)	68.9 ± 5.4 ^b	56.18 ± 7.5 ^c	68.3 ± 6.68 ^b	84.47 ± 14.2 ^a	68.98 ± 7.1 ^b	67.10 ± 9.90 ^{bc}	72.34 ± 9.7 ^b	< 0.01
AST (GOT) (U/L)	9.78 ± 1.18 ^a	4.64 ± 1.25 ^c	8.86 ± 1.32 ^a	8.64 ± 1.04 ^{ab}	7.01 ± 1.13 ^b	8.65 ± 1.84 ^{ab}	9.64 ± 1.49 ^a	< 0.001
ALT (GPT) (U/L)	9.69 ± 3.67 ^{ab}	5.87 ± 0.99 ^c	10.4 ± 1.9 ^{ab}	9.28 ± 2.1 ^{ab}	7.34 ± 3.93 ^{bc}	10.53 ± 1.6 ^{ab}	10.78 ± 1.4 ^a	< 0.05
ALP (U/L)	461.98 ± 40.1 ^{bc}	623 ± 64.9 ^a	486 ± 62.0 ^b	449.5 ± 47.7 ^{bc}	436.3 ± 65.6 ^{bc}	400.5 ± 39.3 ^c	418.4 ± 46.2 ^{bc}	< 0.001
Level of energetic parameters								
ATP (μmol/mg)	1.47 ± 0.11 ^a	0.602 ± 0.03 ^f	0.978 ± 0.05 ^d	1.03 ± 0.07 ^d	1.27 ± 0.03 ^b	0.87 ± 0.05 ^e	1.16 ± 0.05 ^c	< 0.001
ADP (μmol/mg)	0.152 ± 0.007 ^a	0.37 ± 0.02 ^a	0.24 ± 0.01 ^b	0.20 ± 0.01 ^c	0.18 ± 0.005 ^d	0.26 ± 0.02 ^b	0.21 ± 0.008 ^c	< 0.001
AMP (μmol/mg)	0.08 ± 0.008 ^e	0.188 ± 0.01 ^a	0.13 ± 0.01 ^c	0.12 ± 0.01 ^{c,d}	0.108 ± 0.008 ^d	0.15 ± 0.008 ^b	0.12 ± 0.008 ^c	< 0.001
Pi (μmol/mg)	12.82 ± 1.9 ^a	5.34 ± 0.75 ^e	8.12 ± 0.46 ^d	8.47 ± 0.36 ^{c,d}	9.93 ± 0.35 ^b	7.84 ± 0.31 ^d	9.49 ± 0.58 ^{bc}	< 0.001
TA	1.70 ± 0.12 ^a	1.16 ± 0.02 ^e	1.35 ± 0.05 ^d	1.36 ± 0.075 ^c	1.56 ± 0.03 ^b	1.27 ± 0.03 ^d	1.49 ± 0.05 ^b	< 0.001
ATP/ADP	9.65 ± 0.64 ^a	1.65 ± 0.18 ^g	4.05 ± 0.29 ^e	5.06 ± 0.039 ^d	7.21 ± 0.31 ^b	3.41 ± 0.31 ^f	5.64 ± 0.26 ^c	< 0.001
ATP/AMP	18.08 ± 2.47 ^a	3.21 ± 0.26 ^e	7.82 ± 0.83 ^e	8.70 ± 1.27 ^c	11.72 ± 0.97 ^b	5.80 ± 0.47 ^d	9.40 ± 0.99 ^c	< 0.001
AEC	0.90 ± 0.01 ^a	0.68 ± 0.01 ^f	0.82 ± 0.01 ^d	0.83 ± 0.01 ^d	0.87 ± 0.01 ^b	0.78 ± 0.01 ^e	0.85 ± 0.01 ^c	< 0.001
PO	0.77 ± 0.15 ^a	0.31 ± 0.05 ^e	0.50 ± 0.05 ^d	0.60 ± 0.04 ^b	0.73 ± 0.02 ^a	0.44 ± 0.06 ^d	0.59 ± 0.06 ^{bc}	< 0.001
Level of some glycolytic and glycogenolytic enzymes								
Glucose (mg/g)	74.28 ± 6.43 ^a	58.5 ± 7.1 ^c	67.2 ± 3.2 ^{ab}	63.0 ± 7.0 ^{bc}	65.03 ± 5.5 ^{bc}	65.82 ± 6.7 ^{bc}	67.9 ± 4.8 ^{ab}	< 0.01
Glycogen (mg/g)	8.9 ± 1.2 ^b	3.4 ± 0.64 ^c	8.7 ± 1.02 ^b	8.9 ± 0.53 ^b	8.8 ± 0.56 ^b	8.99 ± 0.45 ^{ab}	9.98 ± 0.63 ^a	< 0.001
Glycogen- phosphorylase	0.99 ± 0.03 ^g	3.00 ± 0.12 ^a	1.93 ± 0.05 ^c	1.75 ± 0.08 ^d	1.38 ± 0.11 ^f	2.17 ± 0.11 ^b	1.54 ± 0.05 ^e	< 0.001
PK (μmol/min per mg)	4.00 ± 0.276 ^f	7.81 ± 0.16 ^a	5.51 ± 0.38 ^c	5.34 ± 0.25 ^{c,d}	4.92 ± 0.10 ^e	5.87 ± 0.15 ^b	5.11 ± 0.12 ^{d,e}	< 0.001
PFK (μmol/min per mg)	8.16 ± 0.48 ^e	32.03 ± 2.05 ^a	20.70 ± 1.34 ^b	18.43 ± 0.52 ^b	10.13 ± 1.27 ^d	20.32 ± 0.44 ^b	15.13 ± 2.74 ^c	< 0.001

Data are expressed as means ± SD of ten rats in each group; a, b, c, d, e, f, g means within columns with no common superscripts differ significantly. Unshared superscript letters between groups are the significance values at $P < 0.05$. Statistical analysis is carried out using one way analysis of variance (ANOVA) using CoStat computer program. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; ATP: Adenosine triphosphate; ADP: adenosine diphosphate; AMP: Adenosine monophosphate; AEC: Adenylate energy charge; PO: Potential phosphate; PK: Pyruvate kinase; PFK: Phosphofructokinase; Pi: Inorganic phosphate; TA: Total adenylate.

homogenized in 50 mmol Tris HCL, 1 mmol disodium ethylenediaminetetraacetate dehydrate (EDTA) and 5 mmol MgSO₄ at pH 8.2 and then centrifuged for 10 min at 0 °C. The supernatant was used for enzyme assays while 0.25 g tissue was homogenized in 5 mL Tris-HCL buffer pH 7.6 for detection of PK. On the other hand, 0.25 g tissue was homogenized in 100 mmol maleate-NaOH-buffer (pH 6.6) containing 20 mmol NaF, 1 mmol EDTA, 0.5 mg/mL bovine serum albumin and 10 mmol DL-dithiothreitol for estimation of glycogen phosphorylase.

Biochemical studies

Glucose was determined colorimetrically at 505 nm by the method described by Trinder^[18]. Total protein reacts with Bradford reagent to give a blue complex, which is measured calorimetrically at a wavelength of 595 nm (Bradford^[19]). The albumin level was measured according to the method of Doumas *et al*^[20] using Randox Diagnostic kits. In a buffered solution, bromocresol green forms with albumin; a green colored complex, its intensity is proportional to the amount of albumin present in the sample. ALT was determined according to the method of Reitman and Frankel^[21]. AST was determined according to the method of Reitman *et al*^[21]. ALP activity was measured photometrically^[22].

Energetic parameter evaluation

ATP was assayed following the procedure of Lamprecht

et al^[23]. ADP and AMP were assayed in a single assay system according to the method of Jaworek *et al*^[24].

Calculation of phosphate potential: PO is an alternative index used to indicate the free energy status of the tissues and can be calculated from the concentration of ATP, ADP and Pi according to Van Waarde *et al*^[25] $PO = [ATP]/[ADP] [Pi]$.

Calculation of total adenylates: TA = ATP + ADP + AMP.

Calculation of adenylates energy charge: AEC = $1/2 [ADP + ATP]/[AMP + ADP + ATP]$. Glycogen content was estimated by the method of Carroll *et al*^[26] as the green color formed was read at 610 nm against blank. PK was measured according to the method of King^[27]. PFK was estimated according to the method of Zammit *et al*^[28]. Glycogen phosphorylase was determined according to the method of Hedrick *et al*^[29].

Histological study

Liver samples were removed and placed overnight in fixative containing 10% formalin, liver were paraffin-embedded for hematoxylin and eosin (HE) staining and cut at 5 μm in the longitudinal plane; liver samples from different groups were named as control (I), hypoxia (II), idebenone (III), melatonin (IV), idebenone + melatonin (V), arginine (VI), idebenone + arginine (VII). Sections

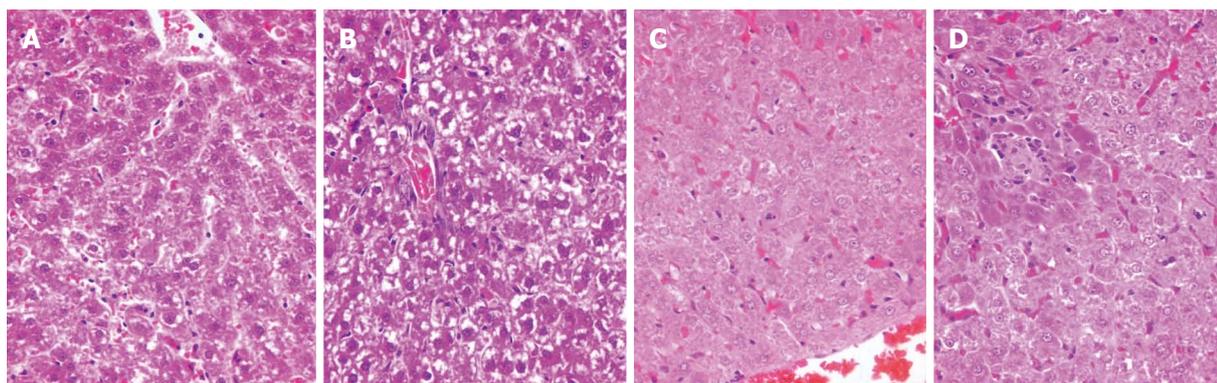


Figure 2 Liver section (hematoxylin and eosin $\times 200$) centrilobular region and periportal region. A: Liver section [hematoxylin and eosin (HE) $\times 200$] centrilobular region; B: Periportal region showed severe morphological changes as a result of giving sodium nitrite; C: Liver section (HE $\times 200$) centrilobular region; D: Periportal region of rats treated with melatonin against sodium nitrite.

were examined under the microscope for the extent of any damage and compared with the control group.

Statistical analysis

The results of biochemical analysis were analyzed using one-way analysis of variance followed by Co-stat computer program. Values of less than 0.05 were regarded as statistically significant.

RESULTS

Biochemical observation

There was a significant reduction in total protein, albumin, AST and ALT of 31%, 18.5%, 52.6% and 39% in the hypoxia group while ALP activity increased in this group (Table 1). All treated groups revealed enhancement of total protein activity of 7.9%, 10.6%, 15.5%, 13.6% and 17% in groups III, IV, V, VI and VII respectively. Albumin was enhanced by 17.6%, 41.6%, 18.6%, 15.8% and 23.5% in groups III, IV, V, VI and VII respectively. AST and ALT activities showed changes of 43%, 40.9%, 24%, 41%, 51% and 46.7%, 35%, 15%, 5%, 50.7% in treated groups III, IV, V, VI and VII respectively. Elevated ALP activity was inhibited by a different amount with different antioxidants.

There was a significant decrease in ATP level in hypoxic rats 59% (Table 1). Treatment of the hypoxic group produced significant amelioration of ATP level by 25.6%, 29%, 45%, 18% and 38% in groups III, IV, V, VI and VII respectively. When we studied hypoxic rats, significant increases of ADP (143%) and AMP (135%) levels were observed. Upon hypoxia, treated rats showed an improvement in ADP and AMP levels by 85.53%, 111.8%, 125%, 72% and 105% respectively for ADP and by 72.5%, 85%, 100%, 47.5% and 85% for AMP and for the same previous treatments respectively. In addition, there was significant reduction in Pi of 58.34% in the hypoxic group. Treated rats showed a significant amelioration of the effects of hypoxia, by 21.7%, 24%, 35.8%, 19.5% and 32% respectively for idebenone, melatonin, idebenone + melatonin, arginine and arginine +

idebenone respectively. At the same time, AEC showed a significant reduction of 24.44%. Significant enhancements of 15.6%, 16.7%, 21%, 11% and 18.9% were observed in treated groups, respectively. Measurements of ATP/ADP, ATP/AMP, PO and TA showed significant reductions in the hypoxic rats of 82.9%, 82%, 59.7% and -31.76%, respectively. Hypoxic groups that received treatment showed significant enhancements in ATP/ADP, ATP/AMP, PO and TA of 24.9%, 35%, 57.6%, 18% and 41%, respectively, for ATP/ADP and of 25.5%, 30%, 47%, 14% and 34%, respectively, for ATP/AMP. Changes in TA detected were 11.18%, 11.76%, 23.53%, 25.29% and 19.41%, respectively, while changes in PO recorded were 24.68%, 37.66%, 54.55%, 16.88% and 36.36% for the same previous mentioned treatments respectively. Glucose and glycogen content showed a marked decrease in the hypoxia group (21% and 61.8% respectively). Treatment with antioxidants changed these decreases to 11.7%, 6.1%, 8.7%, 9.8% and 12.6% respectively, for glucose (Table 1), and 59.9%, 61.7%, 60.7%, 60.9%, and 73.9% respectively for glycogen; while glycogen phosphorylase, PK and PFK enzyme activities showed significant increases in hypoxic rats of 95%, 203% and 292.5% respectively, but these increases were significantly ameliorated after treatment of hypoxic rats with idebenone, melatonin, idebenone + melatonin, arginine and idebenone + arginine. Changes in glycogen phosphorylase were 109%, 126%, 163.6%, 83.8% and 147.5% respectively for each treatment listed above while enhanced levels of PK of 57.5%, 61.8%, 73%, 48.5% and 67.5% were achieved, respectively. Changes in PFK were 138.85%, 166.67%, 268.38%, 143.5% and 207% respectively.

Histological observation

Severe morphological changes were seen in Figure 2A and B particularly in the peri-portal area, compared with Figure 1 control group with normal hepatocyte cells. The hepatocytes were shrunken and vacuolated. The radiating cordlike arrangement of the hepatocytes was disturbed, except in the region around the central veins. Figure 2C and D also showed morphological changes both in the

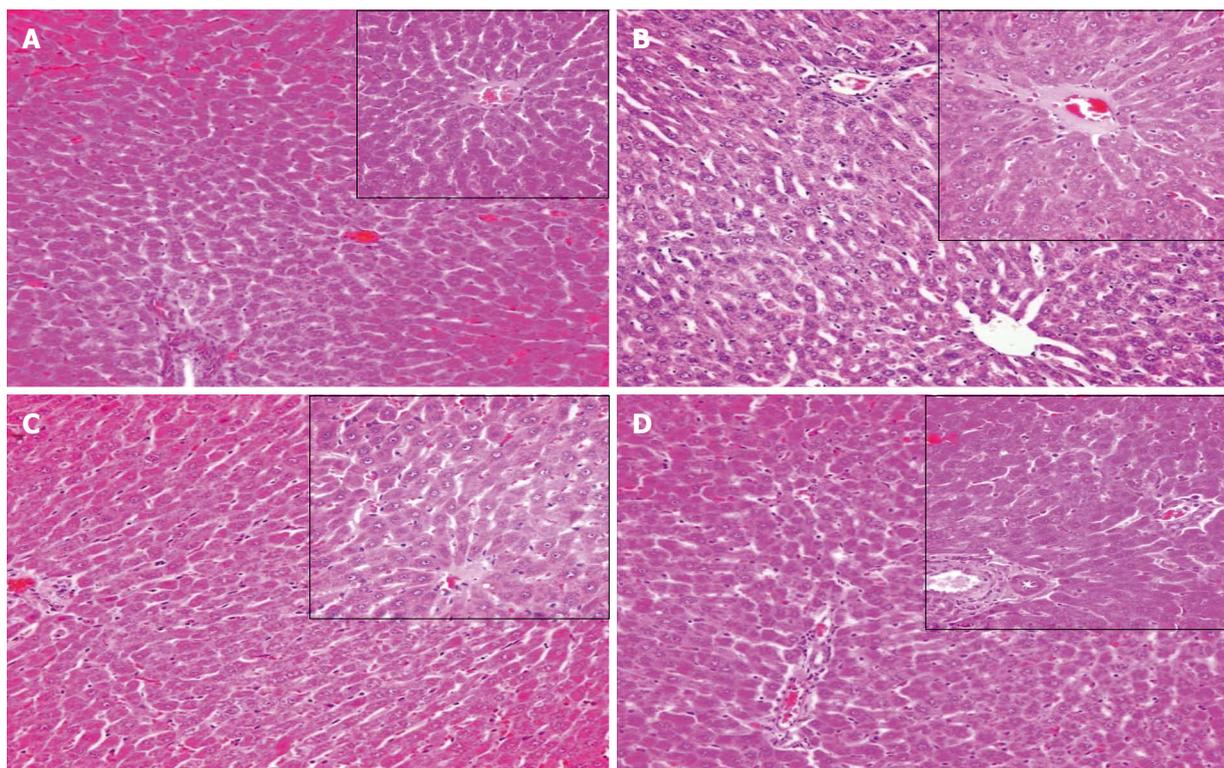


Figure 3 Liver section (hematoxylin and eosin $\times 200$) of rats. A: Liver section [hematoxylin and eosin (HE) $\times 200$] of rats treated with idebenone against hypoxia; B: Liver section (HE $\times 100$ with lateral magnification $\times 200$) of rats treated with idebenone + melatonin against sodium nitrite showing repairing of liver cells; C: Liver section (HE $\times 100$ with lateral magnification $\times 200$) of rats treated with arginine against sodium nitrite revealing normal hepatocyte; D: Liver section (HE $\times 100$ with lateral magnification $\times 200$) of rats treated with idebenone + arginine against sodium nitrite, showing hepatocytes with normal histological.

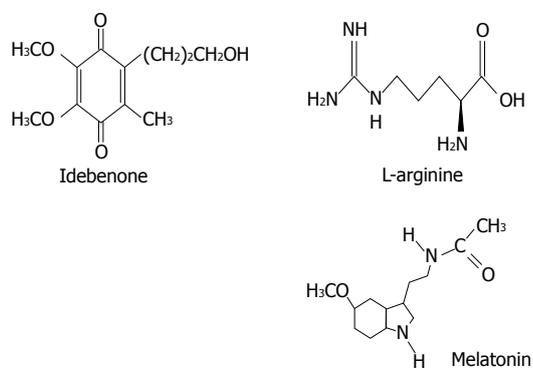


Figure 4 Chemical structures of idebenone, melatonin and arginine.

peri-portal and centrilobular regions. The liver cells were swollen and vacuolated and sinusoidal spaces appeared to be obliterated. Figure 3 did not show any marked difference as compared to the control group.

DISCUSSION

Liver injury causes vascular disorganization and local tissue hypoxia starting early in disease course. In this context, hypoxia acts not only as an aggravating factor of cell damage and inflammation, but also as an inhibitor of liver regeneration, a major stimulus of angiogenesis and fibrogenesis, and a promoter of liver carcinogenesis^[30]. Lack of oxygen causes metabolic cell death; increased

oxygen concentrations carry a risk for oxidative damage to proteins, lipids and nucleic acids, possibly initializing apoptosis or carcinogenesis^[31]. The present results demonstrate a reduction in ATP level in the liver of hypoxic rats, accompanied by an increase in ADP and AMP concentrations. This could easily be correlated with the aerobic-anaerobic transition induced by the hypoxia^[32]. The significant reduction in the Pi concentration in hypoxic rats despite the reduced level of ATP could be explained by the fact that, when liver is subjected to metabolic stress, a large amount of phosphate is trapped due to the presence of an abnormally high level of phosphoryl acceptor^[25]. The ATP/ADP ratio revealed that the energy utilization reaction was higher than the energy-generating reaction in the low oxygen state and, in turn, this could confirm the impairment of oxidative phosphorylation in the liver and reduced metabolism^[33]. The significant decrease in PO during the period of hypoxia confirmed the inhibition of Krebs cycle enzymes and the impairment of the electron transport chain^[34]. Liu *et al.*^[35] reported that hypoxia reduced the level of adenylate energy charge, ATP/ADP and ATP/AMP in collaboration with decreased levels of ATP, ADP and AMP due to an increase in the activity of adenylate kinase enzyme. The significant decrease of TA in hypoxic rats may be explained by the finding of a decrease in the ATP level which is considered to be the main contributing part of TA. In concurrence with the current result, Emerling *et al.*^[36] found that cells exposed to anoxia (0 mL/L O₂) show a decrease

in ATP levels. The intracellular ATP level might indeed direct AMP catabolism either towards IMP or adenosine. Jyoti *et al.*^[37] demonstrated that hypoxia activates AMP-activated protein kinase (AMPK) signalling independent of a decrease in both ratios.

AEC is a linear measure of the ratio of ATP concentration to total adenylate concentration, which ranges in value from 1 in the fully charged state to 0. Paradoxically, high values of AEC are often associated with high toxicant exposures, and low AEC values with low exposure. These discrepancies may be caused by the inability of AEC measurements to adequately evaluate cytosolic adenylate concentrations, which are the critical parameters in enzymatic regulation^[38].

The present results also demonstrated a significant increase in PK, PFK and glycogen phosphorylase in the liver of hypoxic rats. In parallel results Solaini *et al.*^[39] found that the AMPK pathway, which causes increases glycolysis, is driven by enhanced catalytic efficiency of some enzymes, including phosphofructokinase-1 and pyruvate kinase. The marked reduction in glucose and glycogen, while a significant increase in glycolytic enzymes PK, PFK was detected in liver tissue of hypoxic rats, may be due to an increase in metabolic activity in liver of hypoxic rats to compensate the inhibition of Krebs cycle caused by hypoxia. The increase in anaerobic glycolysis might be attributed to the activation of PFK due to decreased citrate formation provision of energy due to inhibition of the Krebs cycle and decreased nicotinamide adenine dinucleotide, oxidised form (NAD)/nicotinamide adenine dinucleotide, reduced form (NADH) (NAD/NADH) ratio due to inhibition of mitochondrial oxidation which favours the conversion of pyruvate to lactate^[40] with respect to glycogen phosphorylase, as glycogenolytic enzyme, it showed an enhanced activity in hypoxic rats which was attributed to degradation of stored glycogen inhibition of translocase, the glucose-6-phosphate transport protein^[41].

Milusheva *et al.*^[42] reported that ATP, ATP/ADP and AEC were significantly decreased in the absence of glucose (glucose deprivation) and ascertained that hypoxia is combined with glucose and glycogen deprivation and inhibition of the glycolysis and glycogenolytic pathways. The lack of glucose is a common factor contributing to the reduction in ATP level and AEC. This agrees with the well-known fact that during anoxia oxidative phosphorylation is impaired while ATP production proceeds *via* anaerobic glycolysis. During metabolic stress, AMPK, a highly sensitive indicator of cellular energy status, leads to an increase in ATP synthesis and inhibition of anabolic pathways to limit ATP consumption during periods of exercise or hypoxia^[43]. AMPK downregulates ATP-consuming pathways, such as glycogen, cholesterol, and fatty acid synthesis^[44]. However, Ou *et al.*^[45] declared that the evidence for increased glucose utilization under hypoxic conditions is equivocal. For example, rats acclimated to chronic hypoxia do not alter the capacity for anaerobic glycolysis in skeletal muscle, but rather increase

the capacity for fatty acid metabolism, possibly sparing carbohydrate metabolism for severe hypoxic conditions. Significant amelioration was detected upon treatment of hypoxic rats with idebenone, melatonin, idebenone + melatonin, arginine and arginine + idebenone with a fluctuating percent of amelioration, where the hypoxic rats receiving combined treatment of idebenone + melatonin showed the best result followed by idebenone + arginine. In agreement with the present findings, idebenone has been reported to preserve non-protein thiols, and inhibit lipid peroxidation in rat brain during post-cardiac arrest reperfusion^[46]. Idebenone also may indirectly reduce oxidative brain stress by elevating nerve growth factor^[47] which acutely blocks reactive oxygen species (ROS) formation in the brain^[48]. On the other hand, idebenone treatment suppressed leukocyte-enhanced cold ischemia/reperfusion injury of liver endothelium through almost complete suppression of the endothelial constitutive nitric oxide synthase mRNA expression after reperfusion^[49]. Moreover, idebenone treatment reduced nitric oxide (NO) generation and apoptosis in hepatocytes treated with toxic bile salt glycochenodeoxycholate. Acute reduction in cerebral oxygen delivery is known to lead to the breakdown of neuronal energy metabolism^[50]. Additionally, high concentrations of ROS, resulting from impaired oxidative phosphorylation or electron leakage, eventually leads to ATP depletion^[51]; this is in contradiction with this study in which the ATP level was markedly reduced in hypoxic liver tissue.

On the basis of these data, we can hypothesise that the induction of melatonin during or post hypoxia could be a defensive mechanism by which hepatic cells contrast these alterations. To support this hypothesis, Nagaoka *et al.*^[38] reported that, melatonin, a lipophilic compound, acts directly or indirectly on ROS production; in fact, melatonin can directly scavenge free radicals or it can induce antioxidant enzymes *via* a specific melatonin receptor. These results could be attained in support of the presenting results that idebenone, melatonin or idebenone + melatonin produced obvious improvement in all the measured parameters in the the liver of hypoxic rats, with a greater effect being seen with combined treatment. Glycogen synthesis, which is located in cytosol, depends on the uridine triphosphate supply and hence on ATP supplies resulting from both mitochondrial oxidative phosphorylation and cytosolic glycolysis^[3]. L-arginine has a protective role against ROS attack due to its direct chemical interaction with superoxide anions^[52]. Data from the l-arginine treated group suggests that a pharmacologic increase in NO levels did not exacerbate the increase in free radical formation. In fact, a high level of NO/or l-arginine itself in the l-arginine treated group may be protective, probably due to their ability to scavenge free radicals as well as inhibit xanthenes oxidase (XO) enzyme^[53]. Idebenone and/or l-arginine treatment ameliorated the depleting effect of nitrite-induced hypoxia on brain ATP content, suggesting that their protective effect may be mediated through improving the cerebral energy metabolism^[54]. In

addition, its role in scavenging ROS and inhibiting lipid peroxidation could be an important factor in improving the mitochondrial respiratory chain. Studies have shown that under cellular low oxygen conditions idebenone prevents the free radical damaging effect and maintains relatively normal cell ATP levels^[55]. The present data support these facts as the decrease in brain ATP content in hypoxic rats was accompanied by a significant increase in brain malondialdehyde (MDA) content and serum uric acid concentrations. L-arginine supplementation has been proven to significantly reduced the increased in cardiac XO activity, MDA levels, and serum uric acid caused by exhaustive exercise in rats^[56].

Hepatocytes in normal rats had a normal chromatin structure with round nuclei. The quantity and the structure of the mitochondria in the cytoplasm was normal. Liver pathophysiology consists of many mechanisms that have an impact on liver damage at different levels. The morphological alterations of liver histology detected in hypoxic rats induced severe alterations in the liver, such as congestion, sinusoidal and lymphatic expansion, regional hepatocellular vacuolization and hepatocyte swelling. These results are in agreement with those of previous studies^[10]. Liver injury was repaired when idebenone, melatonin, arginine, idebenone + melatonin and idebenone + arginine were administered.

In accordance with previous results^[57] which revealed that melatonin exerts a beneficial role in restoring tissue alterations after subjection to hypoxia (Figure 4).

In conclusion, a combination of idebenone + melatonin and idebenone + arginine yields significantly better results than idebenone, melatonin or arginine alone. They are likely responsible for liver tissue protection in hypoxic rats through their action on NO generation, reduction as well as scavenging of ROS, maintenance of normal enzymatic and non-enzymatic antioxidant systems, and improvement of energy production.

Therefore, the current findings may have important implications in the development of therapeutic strategies aimed at manipulating either idebenone + melatonin and l-arginine + idebenone supplementation for amelioration of hypoxic liver injury.

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Expression and function of renal and hepatic organic anion transporters in extrahepatic cholestasis

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Abstract

Obstructive jaundice occurs in patients suffering from cholelithiasis and from neoplasms affecting the pancreas and the common bile duct. The absorption, distribution and elimination of drugs are impaired during this pathology. Prolonged cholestasis may alter both liver and kidney function. Lactam antibiotics, diuretics, non-steroidal anti-inflammatory drugs, several antiviral drugs as well as endogenous compounds are classified as organic anions. The hepatic and renal organic anion transport pathways play a key role in the pharmacokinetics of these compounds. It has been demonstrated that acute extrahepatic cholestasis is associated with increased renal elimination of organic anions. The present work describes the molecular mechanisms involved in the regulation of the expression and function of the renal and hepatic organic anion transporters in extrahepatic cholestasis, such as multidrug resistance-

associated protein 2, organic anion transporting polypeptide 1, organic anion transporter 3, bilirubin translocase, bromosulphophthalein/bilirubin binding protein, organic anion transporter 1 and sodium dependent bile salt transporter. The modulation in the expression of renal organic anion transporters constitutes a compensatory mechanism to overcome the hepatic dysfunction in the elimination of organic anions.

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Key words: Organic anions; Liver; Kidney; Multidrug resistance-associated protein 2; Organic anion transporting polypeptide 1; Organic anion transporter 3; Bilirubin translocase; Bromosulphophthalein/bilirubin binding protein; Organic anion transporter 1; Sodium dependent bile salt transporter

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INTRODUCTION

The kidney and the liver play the major role in the elimination of drugs. No common rules exist for the preference of kidney or liver in the elimination of drugs. While for some compounds one of these two routes is the dominant excretory pathway, for certain other substances both the kidneys and the liver play an important and

overlapping role as excretory organs^[1,2].

The relationship between renal and hepatic excretion of xenobiotics depends on the integrity of the elimination mechanisms. Impairment of liver or kidney function can be followed by compensation *via* the alternative elimination route, changing the relationship between renal and hepatic excretion of drugs^[1-3].

Since 1990, several membrane transport proteins have been cloned and characterized^[4-6]. Organic anion transporters (OATs) are located in the barrier epithelia of diverse organs, where they mediate the absorption and excretion of a wide range of metabolites, signaling molecules, and xenobiotics. The presence of overlapping substrate specificities among the different OAT isoforms suggests a possible role in remote signaling. Substrates excreted through one OAT isoform in one organ are taken up by another OAT isoform located in a different organ, thereby mediating communication between different organ systems. Ahn *et al.*^[7,8] have developed a “remote sensing and signaling hypothesis”. They suggested how the regulation of solute carrier 22 subfamily members (including those of the organic cation, organic carnitine, and unknown substrate transporter subfamilies) can be better understood by considering the organism’s broader need to communicate between epithelia and other tissues by simultaneous regulation of transport of metabolites, signaling molecules, drugs and toxins.

On a mechanistic basis, cholestasis usually is divided into “extrahepatic” and “intrahepatic” forms^[9-11]. The first refers to obstruction of large bile ducts outside the liver, for instance due to gallstones, while the causes of intrahepatic cholestasis lie within the liver. The main clinical manifestations and characteristics of “intrahepatic” and “extrahepatic” cholestasis are similar^[11,12]. It is possible to separate the two kinds of cholestasis by means of a careful evaluation of clinical, serologic, and histologic features. The mechanisms underlying the development of cholestasis in patients can be extremely varied. Intrahepatic cholestasis has multiple causes. Among these, familial cholestasis, drug-induced cholestasis, and many processes that affect bile formation/excretion and finally produce cholestasis, could be cited. Obstruction of the biliary tree due to gallstones or hepatic or biliary tumours causes stagnation of bile flow. Actually, patients with gallstones or tumours in the common biliary tract generally suffer from extrahepatic cholestasis. A well-established model of cholestasis in rodents is obtained by performing double ligation and division of the common bile duct (BDL). In animal models, a decrease in bile flow is associated in general with elevated cholestatic serum markers, altered liver histology and changes in liver functions related to sinusoidal and canalicular transport. Indeed, prolonged cholestasis alters the liver function due to an impaired uptake, changed biotransformation and secretion of compounds^[12]. In fact, cholestasis has been shown to alter the transport of the bile salts and of other organic anions^[9,11,13]. Moreover, altered absorption, distribution and elimination of drugs have been described in

this pathology^[4].

In obstructive jaundice, adaptive mechanisms may permit urinary excretion of those potentially toxic compounds that could not be eliminated by the liver because biliary transport is impaired^[4,10,11]. The modulation in the expression of renal OATs constitutes a compensative mechanism to overcome the hepatic dysfunction in the elimination of organic anions.

The expression and function of several transporters in kidneys and livers from rats with obstructive cholestasis will be reviewed. These transport proteins are as follows: (1) multidrug resistance-associated protein 2 (MRP2); (2) organic anion transporting polypeptide 1 (OATP1); (3) OAT3; (4) bilitranslocase (BTL); (5) bromosulphophthalein (BSP)/bilirubin binding protein (BBBP); (6) OAT1; and (7) sodium dependent bile salt transporter (ASBT).

MRP2, OATP1, OAT3, BTL and BBBP are expressed in kidney and liver, while OAT1 and ASBT are not expressed in liver. Their membrane localization is shown in Figure 1.

MRP2

Several members of MRP are expressed in liver and kidney tissues^[10,14]. They are known to function as ATP-dependent pumps for a broad range of organic anionic substrates. MRP2 is an efflux transporter that mediates the transfer or translocation of a wide variety of potentially toxic endogenous and exogenous compounds, including bilirubin, drugs, and carcinogens, in the form of amphiphilic anionic conjugates by an ATP-dependent mechanism^[10,14]. This efflux pump is also involved in BSP-conjugated derivatives and p-aminohippurate (PAH) transport^[15-18]. It has been localized on the apical membranes of hepatocytes, renal proximal tubular cells and jejunal cells, and is implicated in the excretion of different organic anions^[19].

During cholestasis, the canalicular conjugate export pump MRP2 exhibits a reduced expression that may contribute to impaired hepatic excretion of bile acids and other biliary constituents such as conjugated bilirubin^[20,21]. Thereafter, in obstructive jaundice, adaptive mechanisms may permit the liver to adapt to the higher burden of biliary constituents in part by altering the expression of hepatobiliary transporters. In fact, in order to limit hepatocellular accumulation of toxic compounds, upregulation of hepatocellular basolateral efflux pumps have been described^[10,13,22,23]. Afterwards, alternative elimination routes, such as *via* urine, might provide the only pathway to excrete these compounds from the body.

Under conditions of deficient hepatobiliary secretory function, an induction of renal proximal tubular MRP2 expression has been reported. Lee *et al.*^[13] demonstrated an upregulation in MRP2 protein expression in kidneys as early as one day after BDL. Moreover, renal MRP2 function has been studied by PAH clearance in a rat model of obstructive jaundice by Tanaka *et al.*^[24]. They described a significant increase in PAH clearance 24 h after BDL that

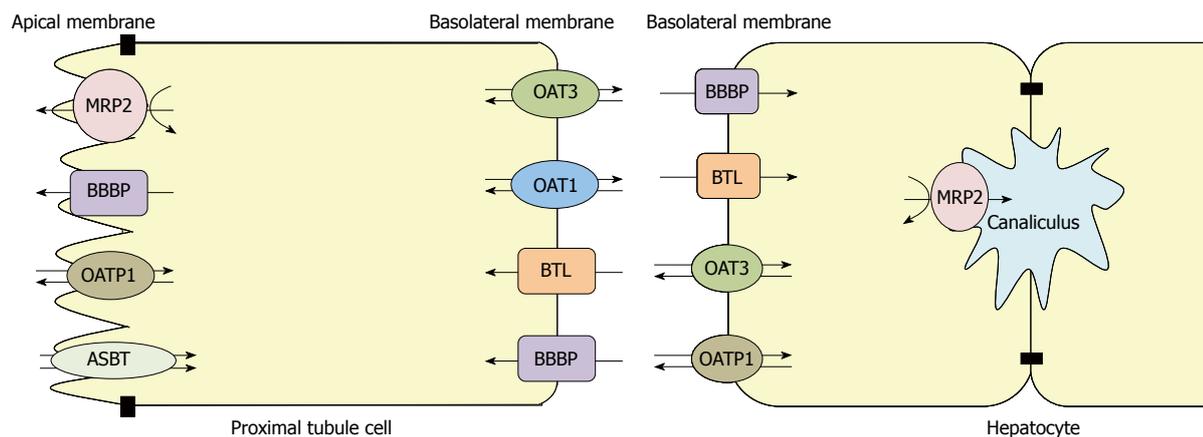


Figure 1 Membrane localization of organic anion transporters in hepatocytes and proximal renal tubule cells from rat. MRP2: Multidrug resistance-associated protein 2; OATP1: Organic anion transporting polypeptide 1; OAT: Organic anion transporter; BTL: Bilitranslocase; BBBP: Bilirubin binding protein; ASBT: Sodium dependent bile salt transporter.

was associated with high levels of renal MRP2 expression. In this study, in contrast to the liver where MRP2 protein and mRNA expression were downregulated after BDL, the protein and mRNA expression of MRP2 were significantly increased in the kidneys 24 h after BDL. They proposed that the increased renal MRP2 expression might be an alternative pathway for accelerating excretion of bilirubin conjugates during obstructive cholestasis. In order to gain insight into the mechanism by which BDL affects renal and hepatic MRP2 expression they also evaluated the effects of synthetic conjugated bilirubin, sulfate-conjugated bile acid, human bile, and unconjugated bilirubin on human MRP2 expression in an *in vitro* system using different cell lines. They described that mRNA expression of MRP2 increased in human renal proximal tubular epithelial cells after treatment with conjugated bilirubin, sulfate-conjugated bile acid or human bile. On the other hand, mRNA expression of MRP2 did not change in a human hepatoma cell line (HepG2 cells) compared with controls after the same treatments. These findings suggest that these substrates might regulate renal MRP2 expression by a cell-specific mechanism.

Downregulation of MRP2 in both liver and intestine from rats undergoing extrahepatic cholestasis might be explained by the increased levels of the inflammatory cytokine interleukin-1 β (IL-1 β) observed in these animals^[20,21]. These increased levels of IL-1 β would lead to decreased binding of RAR α :RXR α nuclear receptor dimer to the promoter region of MRP2 in BDL rats, which in turn downregulates MRP2 expression in the liver. In contrast, BDL rats exhibited upregulation of expression and function of MRP2 in the kidney, which likely results from preserved levels of RAR α :RXR α dimer in this tissue.

Villanueva *et al.*^[25] have reported an increased renal tubular conversion of 1-chloro-2,4-dinitrobenzene to its glutathione conjugate dinitrophenyl-S-glutathione (DNP-SG), followed by subsequent MRP2-mediated secretion into urine that partially compensates for altered liver function in experimental obstructive cholestasis.

At this point, it is noteworthy to mention that we have found increased BSP and PAH renal excretion in our experimental model of BDL in rats^[26-28], which in turn might be also explained by the upregulation of renal MRP2 expression^[29].

Finally, obstructive cholestasis leads to an upregulation of the apical transporter MRP2 in the kidneys that would be involved in the increased secretion into urine of organic anions, including BSP, PAH and DNP-SG. The current data demonstrate the relevance of renal elimination as an alternative pathway, particularly under conditions of impaired hepatobiliary secretory function, as occurs in obstructive cholestasis, for excreting those toxic compounds that the liver could not eliminate by itself from the body. Together, all these findings provide further evidence that transporters are regulated in cholestatic liver disease in a manner that facilitates extrahepatic mechanisms for organic anions excretion.

OATP1

OATP are members of a group of multispecific transporters with a wide spectrum of amphipathic substrates, including endogenous compounds and clinically important drugs^[30]. OATP1, the first member of this group of transporters identified, was isolated from rat liver and shown to mediate Na⁺-independent saturable transport of BSP^[31,32]. Nowadays, the sequences of almost 160 different members of OATP superfamily in over 25 animal species can be found in GenBank^[4].

Several members of this family of polypeptides are expressed in liver and kidneys, among others organs. In hepatic tissue, OATP1 is found at the basolateral membrane of hepatocytes where it plays an important role in the Na⁺-independent uptake of a wide variety of compounds, including bilirubin, BSP and conjugated and unconjugated bile acids^[33]. In contrast, in the kidneys, OATP1 is located at the apical membrane of the proximal tubules, specifically in the S3 segment, and has been

suggested to play a role in the secretion/reabsorption of selected anionic substrates, although its renal physiological function remains unclear^[4,30,32,34]. It could participate in the reabsorption of organic compounds that are filtered, like estradiol-17 β -glucuronide, or in the secretion process of some organic compounds that were taken up into tubular cells across the basolateral membrane^[34,35]. It is interesting to emphasize that renal secretion of BSP, a characteristic substrate for OATP1 transport, is negligible in non-pathological conditions.

Regulation of OATP1 expression and function occurs at both transcriptional and post-transcriptional levels, being tissue specific. Higher OATP1 renal expression in males than in females has been demonstrated in rodents probably as a result of testosterone stimulation and estrogen inhibition. On the other hand, hepatic OATP1 expression is not influenced by sex hormones^[10,34,36,37]. Phosphorylation of this transporter, in serine residues, by extracellular ATP produces its functional down regulation^[38,39]. In addition, protein kinase C (PKC) activation leads to a decreased transport of estrone-3-sulfate in OATP1 expressing *X. laevis* oocytes^[33,40].

The consequences of cholestasis, produced by BDL, are hepatic and systemic accumulation of potentially toxic biliary compounds, which are associated with progressive liver damage and jaundice^[41]. OATP1 hepatic mRNA and protein expression are downregulated in this pathology, with a less pronounced decrease in mRNA expression. This could be due to OATP1 protein having a longer half-life than its mRNA, but the occurrence of a post-transcriptional mechanism cannot be dismissed^[33,42]. It has been proposed that the observed OATP1 down-regulation could be due, at least in part, to a toxic effect of bile constituents retained in the hepatocyte. It was also proposed that this phenomenon might be explained as a protective feedback mechanism to prevent uptake of possible hepatotoxic bile constituents^[42]. In this model, the hepatic mRNA expression levels of cytochrome P450, transporters and conjugation enzymes were found altered. It has been suggested that increased bilirubin and fatty acid levels produced in cholestasis could modify the mRNA and protein levels of the constitutive androstane receptor and peroxisome proliferator-activated receptor α in the nucleus, altering the mRNA expression of several transporters, like OATP1^[43].

In obstructive cholestasis, several proinflammatory cytokines are released in the organism, such as tumor necrosis factor α (TNF- α) and IL-1 β . Cytokine-inactivation studies have demonstrated the existence of a TNF- α -dependent signaling pathway that mediates the down-regulation of the OATP1 gene, at mRNA and protein levels^[33].

Geier *et al.*^[33] also found unaltered kinetic parameters in Na⁺-independent bile salts (cholate and taurocholate) uptake in the presence of decreased OATP1 expression in extrahepatic cholestasis. In addition, they found that other sinusoidal OATPs, OATP2 and OATP4, have preserved expression in cholestatic hepatocytes, suggesting

that, in this pathology, Na⁺-independent bile salts uptake is mediated by OATP2 and OATP4 rather than OATP1, to compensate for its downregulation. This may be related to continuous elimination of other organic anions which also play an important role in determining cholestatic liver injury.

We have shown that BDL rats have a higher OATP1 protein renal expression at apical membranes despite no change in OATP1 abundance in kidney homogenates^[27]. These results could suggest altered OATP1 trafficking, as a result of an increased recruitment of preformed transporters into the membranes where they are functional, or impairment in the internalization of membrane transporters. These results indicate that the kidneys are able to adapt rapidly to obstructive cholestasis, since BSP renal elimination has been shown to increase almost 30-fold during the first day after induction of BDL. Renal adaptation to cholestasis could be due to an upregulation of the transport capacity of OATP1 in the proximal tubules. This increase in OATP1 protein expression at the apical membrane of renal cells may be a compensatory mechanism for reducing injury to hepatocytes or renal epithelia from cytotoxic substances that may accumulate in rats with obstructive cholestasis.

It is also important to emphasize that differential processing and trafficking of the OATP1 transporter in liver and kidney may have important functional and regulatory consequences. A complex series of hormonal changes in kidneys are induced in extrahepatic cholestasis^[44], which could affect OATP1 regulation. Several systemic and local factors are produced at the same time during cholestasis, and the role of such factors in the regulation of channels and transporters in renal cells is still unknown. Most likely, the accumulation of bilirubin, bile acids and other potential toxics existing in this cholestatic model may affect transcriptional regulation (e.g., fetal transcription factor, pregnane X receptor) and post-transcriptional regulatory mechanisms^[45].

In conclusion, the presented data shows that extrahepatic cholestasis induced by BDL in the rat produces renal OATP1 protein units redistribution into apical membranes from renal cells^[27] and a diminished OATP1 expression in the liver^[33,42]. Moreover, this probable adaptation to hepatic injury, particularly in elimination of biliary components, could possibly explain, at least to some extent, the huge increase in BSP renal excretion observed in this experimental model.

OAT3

Human and rat OAT3 is expressed primarily in the kidneys and to a lesser extent in the brain. In rats, OAT3 is also found in the liver^[46]. In the kidneys, immunohistochemistry studies showed that OAT3 protein is localized at the basolateral membrane of proximal tubule cells^[47,48]. Rat OAT3 was also observed in cortical and medullary thick ascending limb of Henle's loop, connecting tubules, and cortical and medullary collecting ducts^[49]. OAT3 was

characterized as an organic anion/dicarboxylate exchanger^[50,51]. This protein has the ability to transport PAH, estrone sulfate (ES), ochratoxin A, estradiol glucuronide, benzylpenicillin, cimetidine, glutarate and taurocholate. Although the selectivity of OAT3 overlaps that of OAT1, the affinity for the different substrates could permit discrimination between both transporters. Several studies demonstrated that ES is a specific substrate for mammalian OAT3 with moderately high affinity, whereas OAT1 interacts little with ES^[52-55]. Accordingly, ES is frequently used as a test substrate for OAT3 activity. Sweet *et al.*^[56] reported reduced uptake of PAH, ES, and taurocholate in renal cortical slices and nearly complete inhibition of transport of the fluorescent organic anion fluorescein in intact choroid plexus in OAT3 knockout mice.

The effects of obstructive cholestasis on the cortical renal expression of OAT3 and the consequences of these effects on the pharmacokinetics and renal excretion of PAH and furosemide (FS) were described by Brandoni *et al.*^[26,57] employing male Wistar rats. Results showed that rats at 21 h after BDL exhibit an enhanced systemic and renal clearance of both PAH and FS. At variance with OAT1 expression (as described below), Western blotting studies showed that OAT3 protein expression was increased significantly only in homogenates and not in basolateral membranes from renal cortex in BDL rats. These results were confirmed by immunohistochemical techniques. The upregulation observed for both OAT1 and OAT3 in this model of acute jaundice, could explain, at least in part, the increased systemic and renal elimination of PAH and FS. In addition, the production of various cytokines and growth factors that may affect gene transcription is associated with extrahepatic cholestasis^[58].

The function and expression of OAT1 and OAT3 were also studied by Brandoni *et al.*^[59] after 3 d of biliary obstruction. It was well documented that serum bile acids and bilirubin levels reach a peak after 3 d of BDL^[13,24,60]. A significantly lower renal clearance of PAH was observed after this period of time in rats with obstructive jaundice. In contrast to OAT1, OAT3 was increased both in homogenates and basolateral membranes from renal tissue after 3 d of BDL. As well as OAT1, both human and rat OAT3 transport PAH with relatively high affinity (87 $\mu\text{mol/L}$ and 65 $\mu\text{mol/L}$ respectively)^[6,47,61,62]. On the other hand, it was described, using *in vivo* and *in vitro* techniques, that OAT3 but not OAT1 has as substrates ES, cholate, and taurocholate^[6,47,56,61-63]. Furthermore, whereas OAT1 is limited to proximal tubules, OAT3 is expressed in different parts of the nephron^[48]. In extrahepatic cholestasis, the high plasma levels of bile acids compete with PAH for OAT3 transport, therefore, upregulation of OAT3 does not compensate for the downregulation of OAT1 in PAH transport^[59,64]. The expression of a number of genes implicated in the transport of bile salts is regulated by bile acids^[65,66]. In this sense, and as another example of substrate-specific regulation, it is possible that OAT3 overexpression would be regulated by high

bile acid levels, while OAT1 expression would be down-regulated.

Urinary excretion is the principal route of excretion of bile acids in obstructive jaundice. It was found that OAT3 mediated the renal secretion of bile acids such as cholic acid, glycocholic acid and taurocholic acid, which are mainly increased during cholestasis^[55,67]. The pharmacokinetic profile of its substrates could also be affected by cholestasis^[67]. Eisai hyperbilirubinemic rats (EHBR) are mutant rats lacking MRP2. Serum and urinary concentrations of total bile acids in EHBR rats are higher than those in wild-type Sprague-Dawley rats. It was described that OAT3 protein in renal plasma membranes was overexpressed in EHBR rats whereas OAT1 expression was unchanged. In addition, rat and human OAT3 transport activities are notably inhibited by diverse bile acids such as chenodeoxycholic acid and cholic acid but OAT1 is not. Cefotiam is also a specific substrate for OAT3. Some authors found that cefotiam clearance was reduced in EHBR despite upregulation of OAT3. This may be due to the inhibition of cefotiam transport *via* OAT3 by elevated serum bile acids. In summary, these results suggested that renal OAT3, but not OAT1, plays a critical role in the adaptative responses to the renal handling of bile acids in cholestasis.

Northern blot analyses demonstrated that OAT3 mRNA is expressed in rat liver, although such expression is not detected in mouse liver^[56,68]. Nevertheless, Buist *et al.*^[69], using branched DNA analysis, reported hepatic OAT3 mRNA expression in mice. In addition, gender differences in OAT3 mRNA expression in liver tissue were shown in both mice and rats^[56,69,70]. It was described also that the hepatic isoform of OAT3 may be the major contributor OAT in the basolateral uptake of organic anions in rats^[71]. Human OAT3 messenger is not expressed in liver.

We have observed in liver plasma membranes from rats with acute obstructive jaundice of 21 h, no differences in OAT3 protein expression when compared with sham rats^[72].

All these results might suggest the involvement of different regulatory mechanisms for OATs in obstructive cholestasis. To minimize liver injury, adaptive or protective responses to damage in the hepatobiliary transport of organic anions would appear, or reduced uptake into hepatocytes or enhanced efflux into the circulation. The observed changes could respond to increased intracellular levels of compounds that are normally excreted in the bile duct or to alterations in the levels of cytokines and other mediators in the liver, as previously described^[13,73,74].

To summarize, OAT3 could play a considerable pathophysiological role in protecting tissues from cholestatic damage by stimulating the renal secretion of bile acids. Indeed, ursodeoxycholic acid (UDCA) has been introduced as cholestatic liver disease therapy^[75]. It is possible that increased serum bile acids and/or administration of UDCA could influence the tubular secretion of

anionic drugs *via* OAT3 as was evidenced for cefotiam. Therefore, more attention should be paid to prevent the occurrence of drug interactions or drug-induced toxicity.

BTL

BTL is a bile pigment transporter that was originally isolated from rat livers^[18,76]. It is also expressed in kidney and stomach. BTL mediates the electrogenic hepatic uptake of cholephilic organic anions, such as BSP and thymol blue, the tetrapyrrole bilirubin, and flavonoids (the anthocyanin malvidin 3-glucoside and the flavonol quercetin)^[18]. BTL has been demonstrated to be involved in the renal transport of BSP, bilirubin and anthocyanins^[77,78]. In this way, BTL contributes to the hepatic and renal excretion of exogenous organic anions (such as BSP), endogenous metabolites (such as bilirubin) and anthocyanins (flavonoid-based pigments present in fruits and vegetables used in the human diet, which have been reported to be positively implicated in human health^[18,78]).

The protein expression and the functional activity of BTL was evaluated in rats with an early phase of acute extrahepatic cholestasis (21 h post BDL), by Western blotting and by measuring BSP electrogenic uptake, respectively, in liver plasma membrane and in renal basolateral membrane vesicles^[28]. No modifications were detected in BTL protein expression and in its activity in liver plasma membrane vesicles from BDL rats. In contrast, extrahepatic cholestasis caused a marked increase in renal BSP uptake, which was due to an increase in V_{max} (capacity). The difference in V_{max} suggests that a higher number of functional carrier units exists in renal basolateral membrane vesicles from BDL rats, which is in agreement with the higher expression of BTL detected in renal basolateral membranes. BDL rats have a higher renal expression of BTL at the basolateral membranes despite no change in kidney homogenates. This suggests an impairment in BTL trafficking.

These results suggest that the complex series of hormonal changes induced in kidneys by extrahepatic cholestasis^[44] might influence the regulation of BTL. The characteristic accumulation of bile acids, bilirubin, and other potential toxins in cholestasis may affect transcriptional and posttranscriptional regulatory mechanisms^[35,79] of BTL in kidneys. In this connection, as has been mentioned earlier, bilirubin, sulfate-conjugated bile acid and human bile upregulate the expression of MRP2 in renal tubular cells but not in liver cells^[24].

In summary, the higher expression and function of BTL observed in renal basolateral membranes from rats with obstructive cholestasis may also contribute to the marked increase in BSP renal excretion described in this experimental model. This would be another compensatory mechanism to overcome the hepatic impairment in the excretion of organic anions.

BSP/BBBP

The transporter protein BBBP has been isolated from

rat liver and described as an organic anion carrier protein involved in the well-known sodium-independent hepatic uptake of BSP and bilirubin^[80-83]. BBBP exhibits electroneutral transport as indicated by experiments in liver plasma membrane vesicles. In kidneys, BBBP has been localized in both apical and basolateral membrane domains, being implicated in PAH tubular transport^[84].

In rats with obstructive cholestasis, we have found an increased expression of BBBP in homogenates and in basolateral membranes from kidney cortex with no change in its apical membrane expression^[29,85,86]. These results might suggest an increase in the synthesis or a decrease in the degradation of this membrane transporter, while the trafficked protein would be preferably directed to the basolateral domain as no changes have been observed in the protein expression of BBBP in the apical membrane. As a consequence of hepatic function impairment, alterations in the renal elimination of organic anions were observed. Connected to this, we have also demonstrated a significant increase in renal excretion of two different organic anions in rats with extrahepatic cholestasis, BSP, an organic anion mainly excreted by the liver and PAH, an organic anion mainly excreted by the kidney. These observations could be explained, at least in part, by the higher basolateral membrane expression of BBBP described in this experimental model of acute cholestasis^[29,85,86].

As has already been mentioned, BBBP is a protein isolated from the sinusoidal membranes of rat liver. In liver plasma membranes from rats with extrahepatic cholestasis of 21 h, we have demonstrated no difference in BBBP abundance compared to sham animals^[85,86]. Modifications in hepatic excretion of organic anions have been demonstrated in rats with extrahepatic cholestasis^[9,10,13]. However, BBBP might not be involved in this observation since no change in its abundance was found in this experimental model in rats.

To this extent, it is noteworthy to remark on the relevant role of this kind of transporter, such as BBBP, in renal elimination of those organic anions excreted mainly by the liver, in the presence of obstructive cholestasis. In fact the increased renal abundance of the organic anion carrier BBBP, observed in rats with obstructive jaundice, might improve renal capacity to eliminate distinct negatively charged compounds, especially those that could not be removed by the liver.

OAT1

OAT1 is a key organic anion/ α -ketoglutarate exchanger^[48,87]. OAT1 is expressed predominantly in the kidneys, and weakly in the brain, and no expression has been described in the liver^[52,53,55,64]. This protein has been immunolocalized at the basolateral surface of the proximal tubule. OAT1 mediates the transport of many compounds (dicarboxylates, nucleotides, prostaglandins, antivirals, loop and thiazide diuretics, β -lactam antibiotics, non-steroidal anti-inflammatory drugs, including the prototypical substrate of the classical pathway, PAH)^[53,55]. Eraly *et al.*^[63] have

generated a colony of OAT1-knockout mice, permitting the elucidation of the role of OAT1 in the context of other potentially functional transporters. They found that the knockout mice manifested a profound loss of organic anion transport (e.g., PAH) both *ex vivo* (in isolated renal slices) as well as *in vivo* (as indicated by loss of renal secretion). The loss of renal secretion in knockout animals resulted in impaired diuretic responsiveness to furosemide^[63]. These results indicate an important role for the OAT1 transporter in the handling of organic anions by the classical pathway.

It has been reported that upregulation of OAT1 protein expression in rats at an early phase of acute obstructive cholestasis might explain the increased renal elimination of PAH and FS^[57]. We have also reported an increase in the systemic clearance of PAH associated with an increase in the abundance of OAT1 in renal cortex homogenates in rats during the early phase of acute extrahepatic cholestasis^[26,88].

FS is a loop diuretic secreted through the organic anion transport system. The capacity of the organic anion transport system to secrete a diuretic determines its intraluminal concentration, which is decisive of the diuretic activity. OAT1 and OAT3 are responsible for FS delivery to its site of action, since these proteins are involved in the renal tubular secretion of this diuretic^[55,89]. The protein expression of OAT1 was significantly increased both in cortical homogenates and in basolateral membranes from kidneys after 21 h of BDL^[26,57]. In fact, clearance and urinary excretion of PAH were both higher in BDL rats. Connected to this, PAH uptake rate^[57] was increased in basolateral membrane vesicles from BDL rats. OAT1 upregulation was also associated with a concomitant increase of systemic and renal FS clearance.

Similar studies were performed by Brandoni *et al.*^[59] after 3 d of obstructive cholestasis (the peak of elevation of serum bile acids and bilirubin). After this time, BDL rats displayed a decrease in the renal elimination of PAH. OAT1 protein expression in kidney homogenates was not modified, but was decreased in basolateral membranes. This study demonstrated, once more, the key role of OAT1 expression in the impaired elimination of PAH after 3 d of BDL. So, the evolution time of obstructive cholestasis has an important impact in the regulation of OAT1.

It has been described that bile acids and high bilirubin levels can activate PKC^[55,90]. We therefore postulate that the peak of elevation of bile acids and bilirubin (demonstrated after 3 d of BDL) can also trigger PKC activation. It has been described that PKC induces OAT1 downregulation through carrier retrieval from the cell membrane. This PKC activation may cause the phosphorylation of caveolin-2, which may induce the internalization of caveolae with OAT1 protein anchored with caveolin, as has been described by Kwak *et al.*^[91].

In summary, in this experimental model during an early phase of acute extrahepatic cholestasis where no evident renal cell injury exists yet, OAT1 upregulation as-

sociated with an increase in renal organic anion elimination was demonstrated. The protein expression of OAT1 was significantly increased both in cortical homogenates and in basolateral membranes from kidneys after 21 h of BDL, which might suggest an increase in the synthesis or a decrease in the degradation of this membrane transporter. In this experimental model, OAT1 upregulation may occur in order to enhance renal secretion of toxic compounds that are not eliminated by the liver in a pathological state. Connected to this, Tanaka *et al.*^[24] found that bilirubin ditaurate, sulfate conjugated bile acids, and some components of human bile upregulate the expression of MRP2 in human renal tubular cells. Moreover, we found an increased ³H-PAH uptake by S2 cells expressing OAT1 incubated with bilirubin ditaurate^[92].

Contrary to what has been described in an early phase of acute extrahepatic cholestasis, OAT1 expression significantly decreased in the basolateral membranes from kidneys after 3 d of BDL^[59]. It has been suggested that there is an increase in the internalization of membrane transporters or an inhibition in the recruitment of preformed transporters into the membranes. This study stressed, once more, the critical role of OAT1 renal expression in the excretion of organic anions, such as PAH, in a model of extrahepatic cholestasis in rats.

ASBT

ASBT is expressed in the ileum, in the cholangiocytes and in the kidneys^[93-96]. Bile acids, after secretion with bile into the small intestine, are nearly completely reabsorbed in the terminal ileum. They return with the portal venous blood to the liver where they are taken up and re-secreted into the bile. About 10%-50% of the reabsorbed bile acids avoid hepatic uptake and enter in the peripheral circulation. Around 10%-30% of the bile acids, present in the blood plasma, are subject to glomerular filtration. Urinary excretion of bile acids is much smaller than the amount filtered due to the tubular reabsorption of the filtered bile acids^[10,97]. Thus, the kidneys also take part in the recirculation process and aid conservation of bile acids. Tubular reabsorption of bile acids is accomplished by ASBT, which is expressed in the apical membrane of proximal tubule cells^[95,96].

Lee *et al.*^[13] have described decreased taurocholate transport in brush border membrane vesicles derived from rat kidneys after 14 d of BDL. The reduced taurocholate uptake was associated with a reduction of renal ASBT protein expression.

After one day of obstructive cholestasis, Schlattjan *et al.*^[98] demonstrated reduced taurocholate transport in proximal tubular cells without changes in the amount of the transport protein in these cells. The diminished taurocholate transport in this early phase of cholestasis may be mediated by a change in the phosphorylation status and hence the activity of ASBT and/or by a redistribution of the transporter between the plasma membrane

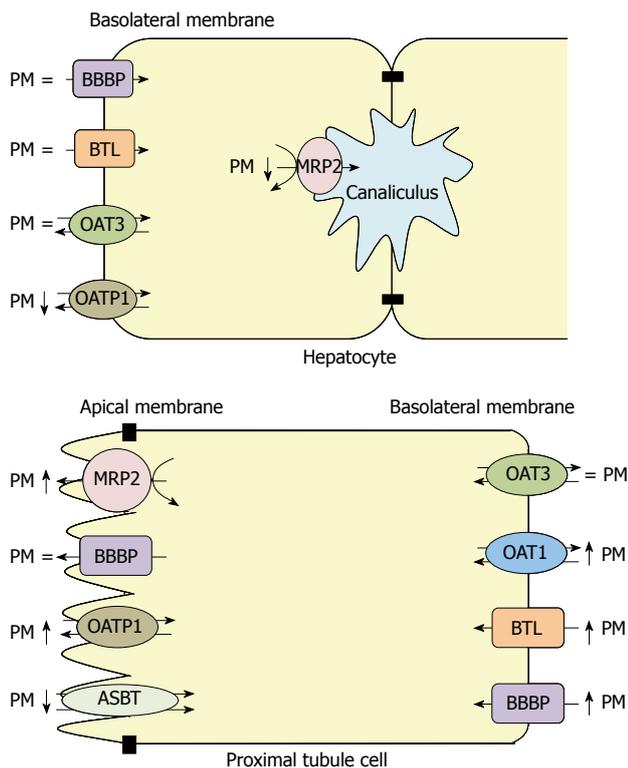


Figure 2 Protein expression of organic anion transporters in hepatocytes and proximal renal tubule cells from a rat with extrahepatic cholestasis of 21 h. PM: Plasma membranes; MRP2: Multidrug resistance-associated protein 2; OATP1: Organic anion transporting polypeptide 1; OAT: Organic anion transporter; BTL: Bilitranslocase; BBBP: Bilirubin binding protein; ASBT: Sodium dependent bile salt transporter.

and intracellular compartments of the proximal tubular cells. These studies have demonstrated that there is a functional adaptive downregulation of renal ASBT leading to enhance renal clearance of bile acids during the early phase of obstructive cholestasis.

Figure 2 shows a summary of the protein expression of the above mentioned transporters in liver and kidney from rats with extrahepatic cholestasis of 21 h.

CONCLUSION

Altered expression of different transporter proteins of organic anions have been described in liver and kidney from rats with extrahepatic cholestasis. MRP2 and OATP1 abundances were decreased while no changes were detected for BBBP, BTL and OAT3 protein expressions in hepatocytes from BDL rats. On the other hand, in proximal tubule renal cells, increased protein expressions were observed for OAT1, BTL, BBBP; MRP2 and OATP1. In contrast, a diminished abundance of renal ASBT was found in this experimental model to prevent reabsorption of excess bile acids that were eliminated in the urine. This pattern in renal cells may be a compensatory mechanism to increase the elimination of compounds that could not be excreted by the liver in this pathological state.

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Current concepts in hepatic resection for hepatocellular carcinoma in cirrhotic patients

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Abstract

Hepatocellular carcinoma (HCC) is one of the most frequent neoplasms worldwide and in most cases it is associated with liver cirrhosis. Liver resection is considered the most potentially curative therapy for HCC patients when liver transplantation is not an option or is not immediately accessible. This review is aimed at investigating the current concepts that drive the surgical choice in the treatment of HCC in cirrhotic patients; Eastern and Western perspectives are highlighted. An extensive literature review of the last two decades was performed, on topics covering various aspects of hepatic resection. Early post-operative and long-term outcome measures adopted were firstly analyzed in an attempt to define an optimal standardization useful for research comparison. The need to avoid the development of post-hepatectomy liver failure represents the "conditio sine qua non" of surgical choice and the role of the current tools available for the assessment of liver function reserve were investigated. Results of he-

patic resection in relationship with tumor burden were compared with those of available competing strategies, namely, radiofrequency ablation for early stages, and trans-arterial chemoembolization for intermediate and advanced stages. Finally, the choice for anatomical versus non-anatomical, as well as the role of laparoscopic approach, was overviewed. The literature review suggests that partial hepatectomy for HCC should be considered in the context of multi-disciplinary evaluation of cirrhotic patients. Scientific research on HCC has moved, in recent years, from surgical therapy toward non-surgical approaches and most of the literature regarding topics debated in the present review is represented by observational studies, whereas very few well-designed randomized controlled trials are currently available; thus, no robust recommendations can be derived.

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Key words: Hepatocellular carcinoma; Hepatic resection; Surgical therapy; Ablation techniques; Transplantation; Survival; Liver failure

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INTRODUCTION

Hepatocellular carcinoma (HCC), the most common primary malignancy of the liver, represents the fifth most common cancer in men and the seventh in women^[1].

The incidence of HCC varies widely in the different geographic areas according to the regional variations in exposure to risk factors for this tumor^[1-14]. Overall, 75%-80% of HCCs are attributable to chronic hepatitis B virus (50%-55%) or hepatitis C virus (25%-30%) infections^[3-7]. Chronic alcohol abuse, obesity, and diabetes have also been recognized as important risk factors, as well as hereditary hemochromatosis, primary biliary cirrhosis and several hereditary metabolic conditions^[8-14]. In all etiologies there is a male gender predominance^[1,8-14]. Most HCCs ensue in a cirrhotic liver, although the association rate between cirrhosis and HCC may range from 60% up to 90% in relation to the relative prevalences of the risk factors, that greatly differ worldwide^[5].

Risk stratification has been proposed to identify patients who benefit from surveillance for HCC occurrence^[5,13]. Indeed, surveillance can detect the tumor at early stages, amenable to curative treatments. The increasing use of surveillance in clinical practice and the advancements in diagnostic ability achieved in the last decades have greatly improved HCC management and patient survival^[15,16]. Liver resection still remains a mainstay of HCC treatment, being a potentially curative approach not only for early stage HCCs but also for some lesions not amenable to liver transplantation. Thanks to the considerable improvements in surgical techniques and perioperative care, the rates of death and complications after liver resection have remarkably decreased over time, giving added value to this procedure^[17,18]. In addition, the long-term survival after liver resection has been improved by the increased accuracy in detecting recurrences at early stages and the availability of potentially curative approaches even for patients no longer amenable to surgical re-treatment^[19]. The present review examines concepts driving the therapeutic choice for HCC toward hepatic resection in cirrhotic patients, and its results in both the Western and Eastern world.

SELECTING ADEQUATE OUTCOME MEASURES

Early outcome measures

The most feared complication of hepatic resection is the development of post-hepatectomy liver failure (PHLF) which is the main cause of perioperative mortality. Currently, there is no standardized definition of PHLF that allows an unequivocal comparison of results from different studies^[20]. This can explain, at least in part, the great variability of PHLF incidence, ranging from 1.2% to 32%, although differences in populations and surgical procedures may have contributed to this disparity^[21-28]. The analysis of the postoperative course of liver tests demonstrates that serum bilirubin and INR ordinarily return within the normal range on postoperative day 5, including patients who have undergone major resections and those with cirrhosis^[29,30]. A first attempt to standardize the definition of PHLF was made by Balzan *et al.*^[31]. In

an unselected population including patients with primary and secondary liver tumors undergoing elective surgery (12% only with cirrhosis), the authors observed that the association of prothrombin time < 50% and serum total bilirubin > 50 $\mu\text{mL/L}$ (2.9 mg/dL) on post-operative day 5 was a strong predictor of mortality ("50-50 criteria"). Namely, patients who met this criterion had a 59% early postoperative mortality rate, compared with 1.2% found in their counterparts. The usefulness of such a definition is boosted by the selection of simple and objective criteria, while its drawbacks are: (1) the lack of a grading system able to segregate several strata at increasing death risk; and (2) the late identification (only on post-operative day 5) of a category with a huge mortality rate. Thus, PHLF definition needs to be better graded and detected earlier to be of clinical utility.

In 2010, the International Study Group of Liver Surgery performed an extensive literature search and proposed to define post-hepatectomy liver failure as "the impaired ability of the liver to maintain its synthetic, excretory, and detoxifying functions, which are characterized by an increased international normalized ratio and concomitant hyperbilirubinemia (according to the normal limits of the local laboratory) on or after post-operative day 5"^[20]. On the basis of this definition, the severity of PHLF should be graded based on its impact on clinical management (as the Dindo-Clavien classification^[32]): grade A requires no change of the patient's clinical management; grade B needs the clinical management of patients to deviate from the regular course but not to require invasive therapy; grade C claims invasive treatment, for example liver transplantation^[20]. Such a grading system overcomes the 50-50 criteria limits and well depicts the post-surgical course of patients; however, it is not liver specific, also being influenced by non-hepatic complications.

Long-term outcome measures

When selecting endpoints for studies, it should be considered that cirrhotic patients with HCC represent a peculiar oncologic category as their prognosis relies not only on the tumor burden but also on the severity of underlying liver disease. According to the guidelines released by the Panel of Experts in HCC-Design Clinical Trials, overall survival should be considered as the primary end-point for phase III trials^[33]. The document discourages the use of cancer-related mortality since it is a more subjective endpoint, particularly in HCC patients, in whom it can be difficult to ascertain the role played on mortality by the concurrent cirrhosis. Since the high rate of HCC recurrence is the main factor affecting survival after partial hepatectomy, many studies report composite endpoints including this adverse event, such as disease-free survival. However, such a composite endpoint can make the results unreliable because imbalance between groups in deaths, resulting from the natural history of cirrhosis, can mask the real benefit

patients with decompensated cirrhosis reporting a better prognostic accuracy of the MELD score^[50]. Comparative data on MELD and ICGR-15 in the field of hepatic resection are warranted.

Hepatic vein portal gradient

The presence of clinical signs of portal hypertension implies a more advanced liver disease and, consequently, a poorer long-term outcome after hepatic resection. The Barcelona Clinic Liver Cancer (BCLC) group recommends hepatic resection only in patients without clinically significant portal hypertension, i.e. with a hepatic vein portal gradient (HVPG) < 10 mmHg^[13]. This is supported by data obtained in a small cohort (77 patients) studied in the 1990s^[51] and without external validation until recently^[52]. Data from 39 patients, 46% of whom with cirrhosis, undergoing hepatic resection after HVPG measurement showed a higher incidence of post-operative liver dysfunction and ascites in patients with HVPG > 5 mmHg^[52]. However, the small sample size and the proposed HVPG cut-off, that is roughly equivalent to the lowest value observed in cirrhotic patients of 6 mmHg^[53], do not help in clarifying the true usefulness of HVPG in the selection of candidates for hepatic resection. It can be said that the HVPG measurement can probably select surgical candidates, belonging to CTP class A, with a very low probability of post-operative hepatic decompensation; however, the drawback is represented by the exclusion of patients that can still benefit from surgery. In fact, there is growing evidence, coming from large Western^[54,55] and Eastern series^[56], that the presence of clinical signs of portal hypertension does not affect early postoperative and long-term survival in selected patients.

The BCLC group defines clinical signs of portal hypertension as the presence of esophageal varices at endoscopy or splenomegaly (major diameter > 12 cm) with a platelet count < 100 000/mm³ and, for these authors, the detection of these signs should contraindicate hepatic resection^[57]. In keeping with modern Western and Eastern perspectives, the presence of portal hypertension should not be considered an absolute contraindication for hepatic resection in patients with well compensated cirrhosis, belonging to Child-Pugh A or with a MELD score < 10^[54,55]. In fact, complications associated with portal hypertension, such as bleeding from variceal rupture and hemostatic disorders caused by thrombocytopenia, can be safely managed by applying appropriate pre- and peri-operative treatments^[28,58].

Future remnant liver volume and extension of hepatectomy

There is general agreement that, for patients without chronic liver disease, the minimal residual liver volume able to prevent severe postoperative hepatic dysfunction ranges from 20% to 30%^[59]. Conversely, the safe limit for liver resection in chronic liver disease and cirrhosis is

not well established. Very few studies, all published in the 1990s, investigated the prognostic role of future remnant liver (FRL) volume in cirrhotic patients. Shirabe *et al.*^[60] analyzed 80 patients with chronic liver disease (50% cirrhotics) who underwent major liver resection and showed that all liver failure-related deaths occurred in patients with a FRL volume < 250 mL/m². The authors therefore concluded that this FRL volume may be considered the safe limit for major liver resections.

More recently, most studies and treatment algorithms have been focused on the extension of hepatectomy as a surrogate of the FRL volume. A decision tree for hepatectomy procedure, very popular in Japan, has been proposed by Makuuchi *et al.*^[61]. This surgical algorithm has indeed improved the operative mortality and morbidity in HCC patients. The decision tree is based on 3 variables: absence/presence of ascites, serum bilirubin level and ICGR-15. Patients with ascites or high bilirubin level are considered not candidates for hepatic resection. In the remaining cases, the maximal extent of hepatectomy is calculated according to the ICGR-15 value as previously reported. Using this decision tree, a post-operative mortality close to zero has been reported^[61-63]. Thus, the ICGR-15 test might be useful for discriminating good and poor risk CTP A patients. There is also some evidence that the MELD score could be used to guide the extent of hepatectomy^[43]. In particular, data from a Western dual-center study suggest that patients with MELD score < 9 could be safely submitted to major hepatectomy with a risk of PHLF below 1%, and that serum sodium can add some information for cases with borderline MELD values (9 and 10): in the presence of a value < 140 mmol/L, resection should be limited to segmentectomy or wedge resection^[43].

Other liver function tests

Several other quantitative estimations of liver function, based on the hepatic clearance of substrates, have been proposed to predict the outcome of resection. Substrates include lidocaine, galactose, aminopyrine, amino acid and methacetin. Such tests have not been shown to be superior to the ICG clearance test in predicting liver failure or complications after surgery, and have never been compared to HVPG or MELD score^[47].

There is an interesting seminal experience, conducted on 72 patients, regarding the predictive role of transient elastography^[64]. The stiffness cut-off selected by receiver operating characteristics (ROC) curve analysis was 25.6 kPa, which gave the best statistical accuracy (sensitivity 71.4%; specificity 88.6%; positive predictive value 55.6%; negative predictive value 93.9%). It should be noted that the positive predictive value was quite low, whereas the negative predictive value was very high. Thus, liver stiffness would adequately identify patients who will not develop post-operative hepatic insufficiency while it has a suboptimal ability in identifying patients that should be excluded from surgical option because of a high risk of

post-hepatectomy failure. In this series, the area under the ROC curve of liver stiffness measurement was not statistically higher than that of ICGR-15, probably as a consequence of large confidence intervals. Given the increasing interest in elastography in relationship with different outcomes of cirrhotic patients, a possible role in pre-operative evaluation of surgical candidates seems reasonable^[65].

TUMOR STAGE

Cancer staging should serve to estimate the prognosis, select the most appropriate primary and adjuvant therapy for each stage, and assist in comparing results of different treatments or coming from different patient series. Ultimately, an accurate cancer stage can help physicians in managing oncologic patients and scientists in exchanging unambiguous information. According to the European Association for Study of the Liver (EASL) recommendations, a staging system for HCC should take into account four issues: tumour burden, degree of liver function impairment, general condition, and treatment efficacy^[13,15]. Indeed, staging of HCC is complex and, currently, there is no universally accepted staging system^[66]. The consensus conference of the American Hepato-Pancreato-Biliary Association (updated in 2010) re-proposed the use in clinical practice of different systems for different patients^[67]: as survival of early stage patients is greatly modified by treatment, prognostic prediction must include treatment-related variables; conversely, as treatment may not be a key predictor in advanced stages, it may not be a crucial variable of a prognostic index for patients with these tumors. Nowadays, clinicians can choose among several staging systems, although it should be underlined that only the BCLC staging system provides a treatment algorithm linked to the HCC stage.

Early stage tumors

According to the BCLC definition, a very early HCC is represented by single nodule < 2 cm, and an early HCC is a tumor fulfilling the Milan criteria at imaging techniques (one nodule \leq 5 cm or 3 nodules each \leq 3 cm, without vascular or lymph nodal invasion)^[57]. In CTP class A patients, survival after hepatic resection for early HCC reaches 70% at 5 years, and up to 90% for very early HCC^[68]; however, whether to prefer, in these patients, hepatic resection over liver transplantation, or percutaneous treatments such as radiofrequency ablation (RFA), still remains a matter of debate^[69,70]. Thus, the actual role of hepatic resection should be viewed in the light of such competing strategies.

The literature comparing the results of hepatic resection versus RFA for early HCC encompasses a number of retrospective studies, some case-control studies and only two randomized controlled trials (RCT), both coming from the Eastern world^[71,72]. The first one, conducted on 161 CTP class A patients with a solitary tumor \leq 5 cm, reports similar survival rates after surgery (90 pa-

tients) and percutaneous treatments (71 patients), with 4-year survival rates of 68% and 64%, respectively (5-year survival rates were not reported)^[71]. Also, DFS was not affected by the treatment adopted either in the whole population or in the subgroups of patients with tumors < 3 cm and between 3.1 cm and 5 cm^[71]. The second RCT was conducted on 230 patients with HCC meeting the Milan criteria, 6.1% of whom belonging to CTP class B^[72]. The authors found that resection (115 patients) was significantly superior to RFA (115 patients) in terms of both 5-year survival (75.7% *vs* 54.8%, respectively) and 5-year recurrence-free survival (51.3% *vs* 28.7%), and this was confirmed in post-hoc analyzes focused on individuals with solitary HCCs \leq 3 cm, those between 3.1 and 5 cm, as well as with multifocal tumors^[72]. Thus, the two RCTs provide conflicting results making it impossible to propose robust recommendations. Nevertheless, when observational studies are also considered, a trend seems to emerge toward better overall and disease-free survivals after resection. In fact, the 5-year survival rate of surgical patients with early HCC can be estimated to be around 70% while the rate of those submitted to RFA is around 60%; the difference is much more striking for the 5-year DFS, the figures being around 60% and 20%, respectively^[69,70]. However, the considerable heterogeneity among studies regarding both patient selection and results does not make it possible to reach definite conclusions on this topic. Pertinently, it should be noted that a recent multicenter prospective cohort study, in patients with a single tumor \leq 2 cm and potentially amenable to hepatic resection, reported a complete response (without local recurrence) in 97% of cases after RFA, and a 5-year survival rate up to 75%^[73]. In another study considering 104 of these patients, the 5-year survival rate achieved with resection and RFA was excellent (> 80%) and equivalent after correction to the one-to-one propensity analysis model for the confounding factors^[74].

Therefore, it can be said that in patients with early HCC, RFA provides a worse DFS as compared with hepatic resection, so that the need for retreatment is greater. Instead, hepatic resection and RFA would achieve similar results in very early HCCs. However, the drawback of RFA in terms of radicality is somehow counterbalanced by lower mortality, morbidity and costs (shorter hospital stay) and the easy repeatability of ablation. On the other hand, Markov models indicate that in HCC early stages, hepatic resection should be considered in the case of RFA local failure^[75] and that surgery provides better quality of life-adjusted survival, due to the lower risk of local recurrent disease requiring retreatment^[76]. Taken together, these observations suggest that hepatic resection and RFA should be considered as complementary rather than competitive treatments. In cases of deep tumor location, that require a removal of a large volume of parenchyma if resected (i.e., major hepatectomy), it is reasonable to consider RFA as the preferred strategy to adopt; conversely, superficial tumor location or tumors adjacent to main vessels or biliary structures, are

much better managed with hepatic resection. Nonetheless, for many experts, recommending RFA as first-line therapy for resectable small HCCs still requires a higher level of evidence^[77]. Such uncertainty is highlighted by the conclusions of the conference of the Japan Society of Hepatology held in 2009^[78]: to the question “Which treatment would you perform for 2-cm sized HCC nodules in patients with Child-Pugh A liver function?” 80% of surgeons responded “resection”, while 68% of non-surgeons responded “RFA”^[78]. Greater agreement was observed when asking about the optimal treatment of 3-cm sized nodules in patients with Child-Pugh A: 95% of surgeons and 79% of non-surgeons responded “resection”^[78].

As already stated, the role of hepatic resection in early stage HCC should be viewed in the light of competing strategies, and liver transplantation (LT) represents the most attractive alternative option because it removes both detectable and undetectable tumor nodules together with the pre-neoplastic cirrhotic background. However, LT use should be viewed in the context of shortage of available grafts, and decisions must consider, together with the benefit for the individual patient, the collective benefit of all potential liver recipients^[79]. Liver transplantation achieves excellent results in patients with limited tumor burden. Patients with HCC fulfilling Milan criteria have a 5-year survival of about 70%, with recurrence in less than 10%. This survival well matches post-transplant survival of most other indications for LT^[80,81]. This is a critical point, recalled by Recommendation No. 7 of the International Consensus Conference on Liver Transplantation for HCC, held in Zurich in 2010, which states that LT should be reserved for HCC patients who have a predicted 5-year survival comparable to non-HCC patients^[79]. When compared to LT, partial hepatectomy would seem to be inferior in terms of long-term survival, but most surgical series rely on patients who underwent resection of a wide spectrum of tumor extent, frequently beyond the Milan criteria. Notably, factors precluding LT, such as large or multifocal tumors and vascular invasion, are often included in series analyzing resection results, and are associated with early recurrence and shorter survival^[82]. There is evidence that hepatic resection and LT can indeed achieve similar post-operative and intention-to-treat survivals in patients respecting Milan criteria^[82]. Thus, when patients with more limited disease are selected, the results of hepatic resection are much more favorable, approaching the 5-year survival rate of 70% reported after LT^[19,83-85]. It should be considered that this figure is the end-result currently achievable thanks to both improved diagnostic imaging and therapies for recurrences, including salvage LT, that have been shown to significantly prolong survival after partial hepatectomy^[19]. Thus, the combination of resection and salvage LT seems to be a reasonable strategy to adopt for resectable HCC within Milan criteria^[86]. This strategy could also increase the proportion of grafts offered to

non-HCC candidates on the waiting list^[87].

Beyond the early stages of the tumor

Beyond the early stages, there is debate on the ability of the current staging systems in segregating patients into homogeneous prognostic strata able to assist clinicians in selecting the optimal treatment strategy. The BCLC intermediate stage (BCLC-B) includes patients in Child-Pugh class A or B, with multi-nodular or large HCC, and preserved performance status^[57]. This definition includes a very heterogeneous patient population, according to either tumor extent (from bifocal HCC to subtotal tumor replacement of liver parenchyma) or liver function (from perfectly compensated to decompensated cases with ascites and hyperbilirubinemia). The recommended treatment modality for this HCC stage by both EASL and American Association for the Study of Liver Diseases guidelines is trans-catheter arterial chemoembolization (TACE). Instead, due to the heterogeneity of this stage, patients are best served when the treatment decision is individualized and taken within a multidisciplinary team^[88,89]. Indeed, retrospective analyses have shown that, in BCLC stage B patients, hepatic resection yielded better survival rates than TACE^[90-92]. Stage B, but even stage C, patients can tolerate hepatic resection showing low mortality, acceptable morbidity, and survival benefits^[90]. The reported 3-year survival rate ranges from 56% to 74% for stage B and from 28.6% to 67% for stage C patients^[90-92]. Especially in stage B, resection is superior to the TACE in terms of survival^[57,91]. A very recent case-control study, conducted on a population of 603 patients (1:2 ratio), has shown that in patients with a portal vein tumor thrombus (PVTT) within segmental branches (type I) or the right or left portal vein (type II), resection provides a significant survival benefit in comparison to TACE^[93]. In particular, in the presence of type I PVTT, the 5-year survival rate was 37.9% after resection and only 3.6% after TACE; in the presence of type II PVTT, the corresponding figures were 17.2% and 0%, respectively. These results suggest a revision of the BCLC recommendations^[89]. Although the BCLC staging classification has been claimed as standard HCC classification in Western regions, its validation across Eastern and Western regions is required and some refinements are probably needed before it can be accepted for universal application. Indeed, most Asian experts state that the BCLC staging system does not satisfy the needs of surgeons and physicians in real clinical practice^[94]: when participants of the Japan Society of Hepatology were asked if they usually follow the BCLC treatment algorithm, 70% responded “no”^[78]. It should also be noted that resection is not excluded as an option for HCCs beyond the early stages in the Asian treatment algorithms^[94] and in real clinical practice about half the physicians include resection as a treatment choice, albeit in cases of advanced HCC^[79,95].

TYPE OF SURGICAL RESECTION

Anatomical vs non-anatomical resection

It remains unclear whether hepatectomy for HCC should be performed as anatomical resection (AR) or non-anatomical resection (NAR). The great majority of recurrences occur in the liver as a result of subclinical metastases, originating from the primary tumor through microscopic vascular invasion and peripheral spread along their intra-segmental branches, which are the most important factors associated with poor prognosis^[19,96,97]. On this basis, the systematic removal of the hepatic segment fed by tumor-bearing portal tributaries, namely the entire functional unit through an AR, was suggested as theoretically more effective for tumor and metastases eradication^[98]. Conversely, most surgeons prefer, in cirrhotic patients, to leave a portion of parenchyma greater than the functional unit, using the NAR, to reduce the risk of postoperative liver failure. Clear evidence of the superiority of one technique over the other is not available, since some studies report a survival benefit of AR^[99-101] that was not manifest in others^[102-104]. Two recent meta-analyses, conducted on observational studies, also reported conflicting results^[105,106]. Importantly, underlying cirrhosis was significantly more common in the NAR patients who also showed more advanced hepatic dysfunction compared with those in the AR group: these features were recently shown by a meta-regression approach to significantly affect results from meta-analyses, that is, patient survival and DFS after AR seem to be superior to NAR because the worse liver function reserve in the NAR group significantly affects prognosis^[107]. Thus, large randomized controlled trials are needed to define the best resective approach to patients with an HCC ensuing in a cirrhotic liver^[107].

Laparoscopic resection

In contrast to other fields of surgery where laparoscopic procedures are routinely performed, laparoscopic hepatectomy is only being performed in a few institutions worldwide; nonetheless, since the first laparoscopic liver resection was described in 1992, there has been an exponential growth of reported laparoscopic liver resections, with almost 3000 cases reported in the English literature so far^[108]. About 50% of them were performed for malignant tumors and, in this group, about half were HCCs. Since 2000, about 500 cases of laparoscopic resection for HCC can be collected from the literature^[109]. Most patients were cirrhotics, but a considerable proportion (about 40%) had pre-cirrhotic chronic hepatitis. Laparoscopic surgery consisted of minor resections (< 3 segments removed) in 90% of cases^[109]. Complications were more frequent after resection for HCC (50%) than for colorectal metastasis (11%), likely due to underlying liver disease^[108]. The 5-year overall survival ranges from 50% to 60%^[108]. These results are comparable to those achieved with open hepatic resection for HCC but the large proportion of patients without cirrhosis suggests

that studies enrolling only cirrhotic patients are still required to adequately compare outcomes in this specific cohort of patients^[109]. Advantages of laparoscopic liver resections are the less aggressive approach, less peritoneal dissection, less bleeding, minimal ascites and decreased post-hepatectomy liver failure^[110,111], extending the indication to liver resection to selected Child B patients^[110]. Moreover, fewer postoperative adhesions after laparoscopic liver resection compared to open liver resection facilitate subsequent salvage LT with decreased morbidity^[112]. On the other hand, the longer learning curve, the greater difficulty in achieving wide resection margins and performing anatomical resections, the difficulties in mobilization and parenchymal transaction, with risk of massive bleeding, are the major obstacles to the widespread diffusion of laparoscopic liver resection. Lastly, lesions located in posterior segments are not good indications for pure laparoscopic approach but suitable for hand-assisted laparoscopic resection^[113,114].

CONCLUSION

Despite improving results of non-surgical approaches, partial hepatectomy still represents a cornerstone for potentially curative treatment of HCC, able to offer long-term survival rates. However, like all the available treatments for HCC, hepatic resection should be considered in the context of multi-disciplinary evaluation of these patients, which increases the chances to cure the tumor and its recurrences, resulting in higher overall survival rates. As tumor recurrence remains the main obstacle in achieving better results in long-term survival after hepatic resection, clinical trials aimed at identifying effective adjuvant therapies are warranted. Regarding types of surgical approaches to HCC, the literature is rich in observational studies but very few well-designed RCTs are currently available; thus, no definitive suggestions can be derived regarding superiority of anatomic versus non-anatomic resection or laparoscopic approach.

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Eosinophil associated genes in the inflammatory bowel disease 4 region: Correlation to inflammatory bowel disease revealed

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Abstract

AIM: To study the association between inflammatory bowel disease (IBD) and genetic variations in eosinophil protein X (EPX) and eosinophil cationic protein (ECP).

METHODS: DNA was extracted from ethylene diamine tetraacetic acid blood of 587 patients with Crohn's disease (CD), 592 with ulcerative colitis (UC) and 300 healthy subjects. The EPX405 (G > C, rs2013109), ECP434 (G > C, rs2073342) and ECP562 (G > C, rs2233860) gene polymorphisms were analysed, by the 5'-nuclease allelic discrimination assay. For determination of intracellular content of EPX and ECP in granulocytes, 39 blood samples was collected and extracted with a buffer containing cetyltrimethylammonium bromide. The intracellular content of EPX was analysed using an enzyme-linked immunosorbent assay. The intracellular content of ECP was analysed with the UniCAP[®] system as described by the manufacturer. Statistical tests for calculations of results were χ^2 test, Fisher's exact test, ANOVA, Student-Newman-Keuls test, and Kaplan-Meier survival curve with Log-rank test for trend, the probability values of $P < 0.05$ were considered statistically significant.

RESULTS: The genotype frequency for males with UC and with an age of disease onset of ≥ 45 years ($n = 57$) was for ECP434 and ECP562, GG = 37%, GC = 60%, CC = 4% and GG = 51%, GC = 49%, CC = 0% respectively. This was significantly different from the healthy subject's genotype frequencies of ECP434 (GG = 57%, GC = 38%, CC = 5%; $P = 0.010$) and ECP562 (GG = 68%, GC = 29%, CC = 3%; $P = 0.009$). The genotype frequencies for females, with an age of disease onset of ≥ 45 years with CD ($n = 62$), was for the ECP434 and ECP562 genotypes GG = 37%, GC =

52%, CC = 11% and GG = 48%, GC = 47% and CC = 5% respectively. This was also statistically different from healthy controls for both ECP434 ($P = 0.010$) and ECP562 ($P = 0.013$). The intracellular protein concentration of EPX and ECP was calculated in $\mu\text{g}/10^5$ eosinophils and then correlated to the EPX 405 genotypes. The protein content of EPX was highest in the patients with the CC genotype of EPX405 (GG = 4.65, GC = 5.93, and CC = 6.57) and for ECP in the patients with the GG genotype of EPX405 (GG = 2.70, GC = 2.47 and CC = 1.90). ANOVA test demonstrated a difference in intracellular protein content for EPX ($P = 0.009$) and ECP ($P = 0.022$). The age of disease onset was linked to haplotypes of the *EPX405*, *ECP434* and *ECP562* genotypes. Kaplan Maier curve showed a difference between haplotype distributions for the females with CD ($P = 0.003$). The highest age of disease onset was seen in females with the *EPX405CC*, *ECP434GC*, *ECP562CC* haplotype (34 years) and the lowest in females with the *EPX405GC*, *ECP434GC*, *ECP562GG* haplotype (21 years). For males with UC there was also a difference between the highest and lowest age of the disease onset (*EPX405CC*, *ECP434CC*, *ECP562CC*, mean 24 years vs *EPX405GC*, *ECP434GC*, *ECP562GG*, mean 34 years, $P = 0.0009$). The relative risk for UC patients with *ECP434* or *ECP562-GC/CC* genotypes to develop dysplasia/cancer was 2.5 (95%CI: 1.2-5.4, $P = 0.01$) and 2.5 (95%CI: 1.1-5.4, $P = 0.02$) respectively, compared to patients carrying the GG-genotypes.

CONCLUSION: Polymorphisms of EPX and ECP are associated to IBD in an age and gender dependent manner, suggesting an essential role of eosinophils in the pathophysiology of IBD.

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Key words: Eosinophil derived neurotoxin; RNase 2; RNase 3; Single nucleotide polymorphism; Inflammation bowel disease; Crohn's disease; Ulcerative colitis

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INTRODUCTION

The concept of inflammatory bowel disease (IBD) comprises the disorders Crohn's disease (CD) and ulcerative colitis (UC). The aetiology of the diseases is unknown, but IBD is currently believed to develop in individuals

with a hereditary predisposition. This is related to the reaction of the immune system to the bacterial challenge of the gut and/or defects in the gut wall, in addition to external environmental factors. IBD usually has an intermittent course of aggravations with common symptoms such as frequent diarrhoea, blood in stool, abdominal pain, and weight loss. The incidence of IBD has increased in the Western world in recent years, and new cases occur primarily in the group between 20 and 40 years of age^[1,2]. CD seems to affect women more than men, while UC appears to be more prevalent among men, but some studies showed no gender differences^[1,3,4]. A bimodal age distribution of disease onset has been reported and debated in population studies of CD and UC^[2,5-8]. Patients with CD and UC have an increased risk of developing colorectal cancer. This risk is more pronounced in patients with UC with an overall 2.7-fold increased risk as compared with the general population^[9].

Under normal conditions, the intestinal mucosa is in a state of "controlled inflammation". Different subsets of T-cells are present in the healthy intestine, as are eosinophils and antigen-presenting cells with a predominance of anti-inflammatory and regulatory cytokine responses that keep the mucosal homeostasis intact^[10]. However, in IBD the balance between mucosal responsiveness and tolerance towards antigens is disturbed, resulting in an exaggerated immune response to the commensal flora. It is well established that neutrophil granulocytes accumulate and infiltrate in the local processes in IBD. In recent years attention has been paid to the involvement of eosinophil granulocytes, since increased numbers of these cells are found in the intestinal mucosa of IBD^[11,12]. Studies now emphasise a key role of the eosinophil granulocyte in the pathophysiology of IBD^[13,14].

The eosinophil participate in a number of biological processes such as wound healing, defence against parasites and allergic inflammation^[15]. The eosinophil granulocyte are characterised by specific cytoplasmic granules containing four major proteins; eosinophil cationic protein (ECP)^[16], eosinophil peroxidase (EPO)^[17], eosinophil protein X (EPX)/eosinophil derived neurotoxin^[18], and major basic protein (MBP)^[15]. Increased intraluminal release of EPX, ECP and EPO has been shown in patients with UC^[19]. The cytotoxic activities of ECP and EPX as well as their potent RNase activities have been well documented^[20] and are generally conceived as part of the host defence against viruses, parasites and helminths^[21,22]. Furthermore both EPX and ECP have been associated with different types of cancer^[23-25]. The presence of eosinophils in the intestinal mucosa of healthy subjects may reflect these host defence activities of the cell. In addition EPX has been identified as a member of the Alarmin family with the capacity to affect various aspects of the dendritic cells^[26], and in aged asthma patients, relative to young, the degranulation of EPX was decreased^[27]. Larger numbers of activated eosinophils, identified as an increased expression of the cell surface receptor CD44, have been reported both in CD and UC, but even more

pronounced in quiescent than in active UC. Findings that might suggest a role of eosinophils in tissue remodelling and repair in IBD^[28,29].

By linkage analysis and genome-wide association studies many genetic variations, linked to IBD, have been found^[30]. The nucleotide sequences of EPX and ECP have a homology of 90%, and both genes have been located to chromosome 14q11.2^[31,32] within the IBD4 locus, located at the 14q11-12 region^[33]. Several single nucleotide polymorphisms (SNPs) were reported in the genes for EPX and ECP^[34]. An intron SNP in the EPX gene, EPX405 G > C (rs2013109) was together with a SNP from the 3' untranslated region in the ECP gene, ECP562 G > C (rs2233860)^[35], shown to be closely linked to the eosinophil content of EPX and ECP (Jönsson *et al.*^[35], to be published). The C-allele of the nonsynonymous missense ECP434 G > C (rs2073342) polymorphism, gives rise to an arginine to threonine shift at amino acid position 97 in the mature protein^[34,36]. As shown with recombinant ECP proteins this amino acid shift resulted in an alteration of the protein and its cytotoxic activity^[37], but with no effect on its RNase activity^[38]. The loss of cytotoxicity of ECP containing threonine at position 97 was confirmed using purified native proteins from genotyped blood donors^[37]. The ECP434(G > C) polymorphism was shown to be associated with the expression of allergic symptoms^[39], and with disease severity in Hodgkin lymphoma^[39] in population based studies as well as to the prevalence and severity of *Schistosoma mansoni* infection in a Ugandan population^[40].

The aim of this study was to investigate the impact of SNPs in the EPX and ECP genes in a cohort of patients with IBD. The hypothesis was that alterations in the cytotoxic activities of ECP and/or the altered expression of EPX might affect the propensity to acquire IBD and also might affect different features of IBD. The influence of polymorphisms in the EPX and ECP genes was first analysed in a pilot cohort of patients with IBD from Uppsala University Hospital and then in a second step in a bigger Swedish cohort. The possible altered expression of EPX was in addition analysed by measuring the intracellular eosinophil content of EPX in a subset of the patients.

MATERIALS AND METHODS

Study population

The patient group consisted of 1179 individuals recruited from Uppsala University Hospital, Sweden (95 CD and 100 UC), and an established Swedish cohort recruited at Karolinska University Hospital and Örebro University Hospital (492 CD and 492 UC). Diagnoses of CD and UC was based on clinical, histological and endoscopic findings, according to standardized criteria^[41]. Bimodal patterns as to age of disease diagnosis were observed for both CD and UC irrespective of gender (Figure 1). The major peak of age at diagnosis of both diseases was seen at 20-25 years, with a second peak around 40-50 years.

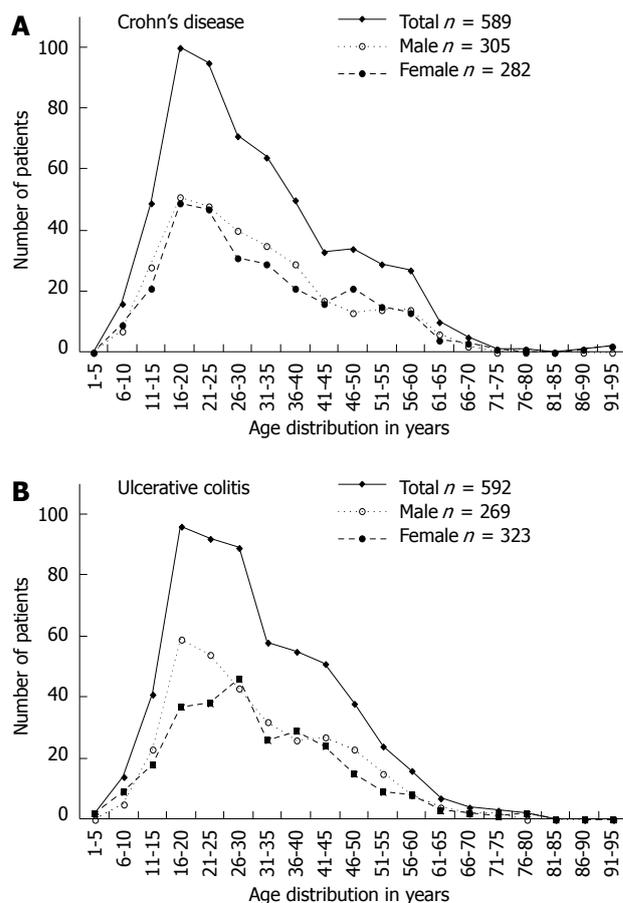


Figure 1 Incidence curves, given as a mean of 5 consecutive years, are shown for the inflammatory bowel disease study cohort. Curves separated by gender and displaying the total inflammatory bowel disease cohort are included.

A group of 300 healthy Swedish blood donors from the Uppsala blood bank served as reference population.

The study was approved by the local Ethics Committees of the Medical Faculties, of Uppsala University and Karolinska University Hospital, Stockholm, and all patients gave their informed consent to participate in the study (Table 1).

DNA extraction

Genomic DNA from all IBD patients and the reference group were extracted from whole ethylene diamine tetraacetic acid (EDTA) blood, by the use of the QIAamp DNA Blood Mini Kit from QIAGEN (QIAGEN Inc, Valencia, CA, United States), according to the manufacturer's description.

Genotyping

All IBD samples and controls were genotyped for the EPX405 (G > C), ECP434 (G > C) and the ECP562 (G > C) polymorphisms by use of the 5'-nuclease allelic discrimination assay. The assay has previously been described in detail^[42]. Oligonucleotide primers were designed by the use of Primer Express[®] (Applied Biosystems, Foster City, CA, United States) based on the se-

Table 1 Clinical characteristic of study populations

	Crohn's disease (n = 587)	Ulcerative colitis (n = 592)	Reference group (n = 300)
Female	282	269	132
Male	305	323	168
Median age ¹	48 (18-95)	48 (14-102)	44 (19-73)
Age at disease diagnosis ²	28 (6-93)	28 (4-78)	

¹Range at sampling; ²Median (range).

Table 2 Primers and probes

EPX405 (G > C):	
Forward primer	5' AAG AGA GCT GAC GTT AGT GCT TAG G 3'
Reverse primer	5' GGT CTT GGT TAT GAC ACA CAC TGT AGT 3'
Probe 1	5' VIC-ACG TTG CAC ACT TT 3'
Probe 2	5' 6-FAM-CGT TCC ACA CTT TG 3'
ECP434 (G > C):	
Forward primer	5' GAG TAG ATT CCG GGT GCC TTT ACT 3'
Reverse primer	5' CGT GGA GAA TCC CGT GGA T 3'
Probe 1	5' VIC-AAA CTG CAG GTA TGC AGA 3'
Probe 2	5' 6-FAM-AAA CTG CAC GTA TGC AG 3'
ECP562 (G > C):	
Forward primer	5' GGT TCC AGT TCA CCT GGA TAC C 3'
Reverse primer	5' GGT ATG GAG ACT GAT GAG GAC AGT 3'
Probe 1	5' VIC-TCA GCA GTC CTC ATC 3'
Probe 2	5' FAM-TCA GCA CTC CTC ATC 3'

EPX: Eosinophil protein X; ECP: Eosinophil cationic protein.

quence of the *EPX* (X16546) and *ECP* (X16545) genes available in GenBank. Sequences for primers and probes are displayed in Table 2.

Polymerase chain reaction (PCR) cycling was carried out in an ABI PRISM 7000 (Applied Biosystems) with the recommended thermal profile of: 50 °C for 2 min, 95 °C for 10 min followed by a total of 40 cycles of 95 °C for 15 s and 60 °C for 1 min. After the PCR cycling the genotypes were determined according to the application Allelic Discrimination of the ABI Prism 7000 SDS software (Applied Biosystems).

Blood cell count

Blood eosinophil count (B-Eos) (reference interval 0.0 × 10⁹/L-0.5 × 10⁹/L) and total white blood cell count (reference interval 3.5 × 10⁹/L-9.0 × 10⁹/L) were determined by an automated haematology analyser (CellDyn Sapphire; Abbott Laboratories, CA, United States) at the Department of Clinical Chemistry, Uppsala University Hospital, Uppsala, Sweden.

Whole blood extraction

The EPX and ECP polymorphisms were first only analysed in the Uppsala cohort. There was a difference in the EPX content between CD and UC patients, which lead to an interest in measuring the intracellular content of EPX and ECP in eosinophils. For the determination of intracellular content of EPX and ECP in granulocytes, EDTA blood was collected from 39 patient samples with

Table 3 Genotype and allelic frequency distribution among 300 healthy individuals and the total cohorts of Crohn's disease and ulcerative colitis

	GG- genotype (%)	GC- genotype (%)	CC- genotype (%)	G-allele (%)	C-allele (%)
Healthy					
ECP562	68	29	3	83	17
ECP434	57	38	5	76	24
EPX405 M ¹	64	32	4	80	20
EPX405 F ¹	53	38	9	72	28
CD					
ECP562	64	33	4	80	20
ECP434	53	40	7	73	27
EPX405 M	55	38	6	74	26
EPX405 F	58	34	8	75	25
UC					
ECP562	64	33	4	80	20
ECP434	55	38	7	74	26
EPX405 M	62	31	7	78	22
EPX405 F	57	38	5	76	24

For ECP434 and ECP562 there was no difference between males and females. ¹In EPX405 there was a difference between the sexes according their genotype ($P = 0.049$) and allelic distribution ($P = 0.021$). UC: Ulcerative colitis; CD: Crohn's disease; EPX: Eosinophil protein X; ECP: Eosinophil cationic protein; F: Female; M: Male.

CD. The subjects were selected based on their *EPX405* (G > C) genotypes (GG = 15, GC = 16, and CC = 8). The method for whole blood extraction using cetyltrimethylammonium bromide has previously been described in detail (Jönsson *et al.*³⁵, to be published). The supernatants were stored at -20 °C until assayed for EPX and ECP concentrations.

Protein assays

EPX whole cell extract was analysed using an enzyme-linked immunosorbent assay (Diagnostics Development, Uppsala, Sweden), according to the recommendations of the manufacturer with minor modifications. The concentration of ECP was analysed with the UniCAP[®] system, (Phadia AB, Uppsala, Sweden) as described by the manufacturer.

Statistical analysis

All the statistical analyses were performed with the Statistica 9.0 software for Windows (StatSoft, Tulsa, United States), and MedCalc software (V.10, Mariakerke, Belgium). The statistical tests in use for the calculations of results were χ^2 and Fisher's exact test, ANOVA, Student-Newman-Keuls test, and Kaplan-Meier survival curve with the Log-rank test for trend. $P < 0.05$ were considered statistically significant.

RESULTS

Genotype distributions in healthy population vs patients with IBD

The distribution of the *EPX405*, *ECP434* and *ECP562* genotypes was investigated in the population of 1179

Table 4 Gender specific difference

		Crohn's disease						Ulcerative colitis						
		Genotype distribution			Allelic frequency			Genotype distribution			Allelic frequency			
	Age at diagnosis (yr)	Patients CD/UC (n)	EPX	ECP	ECP	EPX	ECP	ECP	EPX	ECP	ECP	EPX	ECP	ECP
Female	≥ 40	80/71	NS	NS	NS	NS	0.043	0.034	NS	NS	NS	NS	NS	NS
	≥ 45	42/62	NS	0.009	0.013	NS	0.004	0.007	NS	NS	NS	NS	NS	NS
Male	≥ 40	77/85	NS	NS	NS	NS	NS	NS	0.048	0.025	0.008	0.026	0.024	0.045
	≥ 45	57/52	NS	NS	NS	NS	NS	NS	NS	0.01	0.009	NS	NS	NS

Difference (χ^2 , *P*-values displayed) between the genotype distributions and allelic frequencies, related to age at diagnosis, in individuals with CD or UC compared with the healthy population. UC: Ulcerative colitis; CD: Crohn's disease; NS: Not significant; EPX: Eosinophil protein X; ECP: Eosinophil cationic protein.

subjects with CD ($n = 587$) or UC ($n = 592$) (Table 1).

For comparison the distributions of the same genotypes were investigated in a group of apparently healthy blood donors ($n = 300$). In the healthy reference population males had a higher prevalence of the *EPX405* GC-genotype ($P = 0.049$) and the G-allele ($P = 0.021$) compared to females (Table 3). All continuing data has been calculated gender stratified for the *EPX405* genotype. No difference was seen between genders for the *ECP434* and *ECP 562* genotype in the control cohort (Table 3). No genotype differences were seen between younger ($n = 150$; median age: 31 years; range: 19-43 years) and older ($n = 150$; median age: 54 years; range: 44-73 years) healthy references. At first only the Uppsala cohort with CD ($n = 95$) and UC ($n = 100$) was examined. Here there was a difference between patients with CD and UC ($P = 0.048$) with more *EPX405* GC and CC in CD. These results could not be verified in the whole cohort where no general differences were found between the healthy and the patient cohorts, or between the CD and the UC cohorts, for any of the genotypes. When dividing the patient cohorts by their median (28 years) age at disease diagnosis (ADD) and comparing them, no difference was seen in any of the genotypes for younger *vs* older patients with CD (median ADD: 20 years; range: 6-28 years *vs* median ADD: 40 years; range: 28-93 years) or UC (median ADD: 20 years; range: 4-28 years *vs* median ADD: 40 years; range: 28-78 years). In the total cohort, no difference was seen in the genotype frequencies related to gender in patients with CD or UC. When the patient populations were separated by age at disease diagnosis, according to the Montreal classification^[43] (< 16 years, 17-40 years and > 40 years) and gender, a pattern with significant differences was observed. Since the incidence curve (Figure 1) of women with CD indicated a second peak of disease onset around 45 years the age range > 45 years was also included in the study. Thus, a lower prevalence of the *ECP434* and *ECP562-GG* genotypes and higher prevalence of the *ECP434* and *ECP562-GC* genotypes were found in female patients with CD, compared to the healthy reference population (Table 4). In males similar differences in the prevalence of the *ECP434* and *ECP562* genotypes were observed, but only in patients with UC (Table 4).

For both genders the genotype differences were observed in the older cohort of the population with an age at diagnosis above 40 years (Table 4). In the cohort of males with UC a higher prevalence of the *EPX405* GC-genotype, compared to healthy controls, was observed in patients with an ADD of > 40 years ($P = 0.048$).

Genotype distribution within different cohorts

Among females with CD and ADD of > 45 years, the GC-genotype of the *ECP434* ($P = 0.020$) and *ECP562* ($P = 0.014$) gene polymorphisms were more common than in females with ADD < 45 years. In females with UC, no such differences were observed. In males with UC there was a difference between those with an ADD over and under 40 and 45 years, concerning all three genotypes (40 years; *EPX405* $P = 0.003$, *ECP434* $P = 0.003$ and *ECP562* $P = 0.004$). The differences were, in all genotypes, due to a higher amount of the GC-genotypes among males > 40 years and > 45 years.

In Figure 2, a comparison of the *ECP434* genotype distributions between patients with CD and UC and an age of disease diagnosis of > 45 years is shown. In the comparison the population is separated by gender. Among female patients with CD there was a significantly lower prevalence of the *ECP434GG* genotype and a prevalence of the heterozygote genotype almost twice as high as in those women having UC ($P = 0.010$). This was contrasted by the findings in males, since those patients having CD had a predominance of the *ECP434 GG*-genotype, as compared to patients with UC, who had a predominance of the GC-genotype ($P = 0.004$). Highly significant differences were found in *ECP434* genotype distributions between females and males (> 45 years) with CD ($P = 0.001$) and with UC ($P = 0.001$). Similar patterns were found for the *ECP562* genotypes, but not for the *EPX405* genotypes (not shown).

Relationships of age at disease diagnosis to genotypes and haplotypes

The relationships of the *EPX405*, *ECP435* and *ECP562* genotypes and haplotypes (Table 5) to ADD (females with CD and males with UC) are illustrated in Figure 3. The haplotypes analysed were chosen to include the wild type (WT) (haplotype 1; GG in all three genotypes) and

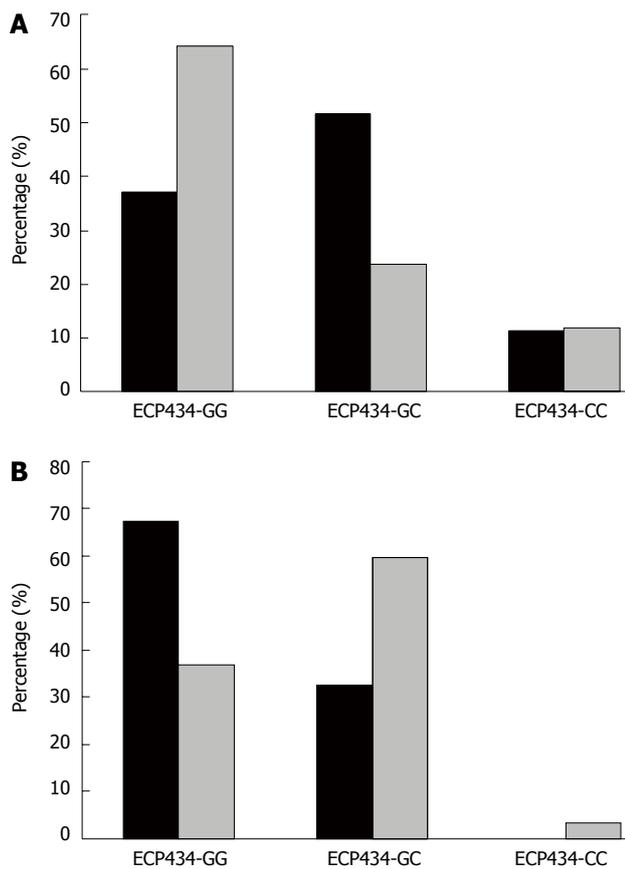


Figure 2 Prevalence of *ECP434* genotypes in Crohn's disease (black bars) and ulcerative colitis (grey bars). A: In females, age at disease diagnosis (ADD) > 45 years, a significant difference between Crohn's disease (CD) and ulcerative colitis (UC) concerning *ECP434* ($P = 0.01$, χ^2) was observed; B: In men, ADD > 45 years a significant difference between CD and UC concerning *ECP434* ($P = 0.004$) was observed. ECP: Eosinophil cationic protein.

the opposite extreme with CC in all genotypes (haplotype 27). The criterion for other haplotypes to be included was to differ from the WT and to contain at least 10 individuals in the group. We found significant associations between the respective genotypes and ADD for men with UC; *EPX405* ($P = 0.007$), *ECP434* ($P = 0.002$) and *ECP562* ($P = 0.010$) with the lowest ADD seen in those carrying the CC-genotypes and the highest ADD in those carrying the heterozygous genotypes (Figure 3C-E). To attain a better understanding of the significance of the genetic diversity of the polymorphisms in the *EPX* and *ECP* genes, four haplotypes were related to ADD. The haplotypes analysed in men with UC were haplotype 1, haplotype 13, haplotype 14 and haplotype 27 (Table 5). For females with CD the haplotypes analysed were haplotype 1, haplotype 4, haplotype 13 and haplotype 27 (Table 5).

As shown in Figure 3A, ADD differed significantly depending on haplotype ($P = 0.0009$) in men with UC and in females with CD ($P = 0.003$) (Figure 3B). In the female cohort extreme outliers (ADD > 80 years) were excluded from the analysis. The ADD was markedly

Gender	Haplotype	EPX405	ECP434	ECP562	Frequency %
Males with UC	1	GG	GG	GG	48
	13	GC	GC	GG	5
	14	GC	GC	GC	20
	27	CC	CC	CC	3
Females with CD	1	GG	GG	GG	46
	4	GG	GC	GG	6
	13	GC	GC	GG	5
	27	CC	CC	CC	5

UC: Ulcerative colitis; CD: Crohn's disease; EPX: Eosinophil protein X; ECP: Eosinophil cationic protein.

lower in males with UC who carried the haplotype 27 as compared to those who carried the haplotype 13. Almost reverse results were obtained in females with CD since ADD in those carrying haplotype 13 was the lowest and haplotype 4 the highest.

Intracellular protein content

As noted in the introduction the eosinophil content of the alarmin *EPX* is associated to the *EPX405* genotypes in healthy and allergic subjects. We therefore investigated the association of the cellular content in a selected subset of patients with CD from the Uppsala cohort. The eosinophil content of *EPX* was closely linked to the *EPX405* genotype with the highest content in the subjects carrying the *EPX405CC* genotype (ANOVA $P = 0.009$) (Figure 4). The eosinophil content of *ECP* was also linked to the *EPX405* genotype (ANOVA $P = 0.022$), but in a reverse mode (Figure 4).

Relationship to dysplasia/cancer

In the study UC cohort there were several patients ($n = 27$; females: 8; males: 19) that through continuous endoscopic examination were shown to have developed dysplasia and/or cancer (D/C) (median age at cancer diagnosis: 49 years; range: 27-67 years). The D/C cohort was compared to all UC patients, females and males who had not developed D/C, since no difference was shown between older and younger UC patients in the mixed cohort. The distributions of the *ECP434* and *ECP562* alleles in patients with UC, with and without findings of D/C of the intestine are shown in Figure 5. The C-allele of both genotypes was more prevalent among patients with D/C (*ECP434* $P = 0.034$ and *ECP562* $P = 0.019$). The D/C patients also differed from the healthy controls (*ECP434* $P = 0.027$ and *ECP562* $P = 0.007$) (Figure 5). The relative risk (RR), for UC patients with the *ECP434* GC or CC genotypes, to develop D/C was 2.5 (95%CI, 1.2-5.4) compared to other patients with UC. In patients with the *ECP562* GC or CC genotypes the RR was 2.5 (95%CI, 1.2-5.8) as compared to UC patients. Considering the whole cohort, no associations to the *EPX405* (G > C) genotypes were detected.

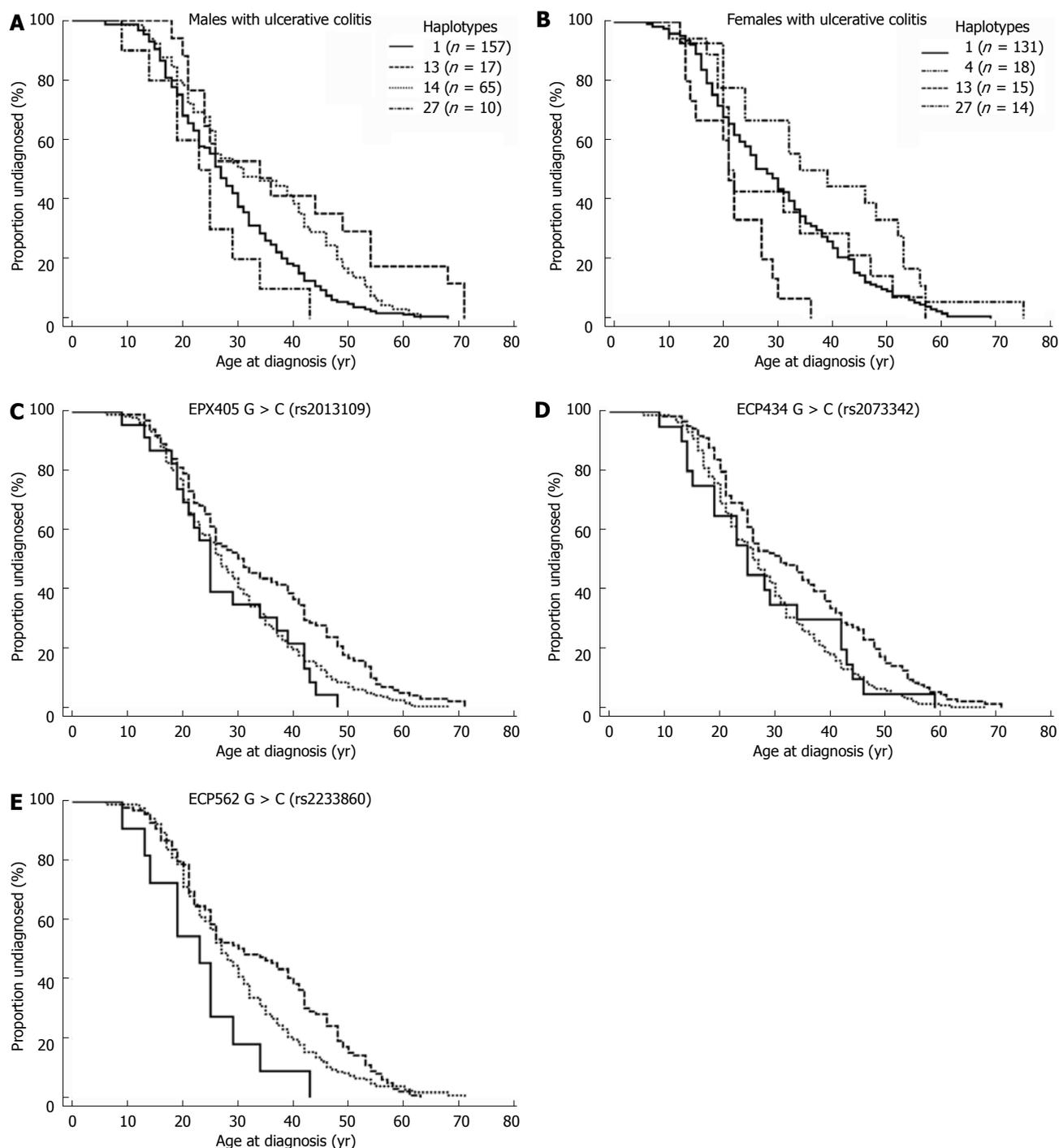


Figure 3 Kaplan-Meier Curve of four groups haplotypes and *EPX405*, *ECP562* genotypes. A: Kaplan-Meier Curve of four groups of haplotypes in male patients with ulcerative colitis (UC) ($P = 0.0009$); B: Kaplan-Meier Curve of four groups of haplotypes in female patients with Crohn's disease ($P = 0.003$); C: Kaplan-Meier curve of the *EPX405* genotype in men with UC ($P = 0.007$); D: Curve of the *ECP434* genotype in men with UC ($P = 0.002$); E: Curve of the *ECP562* genotype in males with UC ($P = 0.01$). Dotted line: GG genotype; Dashed line: GC genotype; Continuous line: CC genotype. ECP: Eosinophil cationic protein; EPX: Eosinophil protein X.

DISCUSSION

Inflammatory bowel disease, including CD and UC, are idiopathic diseases with chronic inflammation of the gut. A key role of the eosinophil in the pathophysiology of IBD has been suggested. Our findings in this report, of intriguing associations between gene polymorphisms of the two major eosinophil granule proteins ECP and EPX,

emphasize this notion. The primary results gained from the Uppsala cohort gave a hint that there was some association between the EPX and ECP polymorphisms and IBD. The associations, however, were in the larger cohort mainly found when the patients were separated into gender, age groups and diagnosis, since the associations were most apparent in older women with CD and in older men with UC. Another interesting observation was the higher

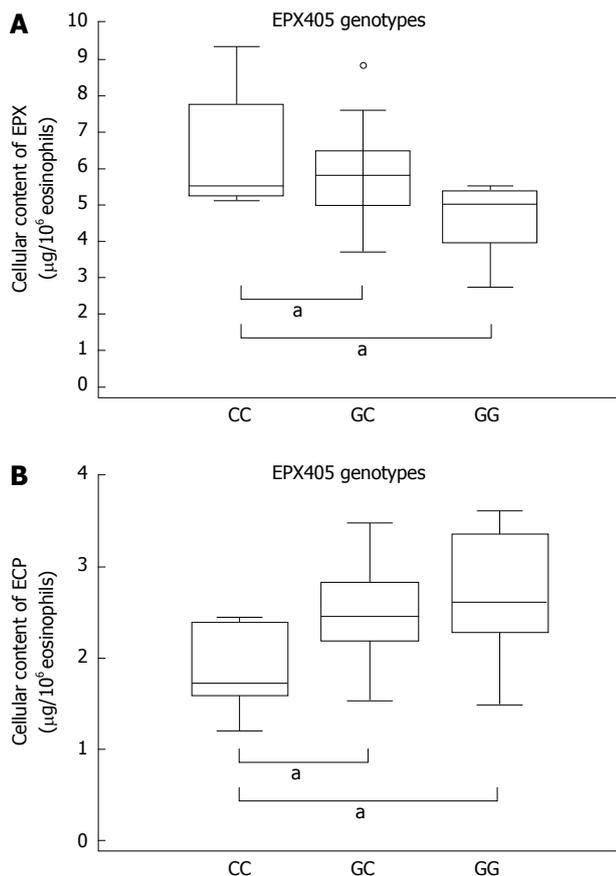


Figure 4 The box plots displays the association between the *EPX405* genotypes and the intracellular content of eosinophil protein X and eosinophil cationic protein in eosinophils. The box plots display the distribution of the protein content within each class of genotype. A: Intracellular content of eosinophil protein X (EPX) (ANOVA $P = 0.009$). $^aP < 0.05$ vs *EPX405* GG genotype (Student-Newman-Keuls test); B: Intracellular content of eosinophil cationic protein (ANOVA $P = 0.022$). $^aP < 0.05$ vs the *EPX405* CC genotype (Student-Newman-Keuls test).

prevalence of the *ECP434*C-allele in those patients with dysplasia/cancer, since this allele codes for the non-cytotoxic variant of ECP. We also confirmed the relationship between the *EPX405* (G > C) polymorphism and the eosinophil content of EPX, since an increased content of EPX was found in subjects carrying the C-allele. The C-allele was also related to the age of disease diagnosis suggesting an impact of the alarmin EPX on the course of the disease in IBD.

In our study, we show that older females with CD had a relatively higher amount of the *ECP434* GC and *ECP562* GC genotypes as compared to healthy individuals, and younger females with CD. Our novel findings show that in healthy individuals and females diagnosed with CD at a young age the GG genotype of *ECP434* and *ECP562* was more dominating than in older women with CD, implying a more cytotoxic ECP and a higher content of ECP. This interesting finding could have a bearing on granuloma formation in CD. Granuloma formation is one of the pathological hallmarks of CD and is detected in the gut wall in 37%-60% of the patients^[44]. Patients with granulomas, and particularly females, are

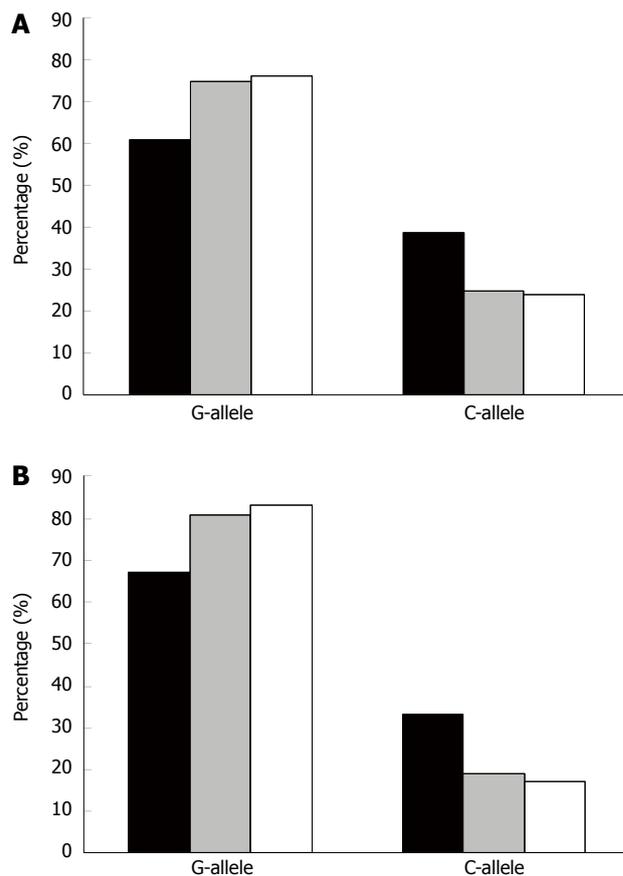


Figure 5 Distribution of allele frequencies, displaying the difference in patients with ulcerative colitis and dysplasia/cancer. Bars represent the allele frequency (%) of *ECP434* (A) and *ECP562* (B) single nucleotide polymorphism in patients with dysplasia/cancer (black bar), all patients with ulcerative colitis (grey bar), and healthy individuals (white bar). ECP: Eosinophil cationic protein.

diagnosed early in their course of disease^[45]. In CD, the production of tumor growth factor (TGF)- β appears critical in CD-associated fibrosis and in the formation of granulomas^[46,47]. ECP is known to promote TGF- β production and migration of fibroblasts^[48,49] and the *ECP434* GG genotype has been linked to increased fibrosis^[40]. All together this suggests a role of ECP in the production of granulomas in younger females with CD. Furthermore high numbers of bacteria, and mainly gram negative bacteria as *Escherichia coli* (*E. coli*), can induce secretion of ECP^[50]. By the use of laser capture microscopy and PCR it was shown that DNA from *E. coli* was present in 80% of granulomas from patients with CD^[51].

There are few studies associating genetic risk factors and gender to falling ill with IBD. For patients with UC, there is a gender-specific risk associated with the interleukin-10 promoter. Females with a particular genotype and haplotype have an increased risk of contracting the disease earlier in life^[52]. In patients with CD the DLG5 R30Q variant was shown to be associated with an increased risk for male patients to develop CD. This risk has been described both in the paediatric^[53] and the adult age group^[54]. We showed that age at diagnosis in females with CD is linked to the *EPX405* genotype. Females

with haplotype 13, including EPX405 GC, are diagnosed earlier in life. The longest span of years before onset of disease was observed in females carrying haplotype 4, including EPX405 GG. The C-allele of EPX405 is linked to a higher content of EPX in the eosinophils. The increased availability of EPX (in combination with unchanged amount of ECP) may result in an earlier disease onset in life. EPX was previously shown to present characteristics of an endogenous alarmin, which strengthens the immune systems Th2 immune response^[26]. CD has often been regarded as a disease with a dominant Th1 immune response, but a Th2 response in the early stages of the disease with active ileitis has been shown^[55,56]. EPX uses the toll-like receptor 2 (TLR2)-MyD88 signal transduction pathway to induce dendritic cell maturation and activation of NF- κ B^[26] and also acts as a chemotactic activator of dendritic cells^[57]. Few of the intestinal dendritic cells in healthy controls express TLR2, whereas in patients with IBD the TLR2 receptor density is increased^[58]. NOD2 also induces nuclear factor κ B (NF- κ B) activation through the MyD88 pathway^[59]. The potentiated activation of NF- κ B through the co-operation of EPX and NOD2 in CD, therefore, is an interesting possibility and may explain the differences found between CD and UC, since the 405C-allele was more common in CD in patients with shorter onset of disease.

In the cohort of older men with UC, the *ECP434* and *ECP562 GC* genotypes were more common than in men with CD, females with UC, younger men with UC and the healthy population. Compared to younger men and the healthy reference population the *EPX405* genotype was also significantly different, with more GC genotype in the older cohort. Thus, older men had a lower content of less cytotoxic ECP and a higher content of EPX. These results are similar to the cohort of older females with CD. Thus, from a genetic point of view, females with CD show similar distributions of the ECP and EPX SNPs as men with UC. These are interesting findings and suggest major gender differences in the pathogenesis of the two diseases.

In men with UC the Kaplan Meier analysis showed the lowest ADD in subjects carrying haplotype 27, (*EPX405 CC*, *ECP434 CC* and *ECP562 CC*) which codes for elevated production of EPX, less cytotoxic ECP and reduced production of ECP protein. The highest ADD was seen in those carrying haplotype 13 (*EPX405 GC*, *ECP434 GC* and *ECP562 GG*), which codes for increased production of EPX and cytotoxic ECP. These findings suggest a protective role of ECP, putatively against the microbial challenge, in the development of UC, whereas the role of the increased availability of EPX may be involved in the initiation of the disease. In contrast to the findings in men with UC, haplotype 13 was associated with the lowest ADD in women with CD, which should result in the increased production of cytotoxic ECP. As noted above this finding may relate to early granuloma formation. Our findings emphasize major differences in the pathophysiology and role of eosinophils

in CD and UC. A notion supported by previous studies showing differences in the morphology and function of eosinophils in CD and UC^[12,27].

Our findings of an association between the *ECP434* and 562 SNPs and UC with complication of dysplasia/cancer suggest a role of ECP in the defence against malignant cell transformation, since the results indicate a lower production of ECP and that the produced protein is non-cytotoxic and unable to kill malignant cells^[37]. The relative risk for a patient with UC and a C-allele in *ECP434* or 562 to develop D/C is 2.5. When separating the cohort due to gender the *EPX405* genotype was also significantly associated to D/C in males (data not shown). It is noticed that the number of patients with D/C was limited in our cohort, but recent studies have pointed out the association of ECP and EPX and cancer^[23-25].

It is important to remember that the polymorphisms *EPX405*, *ECP434* and *ECP562* are in linkage disequilibrium (Jönsson *et al* to be published). Because of the profound linkage disequilibrium between the alleles, it is difficult to determine which allele that is the determining allele.

In conclusion, the present study has identified a gender and age related difference between polymorphisms in the *EPX* and *ECP* genes in CD and UC, and showed that the haplotype distributions are associated to the age of disease diagnosis. We also confirmed a link between the eosinophil content of *EPX* and the *EPX405* gene polymorphism. Finally our results suggested a link between polymorphisms in the *ECP* gene and the risk in patients with UC of developing dysplasia/cancer. The role of neutrophils in IBD is a hallmark of the disease, and the results presented in this study suggest a key role of eosinophil granulocytes and their major granule proteins in the pathophysiology of inflammatory bowel disease.

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COMMENTS

Background

A role of eosinophils is implicated in inflammatory bowel disease. The intraluminal release of eosinophil derived cytotoxic proteins is increased in patients with ulcerative colitis (UC). Larger numbers of activated eosinophils, identified as CD44-high, are seen in quiescent as opposed to active UC. Gene polymorphisms in eosinophil protein X (EPX)/eosinophil derived neurotoxin and eosinophil cationic protein (ECP) affect the eosinophil content of EPX and ECP.

Research frontiers

The cytotoxic activities of ECP and EPX as well as their potent RNase activities have been well documented and are generally conceived as part of the host defence against viruses, parasites and helminths exerted by eosinophils. Polymorphisms in the genes of EPX and ECP are known to be linked to changes of cytotoxic affect and protein content. Increased release of EPX and ECP has been shown in patients with UC.

Innovations and breakthroughs

There are no previous studies focusing on the genetics of the eosinophil cationic proteins and inflammatory bowel disease (IBD). Studies of IBD and genetics in a gender and age dependent manner are few. With the present study, the

authors have combined the two, enhancing the relation between the eosinophils and IBD, and revealing the possible risk for cancer.

Applications

The study results have helped to attain an increased knowledge of the eosinophils role in IBD with focus on age and gender related differences. The results also suggest that the determination of the EPX and ECP genotypes may contribute in patients risk stratification for cancer development.

Terminology

Eosinophil granulocyte: the eosinophil granulocyte is a pro inflammatory cell. The cell participates in wound healing and host defence against parasites and helminths. The cell is also present in the process of the allergic inflammation.

Eosinophil cationic proteins: the cationic proteins are granula proteins secreted by activated eosinophils. The proteins are eosinophil specific and are therefore often used as markers for the eosinophil activities. Single nucleotide polymorphism (SNP): this is a variation in the DNA sequence, occurring when a single nucleotide (A, C, G or T) in the genome, differs between paired chromosomes in an individual or in between individuals. If a SNP is present in the coding region of a gene this may result in an amino acid change, influencing the phenotype of a specific gene.

Peer review

The authors of this paper report that polymorphisms in the EPX and ECP genes are associated with IBD and contribute to the risk for the disease development in a gender-specific manner. Furthermore, these polymorphisms seem to be specifically related to the late-onset disease and to increased risk for colorectal cancer in UC patients. The results of this large study are certainly interesting, especially with the attention paid to gender-and age-stratified analysis which is a typical shortcoming of most of the genetic studies in IBD.

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Glucose-responsive artificial promoter-mediated insulin gene transfer improves glucose control in diabetic mice

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Abstract

AIM: To investigate the effect of insulin gene therapy using a glucose-responsive synthetic promoter in type 2 diabetic obese mice.

METHODS: We employed a recently developed novel insulin gene therapy strategy using a synthetic promoter that regulates insulin gene expression in the liver in response to blood glucose level changes. We intravenously administered a recombinant adenovirus expressing furin-cleavable rat insulin under the control of the synthetic promoter (rAd-SP-rINSfur) into diabetic *Lep^{ob/db}* mice. A recombinant adenovirus expressing β -galactosidase under the cytomegalovirus promoter was used as a control (rAd-CMV- β gal). Blood glucose

levels and body weights were monitored for 50 d. Glucose and insulin tolerance tests were performed. Immunohistochemical staining was performed to investigate islet morphology and insulin content.

RESULTS: Administration of rAd-SP-rINSfur lowered blood glucose levels and normoglycemia was maintained for 50 d, whereas the rAd-CMV- β gal control virus-injected mice remained hyperglycemic. Glucose tolerance tests showed that rAd-SP-rINSfur-treated mice cleared exogenous glucose from the blood more efficiently than control virus-injected mice at 4 wk [area under the curve (AUC): $21\ 508.80 \pm 2248.18$ vs $62\ 640.00 \pm 5014.28$, $P < 0.01$] and at 6 wk (AUC: $29\ 956.60 \pm 1757.33$ vs $60\ 016.60 \pm 3794.47$, $P < 0.01$). In addition, insulin sensitivity was also significantly improved in mice treated with rAd-SP-rINSfur compared with rAd-CMV- β gal-treated mice (AUC: 9150.17 ± 1007.78 vs $11\ 994.20 \pm 474.40$, $P < 0.05$). The islets from rAd-SP-rINSfur-injected mice appeared to be smaller and to contain a higher concentration of insulin than those from rAd-CMV- β gal-injected mice.

CONCLUSION: Based on these results, we suggest that insulin gene therapy might be one therapeutic option for remission of type 2 diabetes.

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Key words: Insulin gene therapy; Synthetic promoter; Glucose-responsive element; Liver-specific promoter; Type 2 diabetes

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INTRODUCTION

Type 2 diabetes is characterized by high blood glucose levels in the context of insulin resistance^[1]. Insulin resistance indicates that the body does not respond appropriately to serum insulin to regulate blood glucose levels^[2]. This phenomenon is mainly caused by metabolic disorders such as obesity, hypertension, non-alcoholic fatty liver disease and elevated serum lipid levels^[3-7]. Several peripheral organs contribute to an inappropriate insulin response in the body, which include the liver, muscle, hypothalamus and adipose tissues^[8]. For example, the liver regulates blood glucose levels by storing glucose as glycogen and releasing glucose in response to serum insulin changes; this regulation is lost in the insulin-resistant state^[9]. In adipocytes, insulin acts to inhibit lipolysis and the release of fatty acids from adipose tissue by decreasing the activity of hormone-sensitive lipase and adipose triglyceride lipase, which is attenuated in the insulin resistant state^[10]. Muscle is another big player in regulating blood glucose levels by taking up serum glucose through GLUT4, a glucose transporter, in response to insulin^[11]. Due to insulin resistance in the peripheral organs, more insulin is required to maintain blood glucose levels in physiological range, which is evidenced by hyperinsulinemia and impaired glucose tolerance prior to overt type 2 diabetes^[12].

When type 2 diabetes is clinically diagnosed, only 50% of normal beta cell function remains^[13]. The United Kingdom Prospective Diabetes Study demonstrated that beta cell function continues to deteriorate over time despite treatment with diet, exercise, metformin, sulfonylurea, or insulin^[14]. Normal beta cells respond to blood glucose level very efficiently; however, they lose this responsiveness when they are exposed to high glucose levels or high lipid levels for prolonged periods^[15]. This loss of responsiveness is easily reversed in the early stages, so it is very important to keep blood glucose levels low to sustain beta cell function during the pathogenesis of type 2 diabetes^[16]. Another consideration for gradual loss of beta cell function is hyperinsulinemia, which is a common characteristic of early stage type 2 diabetes^[12]. Hyper-secretion of insulin from beta cells is beneficial to control blood glucose levels, but it is paradoxically detrimental to the beta cell itself. The increased demand for insulin drives beta cells to synthesize insulin beyond their capacity for protein folding and secretion, resulting in endoplasmic reticulum stress^[17-19]. Therefore, it is very important to relieve the burden on beta cells to preserve cell function during the pathogenesis of diabetes. In this

context, intensive insulin therapy is the most effective approach to achieve this goal^[16]. For example, it has been shown that patients injected once a day with long lasting insulin analogues showed significant improvement in glucose control, lower HbA1c levels and fewer complications related to diabetes^[20]. All of these results suggest that earlier insulin treatment might be important to maintain blood glucose levels in type 2 diabetic patients.

Insulin gene therapy is a novel approach to deliver the insulin gene into a target organ to express insulin under the control of an insulin responsive promoter, thus augmenting or replacing pancreatic insulin production. There have been several attempts to control blood glucose level using insulin gene therapy^[21-27]. However, most studies were unsuccessful because of lack of an effective promoter to control insulin gene expression. Recently, we developed a novel synthetic promoter that regulates insulin gene expression in the liver in response to blood glucose level changes^[28]. When diabetic mice were infected with an adenoviral vector expressing the insulin gene under this synthetic promoter, blood glucose levels were controlled in the normal physiological range for up to 1 mo^[28]. In the present study, we employed the same adenoviral vector system to investigate how insulin supplementation through insulin gene therapy might preserve beta cell function and help control blood glucose levels in a type 2 diabetic animal model.

MATERIALS AND METHODS

Animals

Eight-week-old *Lep^{db/db}* mice were obtained from The Jackson Laboratory (Bar Harbor, ME) or from the South Korea Research Institute of Bioscience and Biotechnology (Daejeon, South Korea). Animals were maintained under specific pathogen-free conditions and provided with sterile food and water *ad libitum* at the Animal Resource Center, Faculty of Medicine, University of Calgary, Canada and at the facility at the Lee Gil Ya Cancer and Diabetes Institute, Gachon University of Medicine and Science. The use and care of the animals used in this study were approved by the Animal Care Committee, Faculty of Medicine, University of Calgary and Lee Gil Ya Cancer and Diabetes Institute, Gachon University of Medicine and Science.

Generation of recombinant adenovirus

Recombinant adenovirus expressing furin-cleavable rat insulin cDNA under the control of the synthetic promoter (rAd-SP-rINS_{fur}, Figure 1A) was produced as described previously^[28] using Transpose-AdTM Adenoviral vector system (Qbiogene, Carlsbad, CA). For generation of adenoviruses expressing β -galactosidase under the control of the cytomegalovirus (CMV) promoter (rAd-CMV- β gal), the lacZ coding region from pSV- β -galactosidase vector (Promega, Madison, WI) was cloned into the PCR259 transfer vector (Qbiogene), which has the CMV promoter to drive transgene expression. Recombinant viruses were generated and amplified on a

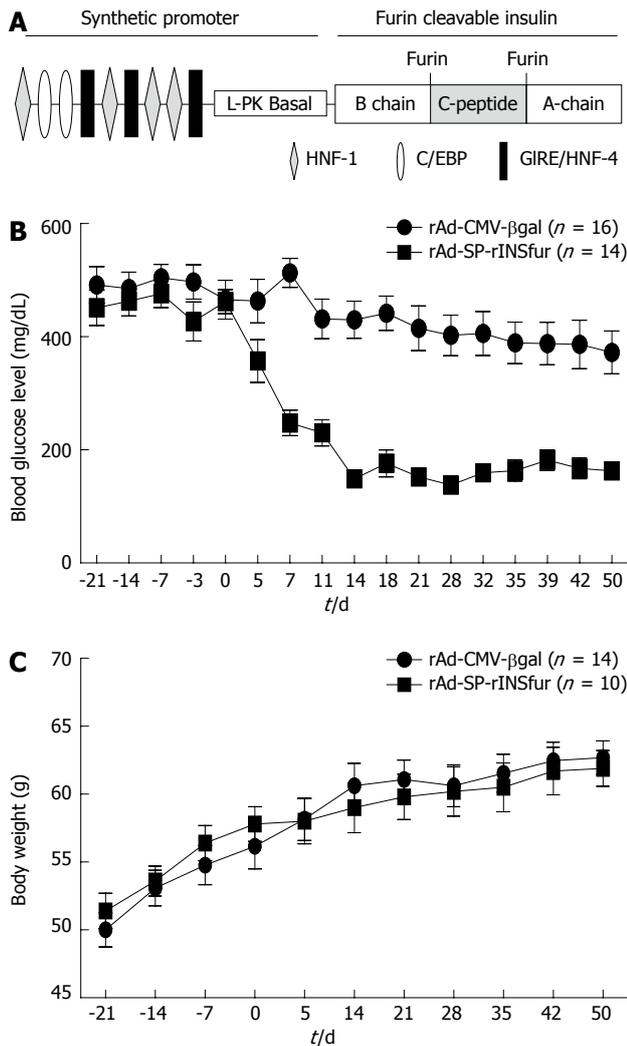


Figure 1 rAd-SP-rINSfur administration reduces high blood glucose levels to normal physiological range in *Lep^{db/db}* mice. A: Schematic diagram of adenoviral construct expressing furin cleavable insulin gene under the control of synthetic promoter. The synthetic promoter is composed of nine transcription factor binding elements upstream of pyruvate kinase basal promoter (L-PK). A furin-cleavable site was introduced at the junction of the B-chain and C-peptide; B: Blood glucose levels; C: Body weights of *Lep^{db/db}* mice treated with rAd-CMV-βgal or rAd-SP-rINSfur. Viruses were injected into experimental animals at 12 wk of age with a dose of 3×10^{10} viral particles, and blood glucose levels and body weights were monitored. Blood glucose levels and body weights were measured between 2-4 pm during the day, and food and water were given *ad libitum* during the experiments. All values are mean \pm SE. Day 0 indicates the day of viral injection. HNF-1: Hepatocyte nuclear factor-1; C/EBP: CCAAT enhancer binding protein; GIRE: Glucose responsive elements; HNF-4: Hepatocyte nuclear factor-4.

large scale in HEK293 cells and purified as previously described^[28]. Viral titer was determined by measurement of optical density at 260 nm.

Blood glucose monitoring and glucose tolerance tests

All mice were given *ad libitum* access to food and water during the experiments. Recombinant adenoviruses were administered intravenously into *Lep^{db/db}* mice at 12 wk of age *via* the tail vein, and blood glucose levels were measured using a One-Touch Profile portable blood glu-

cose monitor (Lifescan, Milpitas, CA, United States). For glucose tolerance tests, animals were fasted for 6 h and then a 1 mol/L glucose solution was administered *via* an intraperitoneal injection at a dose of 2 g/kg body weight. Blood samples were collected from a small cut at the tip of the tail at 0, 15, 30, 60, 90 and 120 min after the glucose load.

Insulin tolerance tests

Animals were fasted for 6 h before injection of insulin (NPH, Lilly, Indianapolis, IN, United States) at a dose of 1.5 U/kg body weight. Blood samples were collected from a small cut at the tip of the tail at 0, 15, 30, 45, 60, 90 and 120 min after the insulin challenge.

Immunohistochemistry

Pancreatic tissues were removed from virus-treated *Lep^{db/db}* mice on day 50 after virus treatment. The samples were fixed with 10% buffered formalin, embedded in paraffin, sectioned at 4.5 μm, and mounted on glass slides. Hematoxylin and eosin staining was performed as previously described^[29]. For immunostaining with anti-insulin antibody, slides were treated with xylene, dehydrated in ethanol, and washed with tap water. Slides were incubated with guinea pig anti-rat insulin antibody for 1 h (1/200 in blocking buffer; DAKO, Carpinteria, CA, United States), washed, and incubated in biotinylated anti-guinea pig antibody (1/300) for 1 h. After washing, horseradish peroxidase-conjugated streptavidin was diluted in blocking buffer (1/300) and added to cells for 1 h, followed by color development using Vector VIP (Vector Laboratories, Burlingame, CA, United States) according to the manufacturer's instructions. After washing with tap water, samples were counterstained with Meyer's hematoxylin solution.

Statistical analysis

All data are presented as mean \pm SE. Differences between groups were evaluated using the Student *t* test, with significance at *P* < 0.05.

RESULTS

Administration of rAd-SP-rINSfur reduces blood glucose levels to normal range in *Lep^{db/db}* mice

The *Lep^{db/db}* mouse is a well-known type 2 diabetic animal model that is homozygous for a point mutation in the gene for the leptin receptor. The leptin hormone plays a key role in regulating energy intake and energy expenditure through its action on the leptin receptor^[30]. Since the *Lep^{db/db}* mouse does not have a functional leptin receptor, it loses its control over food intake, leading to over-nutrition and obesity. To investigate the effect of augmented insulin gene expression on type 2 diabetes, we injected a recombinant adenovirus expressing rat insulin under the control of a synthetic promoter (rAd-SP-rINSfur, 3×10^{10} VP, Figure 1A) into diabetic

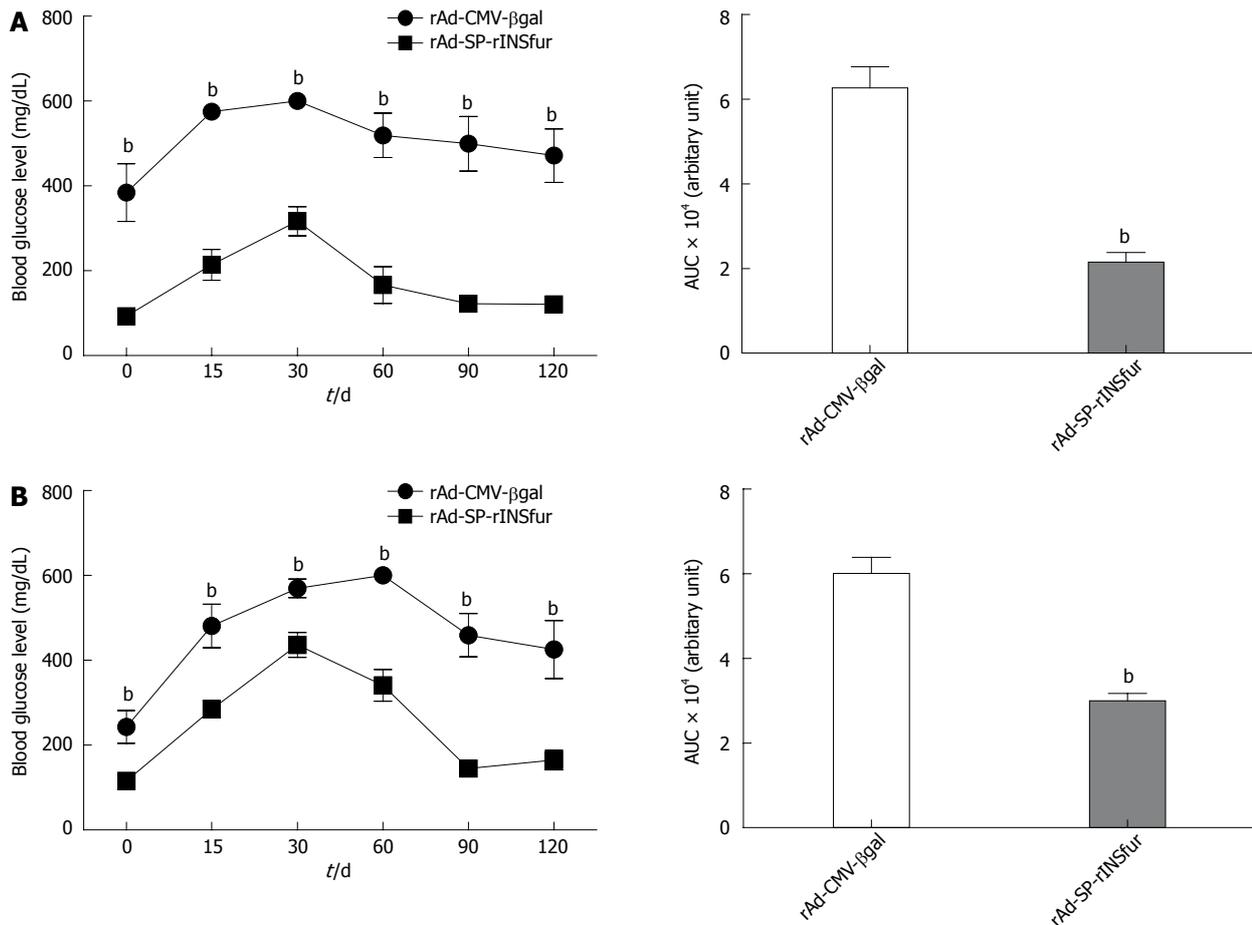


Figure 2 rAd-SP-rINSfur administration improves glucose tolerance in *Lep^{db/db}* mice. Glucose (2 g/kg body weight) was administered by intraperitoneal injection, and blood glucose levels were measured at (A) 4 wk and (B) 6 wk ($n = 4-6$) after virus injection. The area under the curve (AUC) for each glucose tolerance test was quantified. All values are mean \pm SE. ^b $P < 0.01$ vs rAd-CMV-βgal group.

Lep^{db/db} mice ($n = 14$). As a viral control, we injected adenovirus expressing β-galactosidase under the control of the CMV promoter (rAd-CMV-βgal) at same dosage into diabetic *Lep^{db/db}* mice ($n = 16$). Blood glucose levels started to decrease as early as day 5 after rAd-SP-rINSfur infection, whereas there was no significant change in the blood glucose levels of rAd-CMV-βgal-infected mice. Blood glucose levels reached the normal range on day 14 after virus infection, and were maintained up to 50 d after rAd-SP-rINSfur treatment, when animals were sacrificed (Figure 1B). We also checked the body weights during the experimental period. We found that there was no significant difference between the experimental groups (Figure 1C). These results indicate that augmented insulin gene expression maintained blood glucose levels in the normal physiological range but did not affect body weight gain.

rAd-SP-rINSfur administration improves glucose and insulin tolerance in *Lep^{db/db}* mice

Next, we investigated the ability of rAd-SP-rINSfur-treated mice to clear serum glucose during a glucose tolerance test. At 4 and 6 wk after virus injection, there was a significant difference between the groups in the ability

to remove challenged glucose. Mice injected with the control virus showed glucose intolerance, whereas mice treated with rAd-SP-rINSfur showed improved glucose clearance in response to glucose challenge (Figure 2A and B). Since this result indicated improved glucose responsiveness, we also checked insulin sensitivity. In addition to an enhanced ability to clear loaded glucose, the rAd-SP-rINSfur-treated *Lep^{db/db}* mice showed significantly improved insulin sensitivity compared with the *Lep^{db/db}* mice treated with control virus, which showed relative insulin resistance (Figure 3).

rAd-SP-rINSfur administration preserves endogenous islets in *Lep^{db/db}* mice

Since it is likely that the additional insulin expression from the liver in rAd-SP-rINSfur treated *Lep^{db/db}* mice might relieve the burden that was imposed on the beta cells in diabetic insulin-resistant *Lep^{db/db}* mice, we expected that beta cells would be protected in rAd-SP-rINSfur treated mice. In *Lep^{db/db}* mice, it is known that the increased demand for insulin causes beta cell hypertrophy to generate more insulin^[31], which is a typical characteristic of this type 2 diabetic animal model. Consistently, the islets in *Lep^{db/db}* mice injected with rAd-CMV-βgal virus

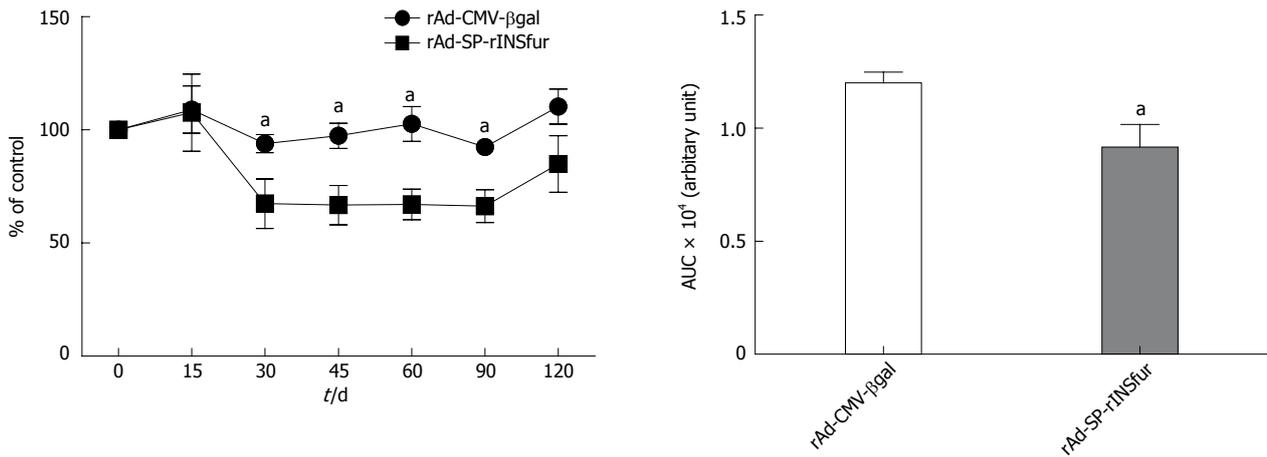


Figure 3 rAd-SP-rINSfur administration improves insulin tolerance in *Lepr^{db/db}* mice. Insulin (1.5 U/kg body weight, *ip*) was administered to mice injected with rAd-CMV-βgal or rAd-SP-rINSfur at 5 wk after virus injection (*n* = 4-6), and blood glucose levels were measured. The area under the curve (AUC) for each insulin tolerance test was quantified. All values are mean ± SE. **P* < 0.05 vs rAd-CMV-βgal group.

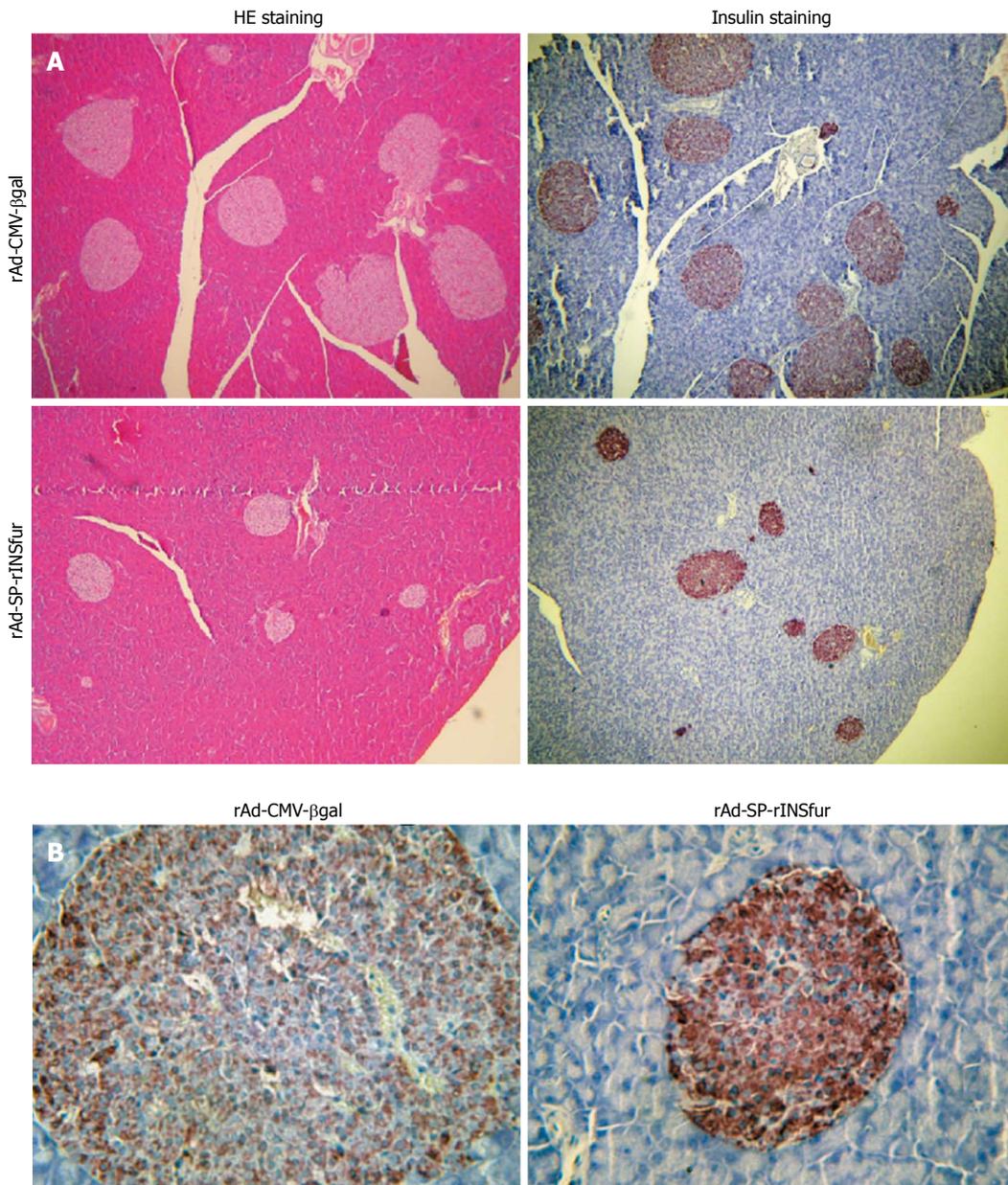


Figure 4 rAd-SP-rINSfur administration preserves endogenous islets in *Lepr^{db/db}* mice. Pancreatic samples were obtained from rAd-CMV-βgal and rAd-SP-rINSfur virus-treated animals on day 50 after virus injection. Tissue sections were prepared and stained with hematoxylin and eosin (HE) or stained with anti-insulin antibody. (A) is 20 × and (B) is 40 × magnification.

were larger than those in rAd-SP-rINSfur-treated mice (Figure 4A). This result suggests that there is less demand for insulin, which will relieve the beta cells from overproduction of insulin. In addition to the size, there seemed to be greater insulin content in the islets from rAd-SP-rINSfur-treated mice (Figure 4B), as shown by darker immunocytochemical staining for insulin, indicating that beta cell function is also preserved by augmented insulin gene expression by insulin gene therapy.

DISCUSSION

The prevalence of type 2 diabetes is increasing in developed and especially in developing countries including South Korea^[32]. In the early period of diagnosis, blood glucose levels can be managed by exercise and oral medication such as rosiglitazone^[13]. However, during progression of disease, these approaches do not control blood glucose levels, and eventually diabetic patients need insulin treatment, as in type 1 diabetes, because the beta cells do not respond to glucose change or the beta cell mass is significantly reduced^[13]. This phenomenon is likely due to prolonged high blood glucose levels (glucotoxicity) and/or high blood lipid levels (lipotoxicity) under insulin resistance status. In addition, beta cells over-produce insulin to compensate for insulin resistance, resulting in endoplasmic reticulum stress, which is known to be one of the main causes of beta cell death in type 2 diabetes. In this context, it is important to find an approach to preserve beta cell function and mass during the early stage of type 2 diabetes. In fact, it was reported that in newly diagnosed type 2 diabetic patients with elevated fasting glucose levels, a short period of intensive insulin therapy helped patients control blood glucose levels in an acceptable range for a long time^[33].

In this study, we demonstrated that additional insulin production by insulin gene therapy improved serum glucose management and glucose tolerance in a type 2 diabetic animal model. In addition to these beneficial effects, there was improved insulin tolerance, indicating that insulin gene therapy is also beneficial for insulin sensitivity. Better glucose management can be explained by increased insulin sensitivity in the liver because of the autocrine action of insulin produced by liver *per se*. In addition, relieving the burden of insulin production would enable beta cells to store more insulin in the vesicles for later needs. We showed that islets from mice injected with rAd-SP-rINSfur appear smaller and appear to contain more insulin compared with control mice, which suggests that glucose tolerance is improved due to enough insulin reservoirs. In addition, lower blood glucose levels are beneficial to preserve beta cell function to secrete insulin in response to serum glucose level changes^[34].

Although there was improved glucose tolerance and insulin sensitivity, these effects were attenuated in the later period of treatment. It is most likely that insulin gene expression gradually decreases due to clearance by

the host's immune system^[28]. Our previous study clearly showed that an insulin gene delivered by gene therapy was cleared by immune surveillance and thus the effect of the gene was transient^[28]. This transient effect can be overcome by using the new generation of adenoviral vectors that lack most of the viral components, enabling them to escape the host's immune system^[35]. Using lentivirus is another option for prolonging the expression period. This viral vector enables the transgene to integrate into the host chromosome for long-term expression of the transgene^[35].

In this study, we successfully improved glucose management in a type 2 diabetic animal model through preservation of beta cells; however, there are several considerations to improve the current study. First, we need to extend the insulin gene expression for a longer time. Second, we need to consider safety. Even though expression of an insulin gene is beneficial to manage the blood glucose level, there is potential risk of hypoglycemia^[36,37]. Therefore, precautions must be taken to avoid any risk that can be encountered in a future clinical study.

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COMMENTS

Background

The prevalence of type 2 diabetes is increasing quickly across the world, and it is widely recognized as one of the leading causes of death in the United States. It is closely related to insulin resistance and subsequent loss of beta cell function and mass.

Research frontiers

When type 2 diabetes is clinically diagnosed, there is still a portion of beta cells with normal function. However, this function continues to deteriorate over time despite treatment with diet, exercise or therapeutic drugs. However, studies have found that intensive insulin therapy improves glucose control and preserves beta cell function in the pathogenesis of diabetes. In this study, the authors demonstrate that early insulin treatment using gene therapy improves glucose levels and insulin resistance in obese diabetic mice.

Innovations and breakthroughs

Recent reports have suggested the importance of intensive insulin therapy in the early stages of type 2 diabetes to improve outcomes by preserving beta cell mass and function. In this study, the authors employed insulin gene therapy to provide extra insulin to the body. The therapy induced the liver to produce insulin, driven by a newly developed synthetic promoter which is glucose responsive. The authors successfully improved glucose management in a type 2 diabetic animal model through preservation of beta cells.

Applications

By showing the efficacy of insulin gene therapy, this study may provide a future strategy for therapeutic intervention in the treatment of type 2 diabetic patients.

Terminology

Insulin gene therapy: Delivering an insulin gene into a target organ in the body to express insulin under the control of a glucose-responsive promoter. The insulin required to maintain blood glucose is then provided by insulin gene expression by the target organ, replacing the need for exogenous insulin injections.

Peer review

The authors demonstrated that administration of adenovirus expressing insulin under the control of synthetic promoter that is responsive to glucose change improved serum glucose management and tolerance as well as insulin

tolerance in a genetically obese animal model.

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Diagnostic role of 18F-fluorodeoxyglucose positron emission tomography for follicular lymphoma with gastrointestinal involvement

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Abstract

AIM: To investigate the capacity for 18F-fluorodeoxyglucose (18F-FDG) positron emission tomography (PET) to evaluate patients with gastrointestinal lesions of follicular lymphoma.

METHODS: This retrospective case series consisted of 41 patients with follicular lymphoma and gastrointestinal involvement who underwent 18F-FDG-PET and endoscopic evaluations at ten different institutions between November 1996 and October 2011. Data for endoscopic, radiological, and biological examinations performed were retrospectively reviewed from clinical records. A semi-quantitative analysis of 18F-FDG uptake was performed for each involved area by calculating the maximum standardized uptake value (SUV-max). Based on the positivity of 18F-FDG uptake in the gastrointestinal lesions analyzed, patients were subdivided into two groups. To identify potential predictive factors for 18F-FDG positivity, these two groups were compared with respect to gender, age at diagnosis of lymphoma, histopathological grade, pattern of follicular dendritic cells, mitotic rate, clinical stage, soluble interleukin-2 receptor levels detected by 18F-FDG-PET, lactate dehydrogenase (LDH) levels, hemoglobin levels,

bone marrow involvement, detectability of gastrointestinal lesions by computed tomography (CT) scanning, and follicular lymphoma international prognostic index (FLIPI) risk.

RESULTS: Involvement of follicular lymphoma in the stomach, duodenum, jejunum, ileum, cecum, colon, and rectum was identified in 1, 34, 6, 3, 2, 3, and 6 patients, respectively. No patient had esophageal involvement. In total, 19/41 (46.3%) patients exhibited true-positive 18F-FDG uptake in the lesions present in their gastrointestinal tract. In contrast, false-negative 18F-FDG uptake was detected in 24 patients (58.5%), while false-positive 18F-FDG uptake was detected in 5 patients (12.2%). In the former case, 2/19 patients had both 18F-FDG-positive lesions and 18F-FDG-negative lesions in the gastrointestinal tract. In patients with 18F-FDG avidity, the SUVmax value of the involved gastrointestinal tract ranged from 2.6 to 17.4 (median: 4.7). For the 18F-FDG-negative ($n = 22$) and -positive ($n = 19$) groups, there were no differences in the male to female ratios (10/12 *vs* 4/15, $P = 0.186$), patient age (63.6 ± 2.4 years *vs* 60.1 ± 2.6 years, $P = 0.323$), presence of histopathological grade 1 *vs* 2 (20/2 and 17/2, $P = 1.000$), follicular dendritic cell pattern (duodenal/nodal: 13/5 *vs* 10/3, $P = 1.000$), mitotic rate (low/partly high, 14/1 *vs* 10/3, $P = 0.311$), clinical stage according to the Ann Arbor system (stages I E and II E/other, 15/7 *vs* 15/4, $P = 0.499$), clinical stage according to the Lugano system (stages I and II-1/other, 14/8 *vs* 14/5, $P = 0.489$), soluble interleukin-2 receptor levels (495 ± 78 *vs* 402 ± 83 , $P = 0.884$), LDH levels (188 ± 7 *vs* 183 ± 8 , $P = 0.749$), hemoglobin levels (13.5 ± 0.3 *vs* 12.8 ± 0.4 , $P = 0.197$), bone marrow involvement (positive/negative, 1/8 *vs* 1/10, $P = 1.000$), detectability by CT scanning (positive/negative, 1/16 *vs* 4/13, $P = 0.335$), and FLIPI risk (low risk/other, 16/6 *vs* 13/6, $P = 0.763$), respectively in each case.

CONCLUSION: These findings indicate that it is not feasible to predict 18F-FDG-avidity. Therefore, 18F-FDG-PET scans represent a complementary modality for the detection of gastrointestinal involvements in follicular lymphoma patients, and surveillance of the entire gastrointestinal tract by endoscopic examinations is required.

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Key words: Follicular lymphoma; Gastrointestinal endoscopy; 18F-fluorodeoxyglucose positron emission tomography; Gastrointestinal lymphoma; Duodenal neoplasm

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INTRODUCTION

To date, 18F-fluorodeoxyglucose (18F-FDG) positron emission tomography (PET) has been widely used for the initial staging of various malignant diseases, as well as for the evaluation of therapeutic responses^[1-5]. Similar to other types of lymphomas^[6-10], follicular lymphoma exhibits a high avidity for 18F-FDG^[11-15]. Correspondingly, the percentage of patients with 18F-FDG-avid follicular lymphoma has been reported to range from 91% to 100%^[16-20]. Furthermore, nodal and extranodal lesions not detected by other modalities have been identified using 18F-FDG-PET, thereby resulting in a significant advance in lesion management^[18-22]. The usefulness of post-treatment 18F-FDG-PET during the follow-up period in cases of follicular lymphoma has also been noted, with positive 18F-FDG accumulation suggesting an adverse outcome^[23-25]. Consequently, 18F-FDG-PET is considered a valuable tool for the management of nodal follicular lymphoma.

Despite these advantages, few studies have addressed the use of 18F-FDG-PET for gastrointestinal involvement of follicular lymphoma. In 2004, Hoffmann *et al*^[26] reported eight cases of follicular lymphoma localized in the duodenum, and 18F-FDG did not accumulate in any of those patients. Subsequently, several authors described follicular lymphoma patients with positive 18F-FDG uptake in the gastrointestinal tract, including the stomach, duodenum, jejunum, ileum, cecum, and colon^[27-30]. However, a limited number of these cases have been reported. Therefore, the sensitivity of 18F-FDG-PET for gastrointestinal lesions of follicular lymphoma has not been sufficiently evaluated.

In this study, 18F-FDG-PET results and clinical characteristics of 41 follicular lymphoma patients with gastrointestinal involvement were retrospectively examined. Based on these results, the role of 18F-FDG-PET in the management of these cases was evaluated.

MATERIALS AND METHODS

A database search performed at the Department of Pathology of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences identified follicular lymphoma patients with gastrointestinal involvement ($n = 80$) treated at ten collaborating institutions between November 1996 and October 2011. The diagnosis of follicular lymphoma was made according to World Health Organization (WHO) classifications^[31,32].

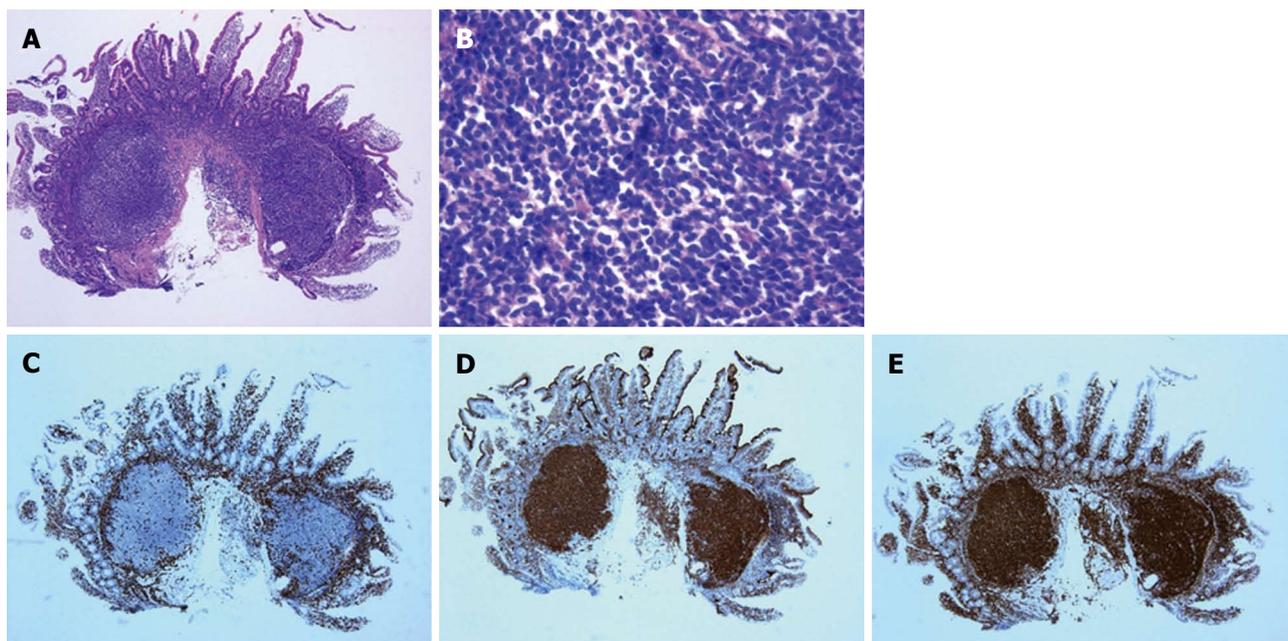


Figure 1 Typical histological features of follicular lymphoma. A, B: Small cleaved cells that infiltrated the duodenal mucosa and formed lymphoid follicles are present (hematoxylin and eosin staining); C: Representative immunohistochemical staining of lymphoma cells negative for CD3; D: Lymphoma cells positive for CD10; E: Lymphoma cells positive for BCL-2. All of the images shown are at 40 × magnification, except for panel B which is at 400 × magnification.

A histological diagnosis was based on morphologic and immunophenotypic analyses of endoscopically biopsied specimens or surgically resected specimens (Figure 1). Histopathological grading was also determined according to WHO criteria^[31]. Patients with grade 3 follicular lymphoma were excluded from this study since these cases are typically managed as diffuse large B cell lymphoma^[31]. A subset of the 80 patients examined were also subjects of our previous studies^[33-36].

Of the 80 patients identified, 30 were excluded since they did not undergo 18F-FDG-PET. Nine patients were further excluded from this study, and these included: four patients that received systemic chemotherapy and remained in complete remission during their 18F-FDG-PET examination, four patients that underwent surgical resection of gastrointestinal lesions and 18F-FDG-PET scans were postoperatively performed, and one patient that underwent 18F-FDG-PET during their initial staging, and later was diagnosed with follicular lymphoma by laparotomy. In the latter case, the patient was not endoscopically evaluated preoperatively. Therefore, a total of 41 patients were enrolled in this study, and data regarding endoscopic, radiological, and biological examinations performed were retrospectively reviewed from their clinical records. Gastrointestinal involvement was defined by gross findings of endoscopic examinations which included esophagogastroduodenoscopy, colonoscopy, double-balloon enteroscopy, and/or video capsule endoscopy. Typically, small, whitish polypoid nodules up to 2 mm in diameter were observed^[37,38]. In cases involving an atypical endoscopic appearance, a histopathological assessment of biopsy specimens was performed to confirm a diagnosis of gastrointestinal involvement.

For all patients, 18F-FDG-PET was performed after patients had fasted for at least 4 h, and it was confirmed that their serum glucose levels were below 150 mg/dL. Moreover, 18F-FDG-PET was preceded by a low-dose computed tomography (CT) scanning, and these scans were used to correct attenuation and to localize anatomical variations visualized in 18F-FDG-PET. The time to initiation of 18F-FDG-PET following the intravenous administration of 18F-FDG also varied according to institution (i.e., 60, 90, or 120 min). A semi-quantitative analysis of 18F-FDG uptake was then performed for each involved area by calculating the maximum standardized uptake value (SUVmax).

Based on the positivity of 18F-FDG uptake in the gastrointestinal lesions analyzed, patients were divided into two groups. To identify potential predictive factors for 18F-FDG positivity, patient gender, age at diagnosis of lymphoma, clinical stage, bone marrow involvement, histopathological grade, soluble interleukin-2 receptor (sIL-2R) levels, lactate dehydrogenase (LDH) levels, and hemoglobin levels were evaluated. CD21 and CD23 staining were also performed to characterize duodenal patterns and nodal patterns of follicular dendritic cells present, as previously described^[35]. To estimate the mitotic rate, Ki-67 staining was performed. Positive staining of 0%-5% of cells was classified as a low mitotic rate, while > 5% positivity was classified as high mitotic activity. The Lugano staging system for classification of gastrointestinal lymphoma^[39,40], and the classical Ann Arbor staging system for nodal lymphoma^[41], were used to determine patients' clinical stages. The follicular lymphoma international prognostic index (FLIPI) was used as well^[42]. Despite FLIPI-2 recently being introduced as an updated

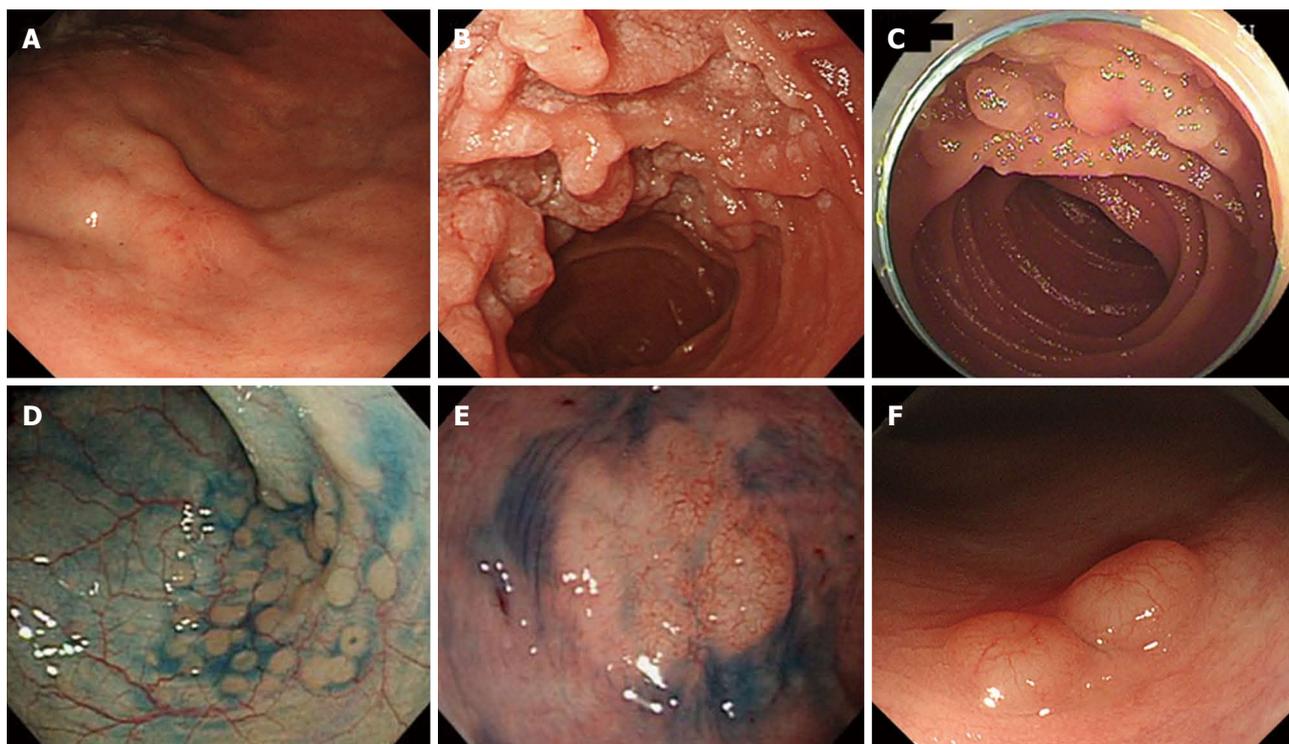


Figure 2 Endoscopic images of follicular lymphoma. A: A gastric lesion with thickened rugae exhibiting a slight redness; B: Typical features of the whitish polypoid granules observed in the duodenum; C: Whitish polypoid lesions in the jejunum; D: Indigo carmine contrast was used to emphasize the slightly elevated small polyps present in the colon; E: An elevated lesion with a flat surface and a 20 mm diameter was observed in the rectum; F: In another patient, polypoid lesions exhibiting hypervascularity on the surface were detected in the rectum.

prognostic index^[43], it was not employed in this study since beta-2-microglobulin levels were not available for all of the patients. In some patients, CT scanning was separately performed from 18F-FDG-PET, and the detectability of gastrointestinal lesions by CT scanning was also analyzed.

For comparisons of the two groups, statistical analyses were performed by JMP 8.0.1 software (SAS Institute, Cary, NC, United States), which included *t*-tests, χ^2 tests, and *F*-tests. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

For detection of gastrointestinal lesions, an esophago-gastroduodenoscopy was performed for all 41 patients included in this study. However, for one patient with colonic follicular lymphoma, the results of the esophago-gastroduodenoscopy performed at another referral center were not available. Colonoscopies were also performed for 29/41 patients. For these patients, the small intestines were evaluated by double-balloon enteroscopy ($n = 7$), video capsule endoscopy ($n = 6$), or both methods ($n = 3$). None of the patients had esophageal involvement. Furthermore, one patient had involvement of the stomach which manifested with thickened rugae observed to have a slight redness to its surface (Figure 2A). Involvement of the duodenum was identified in 34 patients, with the duodenal lesions present in 32 of these patients exhibiting an

accumulation of small, whitish polypoid nodules (Figure 2B). For the other two patients, one presented with erosions having peripheral whitish mucosa in the duodenum, while the other had confluent whitish granules in the duodenum. These atypical macroscopic features were previously described^[44,45]. Jejunal involvement was identified in six patients. Ileal lesions were found in three patients. In the jejunum and ileum, small whitish polypoid nodules, or ulcerative tumors, were observed (Figure 2C). The cecum was found to be involved in two cases, the colon in three patients, and the rectum in six patients. For cases involving the cecum and the colon, multiple polypoid lesions of various sizes were identified (Figure 2D). Moreover, rectal involvement varied in morphology, with nodules, polyps, submucosal tumors, and laterally spreading tumors observed (Figures 2E, F).

Representative images of 18F-FDG-PET performed are shown in Figures 3-6. In addition, the results of 18F-FDG accumulation in the gastrointestinal tract are summarized in Table 1. The sensitivity of 18F-FDG-PET for the detection of gastrointestinal lesions ranged from 33.3% to 100%. It is noteworthy that sensitivity for duodenal involvement was only identified in 35.3% of cases, while the duodenum was the most frequently affected site^[41]. In total, 19 of the 41 patients (46.3%) exhibited true-positive 18F-FDG uptake in the involved gastrointestinal tract. Furthermore, two of these cases had both 18F-FDG-positive lesions and 18F-FDG-negative lesions present in the same gastrointestinal tract. In one patient,

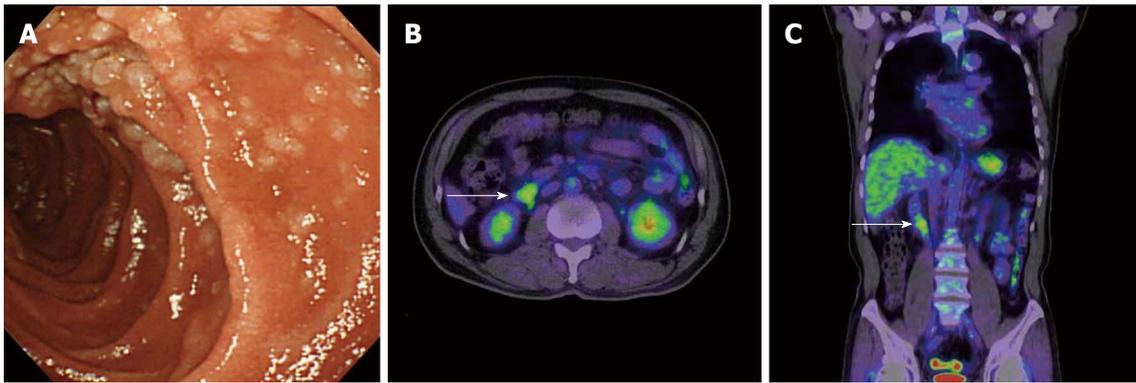


Figure 3 A 60-year-old male patient with duodenal follicular lymphoma without nodal lesions (Lugano system stage I, grade 1). A: An esophagogastroduodenoscopy detected features typical of primary duodenal follicular lymphoma, including small whitish nodules; B, C: 18F-fluorodeoxyglucose positron emission tomography detected tracer uptake in the duodenal second portion (indicated with arrows).



Figure 4 A 65-year-old female patient with duodenal and jejunal follicular lymphoma and intra-abdominal lymph node involvement (Lugano system stage II-1, grade 1). A: An esophagogastroduodenoscopy revealed small whitish nodules present in the duodenum; B: Video capsule endoscopy also identified small nodules present in the jejunum; C, D: 18F-fluorodeoxyglucose positron emission tomography detected tracer uptake in the duodenum (C) and the jejunum (D).

Table 1 Accumulation of 18F-fluorodeoxyglucose detected in the gastrointestinal tract

	Positive involvement (defined by endoscopy)	True-positive 18F-FDG uptake	False-positive 18F-FDG uptake	Sensitivity (%)
Esophagus	0	0	0	NA
Stomach	1	1	1	100.0
Duodenum	34	12	0	35.3
Jejunum	6	5	0	83.3
Ileum	3	1	1	33.3
Cecum	2	2	0	100.0
Colon	3	1	1	33.3
Rectum	6	3	2	50.0

18F-FDG: 18F-fluorodeoxyglucose; NA: Not available.

the duodenum, ileum, cecum, colon, and rectum were endoscopically involved, yet 18F-FDG only accumulated in the ileum, cecum, and colon (Figure 5). Another patient had involvement of the duodenum and ileum, yet only the ileal lesion accumulated 18F-FDG. On the other hand, false-positive 18F-FDG uptake was identified in five patients (12.2%), and false-negative 18F-FDG uptake was found in 24 patients (58.5%).

Thirty-four patients underwent CT scanning separate from 18F-FDG-PET. Gastrointestinal lesions de-

tected by CT involved the duodenum in two cases, the jejunum in one case, and the ileum in two cases. In all cases, gastrointestinal involvement was accompanied by a thickness of the intestinal wall. In 13 cases, gastrointestinal lesions that were not detected by CT scanning were identified by 18F-FDG-PET. Moreover, 18F-FDG-PET detected affected extra-gastrointestinal organs that were not diagnosed by CT scanning. These included the pleura, diaphragm, and adrenal gland in one patient, the pharynx in another, and diffuse splenic invasion in a third patient. Furthermore, regarding the latter patient, the results of 18F-FDG-PET resulted in an upgrade of the Ann Arbor clinical staging from II E to III ES, and in the Lugano system from stage II-2 to stage IV (Figure 6). In regard to 18F-FDG-avid patients, the SUVmax value of the involved gastrointestinal tract region ranged from 2.6 to 17.4 (median: 4.7). Moreover, while one patient had a relatively high SUVmax value of 17.4, the other patients had the SUVmax values less than 10.

A comparison of patients with positive 18F-FDG uptake by their gastrointestinal lesions *vs* those with negative 18F-FDG uptake had no difference in their clinical backgrounds (Table 2). No differences in patient gender, age at the time of diagnosis of lymphoma, clinical stage according to the Ann Arbor and Lugano systems, bone

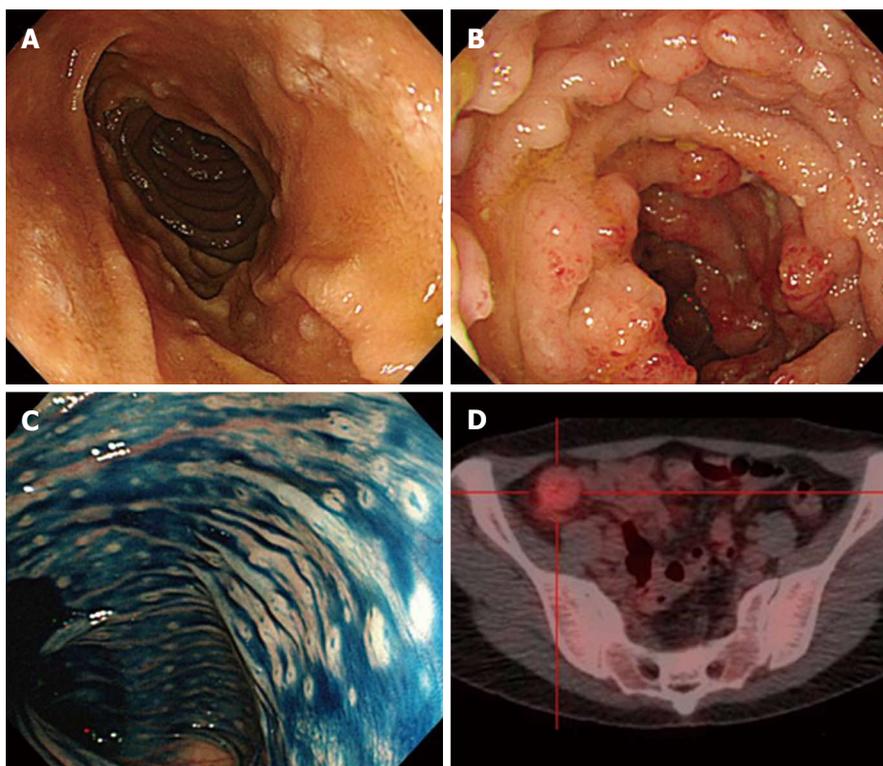


Figure 5 A 37-year-old female patient with systemic follicular lymphoma and extended gastrointestinal involvement from the duodenum to the rectum (stage IV, grade 1). A: An esophagogastroduodenoscopy detected small whitish nodules present in the duodenum; B, C: A colonoscopy revealed multiple polyps present in the ileum (B), cecum, colon (C), and rectum. Jejunal involvement was confirmed by video capsule endoscopy; D: During 18F-fluorodeoxyglucose positron emission tomography, tracer uptake was only noted in the ileum and colon.

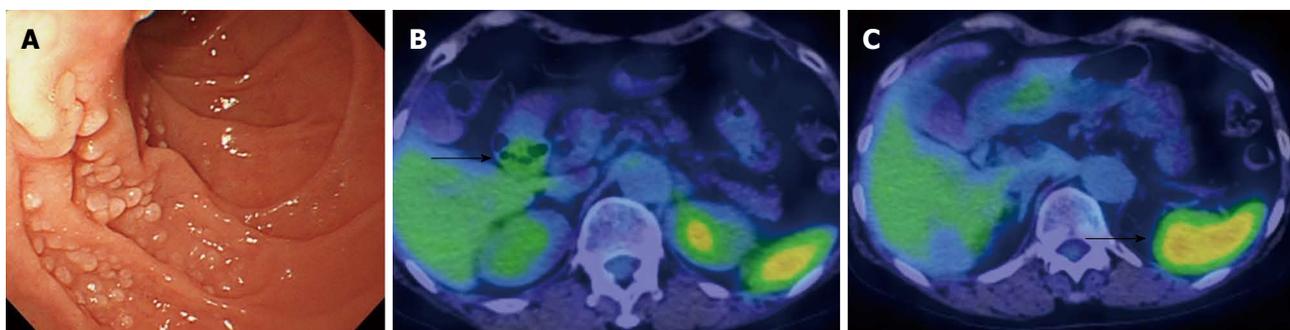


Figure 6 A 61-year-old female patient with follicular lymphoma and duodenal involvement (Lugano system stage IV, grade 1). A: In the duodenum, small whitish nodules were observed; B: 18F-fluorodeoxyglucose positron emission tomography (18F-FDG-PET) detected tracer uptake in the duodenum (indicated with an arrow); C: 18F-FDG-PET identified diffuse lymphoma infiltration into the spleen (indicated with an arrow), which was not detected by computed tomography scanning. In this case, these results upgraded the clinical stage.

marrow involvement, histopathological grade, pattern of follicular dendritic cells, mitotic rate, sIL-2R, LDH, hemoglobin levels, FLIPI, and detectability of gastrointestinal lesions by CT scanning were found between the two patient groups.

DISCUSSION

To the best of our knowledge, this is the largest study on the sensitivity of 18F-FDG-PET for gastrointestinal involvement of follicular lymphoma. Nineteen of our 41 patients showed true 18F-FDG uptake in the involved gastrointestinal tract, resulting in a sensitivity of 46.3%.

In contrast, false-negative 18F-FDG uptake was detected in 24 patients (58.5%), 22 patients (53.7%) exhibited false negative 18F-FDG uptake, and 2 patients (4.9%) showed both true positive and false negative 18F-FDG uptake in gastrointestinal lesions. These results indicate that 18F-FDG-PET is not a reliable imaging tool for evaluating the involvement of the gastrointestinal tract, since greater than half of the patients showed false-negative lesions. It is well-known that follicular lymphoma often affects multiple gastrointestinal tracts. For example, in our previous report, 46 of 54 duodenal follicular lymphoma patients (85.2%) who underwent whole gastrointestinal tract surveillance had extensive involvement within the

Table 2 Clinical backgrounds of the study subjects (mean \pm SD)

	Negative FDG uptake in GI tract	Positive FDG uptake in GI tract	P value
No. patients (<i>n</i>)	22	19	
Male/female	10/12	4/15	0.186
Age (yr) at diagnosis of FL	63.6 \pm 2.4	60.1 \pm 2.6	0.323
WHO grade			1.000
Grade 1	20	17	
Grade 2	2	2	
Follicular dendritic cell pattern			1.000
Duodenal	13	10	
Nodal	5	3	
Mitotic rate			0.311
Low	14	10	
Partly high	1	3	
Ann Arbor system staging			0.499
I E	12	11	
II E	3	4	
III ES	1	0	
IV	6	4	
Lugano system staging			0.489
I	12	10	
II-1	2	4	
II-2	1	1	
IV	7	4	
sIL-2R	495 \pm 78	402 \pm 83	0.884
LDH	188 \pm 7	183 \pm 8	0.749
Hb	13.5 \pm 0.3	12.8 \pm 0.4	0.197
Bone marrow involvement			1.000
Positive	1	1	
Negative	8	10	
Detection of GI lesions by CT			0.335
Positive	1	4	
Negative	16	13	
FLIPI risk			0.763
Low	16	13	
Intermediate	3	3	
Poor	3	3	

FDG: Fluorodeoxyglucose; GI: Gastrointestinal; FL: Follicular lymphoma; sIL-2R: Soluble interleukin-2 receptor; CT: Computed tomography; FLIPI: Follicular lymphoma international prognostic index; LDH: Lactate dehydrogenase; WHO: World Health Organization.

small intestine, predominantly in the jejunum (i.e., 40 of 54 patients, 74.1%)^[44]. These data are consistent with those reported in other studies where the percentage of patients with multiple lymphoma lesions in the small intestine ranged from 66.7% to 100%^[29,46-49]. Therefore, in combination, these results suggest that 18F-FDG-PET represents a complementary method for assessing gastrointestinal involvement of follicular lymphoma.

Regarding the false-negative 18F-FDG-PET results obtained in this study, there are many conditions to consider. First, some of the lymphoma lesions may have been too small to be detected by 18F-FDG-PET^[28]. For example, nodes less than 1 to 1.2 cm in diameter have previously been shown to exhibit a false-negative 18F-FDG uptake^[17,50]. In addition, the representative endoscopic features of gastrointestinal follicular lymphoma, especially in small intestinal cases, include the presence of small, whitish polypoid nodules up to 2 mm in diam-

eter^[37,38]. Generally, these lesions remain small and rarely form bulky tumors. In the present report, gastrointestinal lesions could not be detected by CT scanning in 29 of 34 cases (85.3%). In contrast, CT scanning was able to detect gastrointestinal lesions in five cases, four of which showed 18F-FDG uptake. Therefore, although the sensitivity of CT scanning did not statistically correlate with 18F-FDG avidity, small tumor volume appears to have contributed to the false-negative 18F-FDG uptake results obtained. Second, the intensity of 18F-FDG uptake in follicular lymphoma is relatively low compared with aggressive lymphomas^[50,51]. A SUVmax value is a relative quantification of local radiotracer accumulation. Schöder *et al.*^[50] reported SUVmax values for nodal lesions in indolent and aggressive lymphomas to be 7.0 \pm 3.1 and 19.6 \pm 9.3, respectively ($P < 0.01$). They also noted that a SUVmax value > 10 excluded indolent lymphoma with a specificity of 81%. In the present study, all but one patient had a SUVmax value < 10 , and the results of the present findings are in concordance with data reported in an earlier study^[50]. Consequently, small tumor volume and low tracer uptake intensity represent factors that can contribute to false-negative findings.

False-positive FDG uptake by the gastrointestinal tract is another disadvantage associated with 18F-FDG-PET. In this study, five patients had a false-positive 18F-FDG uptake detected in the gastrointestinal tract. Previously it was proposed that physiological peristaltic activity, normal gastrointestinal lymphoid tissue, and granulomatous or inflammatory conditions such as enterocolitis, Crohn's disease, tuberculosis, hemorrhoids, or diverticulitis can cause false-positive 18F-FDG uptake in the gastrointestinal tract^[52]. However, physiologic uptake in the gastrointestinal tract is usually diffuse and its intensity generally moderate. In contrast, intense 18F-FDG uptake can be detected under granulomatous or inflammatory conditions, and even in constipated patients^[53-55]. Therefore, both false-positive, and false-negative, 18F-FDG-uptake represent potential pitfalls in evaluating possible lymphoma involvement of the gastrointestinal tract.

Despite these disadvantages, there are several benefits to the application of 18F-FDG-PET to follicular lymphoma patients with gastrointestinal involvement. First, as demonstrated in the present study, 18F-FDG-PET was able to detect a greater number of involved gastrointestinal sites than CT scanning. For example, 34 patients underwent both CT scanning and 18F-FDG-PET. Of these, 13 (38.2%) had gastrointestinal lesions that were not detected by CT scanning. Second, 18F-FDG-PET was able to detect extra-gastrointestinal sites of involvement. For example, extra-gastrointestinal sites were detected in three patients, and this resulted in an upgrade of clinical stage for one of these patients (Figure 6). Similarly, previous studies of nodal cases found that the clinical staging of follicular lymphoma patients needed to be modified in 18% to 31% of cases based on 18F-FDG-PET results^[17,19,22]. This aspect is particularly

vital for stage I or stage II patients under consideration for radiotherapy as a curative treatment, since most treatment failures occur outside the involved field of radiotherapy^[56]. Thirdly, 18F-FDG-PET can be used to evaluate treatment response if a patient has 18F-FDG-avid gastrointestinal lesions. In nodal follicular lymphoma, disappearance of 18F-FDG accumulation after completion of treatment has been associated with a favorable outcome^[23-25]. Although the cost and benefit (e.g., sensitivity, specificity, and patient acceptability) of 18F-FDG-PET *vs* other modalities, including the combination of CT scanning and endoscopic examinations, remains to be investigated, 18F-FDG-PET represents an option for assessing the therapeutic effect in cases of gastrointestinal involvement of follicular lymphoma.

There were also several limitations associated with this study. First, not all of the patients underwent endoscopic surveillance for the entire gastrointestinal tract. In particular, the small intestine was not evaluated in 25 patients, although multiple sites, including the jejunum and ileum, were frequently involved^[34]. As a result, an overestimation of 18F-FDG-PET sensitivity may have occurred. Second, 18F-FDG-PET was performed under different conditions since the patients included had been treated at various institutions. For example, the period of time between the intravenous administration of 18F-FDG and the initiation of 18F-FDG-PET varied between 60 and 120 min. It is possible that other differences in methodology among the participating institutions may have affected the positivity of 18F-FDG uptake and SUVmax values as well^[57,58].

In conclusion, 19 of 41 follicular lymphoma patients (46.3%) exhibited true-positive 18F-FDG uptake in the involved gastrointestinal tract. In contrast, false-negative 18F-FDG uptake was detected in 24 patients (58.5%). There were also no differences found between the 18F-FDG-PET-positive group and the 18F-FDG-PET-negative group based on the clinical backgrounds of the patients examined, suggesting that it is not feasible to predict 18F-FDG-avidity. However, 18F-FDG-PET may facilitate the detection of gastrointestinal and extra-gastrointestinal sites of involvement. Therefore, we propose that 18F-FDG-PET represents a complementary method for the detection of gastrointestinal lesions of follicular lymphoma. However, endoscopic examinations should be performed to monitor the entire gastrointestinal tract.

COMMENTS

Background

18F-fluorodeoxyglucose (18F-FDG) positron emission tomography (PET) has been widely used for the initial staging of various malignant diseases. Similar to other types of lymphomas, follicular lymphoma exhibits a high avidity for 18F-FDG. The percentage of patients with 18F-FDG-avid follicular lymphoma has been reported to range from 91% to 100%. On the other hand, few studies have addressed the use of 18F-FDG-PET for gastrointestinal involvement of follicular lymphoma.

Research frontiers

Follicular lymphoma is the second most common form of non-Hodgkin's lymphoma in Western countries. Moreover, the number of patients newly diag-

nosed with gastrointestinal follicular lymphoma has been increasing. In the area of management of gastrointestinal follicular lymphoma, the research hotspot is what role does 18F-FDG-PET play in the initial staging.

Innovations and breakthroughs

In 2004, Hoffmann *et al* reported eight cases of follicular lymphoma localized in the duodenum, and they noted that 18F-FDG did not accumulate in any of those patients. Subsequently, several authors described follicular lymphoma patients with positive 18F-FDG uptake in the gastrointestinal tract including the stomach, duodenum, jejunum, ileum, cecum, and colon, but the number of reported cases is limited. Taken together, these inconsistent results indicate that the sensitivity of 18F-FDG-PET for gastrointestinal lesions of follicular lymphoma has not been sufficiently evaluated. Here, the authors provide the first report of the sensitivity of 18F-FDG-PET for the detection of gastrointestinal involvement in follicular lymphoma patients, 46.3%.

Applications

This study results indicates that 18F-FDG-PET may represent a complementary modality for the management of patients with gastrointestinal follicular lymphoma.

Peer review

The authors assessed 41 patients with follicular lymphoma and gastrointestinal involvement, who underwent 18F-FDG-PET and endoscopic evaluations. They demonstrated 46.3% true-positive of 18F-FDG PET in the lesions of the involved gastrointestinal tract. False-negative 18F-FDG uptake was 58.5%, and false-positive was 12.2%. They emphasized on the complementary role of 18F-FDG-PET scan in the diagnosis of gastrointestinal involvements in follicular lymphoma patients. This is a good descriptive study in this query which there is just limited available data.

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Clonal evolution of hepatitis B virus polymerase gene mutations during lamivudine-adeфовir combination treatment

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Abstract

AIM: To identify hepatitis B virus polymerase gene mutations during antiviral therapy using lamivudine-adeфовir sequential monotherapy followed by lamivudine-adeфовir combination therapy.

METHODS: The patient cohort included four adult chronic hepatitis B patients who had undergone sequential monotherapy, first with lamivudine (LMV) and then, after developing viral breakthrough, with adefovir (ADV) therapy. All of the patients had non-response or viral breakthrough after LMV-ADV sequential monotherapy, which resulted in the switching of their antiviral regimen to LMV-ADV combination therapy. Eleven serum samples from the four patients who

showed non-response to rescue LMV-ADV combination therapy were collected sequentially at a time before the antiviral treatment and then during the LMV monotherapy, ADV monotherapy, and LMV-ADV combination therapy. For the genotypic analysis, the whole 1310-bp polymerase gene region was amplified, cloned and sequenced.

RESULTS: All patients had been previously treated with 100 mg of LMV once daily for a 15- to 26-mo period. The emergence of resistance mutations to LMV, such as rtM204V/I and/or rtL180M, were found in all patients. Their antiviral regimens were switched to ADV monotherapy as the second line treatment. All patients had viral breakthrough or non-response after the LMV-ADV sequential monotherapy. ADV-resistant mutations were detected after 13 to 19 mo of LMV-ADV sequential monotherapy. The rtA181V/T mutations were predominantly identified during the ADV treatment in the LMV-resistant patients. Twenty-seven of 38 clones were combined with an amino acid change at rt181; three clones had mutations in rt236 and one clone had a combined mutation. The rtA181V/T mutations were not suppressed by the LMV-ADV combination therapy. Thirty-nine of 64 clones showed an rtA181V/T mutation and six clones showed combined mutations in rt181 and rt236. Mutations in rt204 re-emerged during the combination treatment. The rt181 and rt204 mutations did not co-exist in one clone.

CONCLUSION: Add-on lamivudine therapy with adefovir for adefovir resistance may not suppress the pre-existing adefovir-resistant mutation that develops during lamivudine-adeфовir sequential monotherapy.

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Key words: Hepatitis B virus; Lamivudine; Adefovir; Mutation; Drug resistance

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INTRODUCTION

Chronic infection with hepatitis B virus (HBV) can result in cirrhosis and hepatocellular carcinoma. The ultimate goal of antiviral therapy for chronic hepatitis is to prevent its devastating complications of decompensated liver cirrhosis and hepatocellular carcinoma. In recent years, the treatment of chronic hepatitis B (CHB) has been improved by introducing nucleos(t)ide analogs (NAs) such as lamivudine (LMV), adefovir (ADV), entecavir (ETV), telbivudine (LDT), and tenofovir (TDF)^[1-5]. However, drug-resistant HBV mutants frequently arise during treatment with NAs, leading to treatment failure and progression to liver disease^[6]. ADV, a phosphate acyclic nucleotide analog of adenosine monophosphate, is a potent inhibitor of HBV reverse transcriptase in the wild-type HBV, and in lamivudine-, telbivudine-, and entecavir-resistant mutants^[7,8]. Switching to ADV has become the most widely-used option since the emergence of LMV-resistant mutations, especially in Asian countries^[8]. The 5-year cumulative probability of genotypic resistance and viral breakthrough was 65.6% and 61.8%, respectively, in LMV-resistant CHB patients^[3]. Unfortunately, ADV resistance occurs more frequently in the second-line treatment of lamivudine-resistant patients than in treatment-naïve patients^[4]. According to large-scale clinical studies of ADV, the rtA181V and rtN236T mutations associated with resistance to ADV were identified in the HBV DNA polymerase gene^[5]. *In vitro* studies demonstrated that those two mutations decreased susceptibility to ADV by 4.3- to 23-fold^[8,9]. Other minor mutations resistant to ADV, such as rtS85A, rtT184S, rtQ215S, rtP237H, rtN238D, and rtM250L, have been reported, but the significance of these mutations is unclear^[4,10,11]. In 2006, it was reported that a novel ADV-resistant HBV variant rtI233V (substitution of isoleucine by valine) was reported to be a cause of primary resistance to ADV^[12,13]. Studies also showed that rtI233V was sensitive to TDF *in vitro*. However, this result was not confirmed in recent studies. To our knowledge, little is known about clonal analysis in patients who successively received LMV monotherapy, ADV monotherapy, and LMV-ADV combination therapy. We investigated the clonal evolution of genetic mutations in the HBV polymerase gene in non-responders to LMV-ADV sequential monotherapy followed by LMV-ADV combination therapy.

MATERIALS AND METHODS

Patients

From January 2003 to July 2007, four adult chronic hepatitis B patients who showed non-response during LMV-ADV combination therapy were identified at Konkuk University Medical Center in Seoul, South Korea. All patients had undergone sequential monotherapy, first with LMV and then, after developing viral breakthrough, with ADV therapy. All of the patients had non-response or viral breakthrough after the LMV-ADV sequential monotherapy which resulted in the switching of their antiviral regimen to LMV-ADV combination therapy. The patients were then followed from when they were switched to an antiviral regimen. These patients gave written informed consent for the treatment of CHB and testing for antiviral resistance. Approval for this study was obtained from the Institutional Review Board at the Konkuk University Medical Center.

Before ADV plus LMV, the clinical and laboratory data of each patient were analyzed, including the quantification of serum HBV DNA levels. After the initiation of LMV-ADV combination therapy, clinical, biochemical, and virological parameters, such as aspartate aminotransferase, alanine aminotransferase (ALT), and serum HBV DNA levels, were monitored every month up to three months. A clinical diagnosis of liver cirrhosis was based on imaging findings, using methods such as abdominal computed tomography or magnetic resonance imaging, together with compatible clinical features such as esophageal varices or thrombocytopenia. The severity of liver cirrhosis was classified according to the Child-Pugh score.

Definitions

Viral breakthrough was defined as a $\geq 1 \log_{10}$ IU/mL increase in HBV DNA from the nadir on two consecutive occasions after an initial virologic response or an initial decline in HBV DNA by $\geq 1 \log_{10}$ IU/mL. Non-response was defined as a decrease in serum HBV DNA $\leq 2 \log_{10}$ IU/mL after at least 24 wk of therapy.

HBV DNA quantification

Serial samples were collected from each patient at the time of initiation of each antiviral agent, every three months during treatment and at the time of viral breakthrough and stored frozen at -80°C . The serum HBV DNA levels were quantified by real-time polymerase chain reaction, with a lower limit of detection of $2.6 \log_{10}$ copies/mL (approximately 400 copies/mL). The HBV DNA quantification was also performed before cloning and sequencing using the COBAS TaqMan HBV test (Roche Molecular Systems Inc, Pleasanton, CA, United States).

Detection of antiviral-resistant mutations

Using the stored serum samples, HBV DNA polymerase mutations were analyzed using a matrix-assisted laser desorption/ionization time of flight mass spectrometry

Table 1 Clinical features of the chronic hepatitis B patients treated with lamivudine-adefovir sequential monotherapy followed by lamivudine-adefovir combination therapy

	Patient 1	Patient 2	Patient 3	Patient 4
Baseline characteristics				
Gender	Male	Male	Male	Male
Age (yr)	39	58	51	42
Liver disease	Cirrhosis	Cirrhosis	Cirrhosis	Cirrhosis
Initial viral load (copies/mL)	3.0×10^7	3.3×10^7	7.0×10^8	5.3×10^8
HBeAg	+	+	+	+
During LMV mono-therapy				
Duration of LMV (mo)	26	22	15	25
LMV-resistant mutants ¹	M204I + L180M	M204V	M204I + L180M	M204I + L180M
Response to LMV	VT	VT	VT	VT
During ADV mono-therapy				
Duration of ADV (mo)	18	16	19	13
ADV-resistant mutants ¹	A181V	A181V/T	A181V	A181V
Response to ADV	NR	NR	NR	NR
During LMV-ADV combination therapy				
Viral load at the start of				
LMV-ADV (copies/mL)	8.0×10^7	8.0×10^7	1.2×10^5	3.1×10^6
Duration of LMV-ADV (mo)	9	13	11	15
Resistant mutants ¹	A181V	A181T + M204I	A181V	A181V
Response to LMV-ADV	NR	NR	NR	NR
Current treatment	LMV + ADV	LMV + TDF	LMV + TDF	LMV + TDF

¹RFMP analysis was used for the detection of resistant mutations in the hepatitis B virus polymerase gene. NR: Non-response; VT: Viral breakthrough; LMV: Lamivudine; ADV: Adefovir; TDF: Tenofovir.

(MALDI-TOF MS)-based genotyping assay, termed a restriction fragment mass polymorphism (RFMP; GeneMatrix, Yongin, South Korea)^[14]. LMV-resistant mutations at rt180 or rt204, and ADV-resistant mutations at rt181 or rt236, were detected using this method.

Cloning of hepatitis B viral polymerase gene

For the genotypic analysis, the entire 1310-bp polymerase gene region was amplified, cloned, and sequenced. To analyze the complete sequence of HBV reverse transcriptase (RT) from four patients with resistant mutations during LMV-ADV combination therapy, we isolated HBV DNA from the patients' sera at the time of viral breakthrough during the antiviral therapy. DNA was extracted using a QIAamp MinElute virus spin kit (Valencia, CA, United States) according to the manufacturer's protocol. To generate HBV1.2mers harboring patient-derived RT mutations, we amplified the RT region of the HBV genome using the following primers: forward, 5'-AAT CTT CTC GAG GAC TGG GGA CCC TGC ACC-3' (the *Xho* I site is underlined) and reverse, 5'-GAG CAG CCA TGG GAA GGA GGT GTA TTT CCG-3' (the *Nco* I site is underlined)^[15]. The purified products were digested with *Xho* I and *Nco* I, after which the wild-type RT sequence of the HBV1.2mer was swapped for the patient-derived sequence. All clones were confirmed by sequencing.

RESULTS

Characteristics of the patients enrolled in this study

Four patients were included in this study. The age of the patients was between 39 and 58 years; all were men who had cirrhosis. All patients had been previously treated with

100 mg of LMV once daily for a 15- to 26-mo period. The emergence of resistant mutations to LMV, such as rtM204V/I and/or rtL180M, was found in all patients. Their antiviral regimens were switched to ADV monotherapy (10 mg/d) as the second line treatment and no patient required a dose reduction. All patients had viral breakthrough or non-response after the LMV-ADV sequential monotherapy (Table 1). ADV-resistant mutations, including rtA181V/T or rtN236T, were detected after 13 to 19 mo of LMV-ADV sequential monotherapy. LMV was added to the ongoing ADV treatment as a salvage therapy.

Virologic response during lamivudine-adefovir combination therapy

The outcome of the LMV-ADV combination therapy in the patients with ADV resistance is demonstrated in Table 1. The duration of the combination therapy was 9 to 15 mo. Patient 1 had viral breakthrough after 26 mo of LMV monotherapy. LMV was stopped and the patient was switched to ADV. Non-response occurred after 18 mo of ADV monotherapy. Patient 2 experienced non-response after 16 mo of second-line ADV monotherapy. Patient 3 had viral breakthrough after 15 mo of LMV monotherapy. Non-response occurred after 19 mo of second-line ADV monotherapy. Patient 4 experienced non-response after 13 mo of second-line ADV monotherapy. One patient continued to receive LMV-ADV combination therapy and three patients were switched to TDF-LMV combination therapy as a salvage therapy.

Evolution of HBV polymerase mutations during antiviral treatment

Clonal analyses were performed in four patients harbor-

ing antiviral resistant mutants at the time of viral breakthrough during the antiviral therapy. From eleven serum samples, we obtained and analyzed a total of 38 clones of the HBV polymerase gene during the ADV monotherapy and a total 64 of clones during the LMV-ADV combination therapy. The sequences were compared against the sequence from genotype C HBV (NCBI GenBank accession no. GQ872210), a wild type HBV genome isolated from the serum of a 25-year-old HBeAg-positive asymptomatic HBV carrier in our hospital.

During the ADV monotherapy, 27 of 38 clones were combined with an amino acid change at rt181. Four clones had a single mutation at rt236, one of which showed an rtA181T + N236T double mutation. During the treatment period with LMV-ADV combination therapy, 39 of 64 clones showed an rtA181V/T mutation. The viral quasispecies evolved with the extension of the combination therapy, followed by LMV-ADV sequential monotherapy (Figure 1). In all patients, clonal analyses during the LMV-ADV combination therapy revealed the maintenance of the rtA181V/T mutant that emerged during the ADV monotherapy (Figure 2). The combined mutation of rtA181V/T + N236T was detected in seven clones. The LMV-resistant mutation rtM204I reemerged in two clones. The combined LMV-resistant mutation rtM204I/V + L1801M was also detected in seven clones. However, the rt181 and rt204 mutations did not co-exist in the same clone.

The clinical course of four patients in whom the clonal analyses were performed during the antiviral treatment is illustrated in Figure 2. In patient 1, the previously noted resistant mutations to LMV, rtM204I, and rtL180M were suppressed, but a resistant mutation to ADV, rtA181V, was detected after the second-line ADV monotherapy. That ADV-resistant mutation remained detectable after 4 mo of combination therapy and a resistant mutation to LMV, rtM204I, was also detected. In patient 2, a resistant mutation to LMV, rtM204V, was suppressed, but a resistant mutation to ADV, rtA181V/T, was detected after the LMV-ADV sequential monotherapy. After 3 mo of LMV-ADV combination therapy, a resistant mutation to ADV remained detectable. In patient 3, the previously noted resistant mutations to LMV, rtM204I and rtL180M were suppressed, but a resistant mutation to ADV, rtA181V/T, was detected after 18 mo of second-line ADV monotherapy. After 10 mo of LMV-ADV combination therapy, a resistant mutation to ADV, rtA181V, remained detectable. In patient 4, resistant mutations to LMV, rtM204V and rtL180M, were suppressed, but a resistant mutation to ADV, rtA181V, was detected after the LMV-ADV sequential monotherapy. After 13 mo of LMV-ADV combination therapy, a resistant mutation to ADV remained detectable, and a resistant mutation to LMV, rtM204I, was also detected.

DISCUSSION

Sequential NA monotherapy can promote the selection for multidrug-resistant HBV mutants, but little is known

about the multidrug-resistant HBV based on clonal analysis. We analyzed the full-length genomic sequence of HBV polymerase in patients who successively received LMV monotherapy, ADV monotherapy, and LMV-ADV combination therapy. This study demonstrated that an ADV mutant, rtA181V/T, was not suppressed by LMV-ADV combination therapy.

Seven drugs are approved by the Food and Drug Administration in the United States for the treatment of HBV infection: interferon alpha, pegylated interferon α -2a, LMV, ADV, ETV, LDT, and TDF^[1]. The treatment of chronic hepatitis B has been improved by the introduction of these NAs. However, drug-resistant HBV mutants frequently arise after the extended use of NAs, leading to treatment failure and progression to liver disease^[16]. The drug resistant mutations selected with one agent may affect the efficacy of the other NAs^[17]. Several major HBV-evolutionary NA-resistance pathways have been characterized^[17]. The rtM204V/I pathway is responsible for the resistance to the L-nucleosides, such as LMV, LDT, and ETV^[17]. The rtN236T pathway is responsible for ADV and TDF resistance^[17]. The rtA181T/V pathway is associated with resistance to LMV and ADV, and is a potential multidrug resistance pathway^[17].

LMV resistance increases progressively over the course of treatment; 14%-32% of patients become resistant to the drug each year after the treatment is initiated, and more than 80% are resistant after 48 mo of treatment^[6,18]. The resistance to LMV was mediated primarily by mutations rtM204I/V \pm rtL180M and rtA181T/V^[6,19]. Viral replication levels may be increased by compensatory mutations, such as rtL80V/I, rtI169T, rtV173L, rtT184S/G, rtS202I, and rtQ215S^[6,20-23].

In treatment-naïve patients, the cumulative percentage of patients who showed ADV resistance has been reported to be 0% in year 1, 3% in year 2, 11% in year 3, 18% in year 4, and 28% in year 5^[4]. The cumulative percentage of patients who showed ADV resistance after ADV monotherapy in the LMV-resistant patients has been reported to be 18% in year 1 and 25% in year 2^[4,14]. The resistance to ADV was initially associated with the rtA181T/V and rtN236T substitutions^[6,24-26]. The rtN236T mutation does not significantly affect the sensitivity to LMV, LDT, or ETV, but does decrease the efficacy of TDF *in vitro*^[6,27]. The rtA181T mutation has been observed in patients who developed viral resistance to LMV or to ADV following LMV breakthrough^[19,28]. The other mutation, rtA181V, has been observed in patients who developed ADV resistance^[4,14,29-31]. Villet *et al*^[24] suggested that a single amino acid change at position rt181 induced cross-resistance to LMV and ADV. They also reported that the rtA181V/T substitution induced a decreased susceptibility not only to the L-nucleoside LMV, but also to the alkyl phosphonates ADV and TDF^[6,24].

According to a recently reported meta-analysis, the therapeutic potential of the combination of ADV with LMV is beneficial for treating LMV-resistant CHB patients with ADV-associated mutations^[32]. Switching to ADV monotherapy has been widely used in CHB patients

Antiviral agent (sampling time)	Number of clones	Amino acid sequence at RT position									
		75 91	37 47	163	189	210	230	237	241	257	
GQ872210 1-1-1 and 6 1-1-5 1-1-10 1-1-14 and 15 1-1-16 1-1-17 1-1-18 1-1-19	21 36 38 46	53 54 55 60 66 91	106 109 118 121 122 124 130	132 134 135 148 149 180 181 199	200 204 214 219 221 223 224 229	236 256	257 259 266 269 271 278 285 291 299	305 323 337 344			
	A N T F S T H K L I	S P T N I Y Q	L D S Y K	L A L A M V S F S I L V							
	C	C	E	V V	Y A						
	C	C	E	V V	Y A						
	C	C	E	V V	Y A						
	C	C	E	V V	Y A						
	C	C	E	V V	Y A						
	C	C	E	V V	Y A						
	C	C	E	V V	Y A						
	C	C	E	V V	Y A						
	C	C	E	V V	Y A						
	C	C	E	V V	Y A						
	C	C	E	V V	Y A						
	C	C	E	V V	Y A						
	C	C	E	V V	Y A						
	C	C	E	V V	Y A						
	LMV + ADV (4 mo)	1-2-2									
		1-2-3									
		1-2-7									
	1-2-8										
	1-2-9 and 14										
	1-2-10										
	1-2-12										
	1-2-16										
	1-2-20										

Antiviral agent (sampling time)	Number of clones	Amino acid sequence at RT position									
		3 17 75	91	163	189	210	230	237	241	257	
GQ872210 2-1-5 2-1-6 and 12 2-1-7 2-1-8 and 13 2-1-9 2-1-10 2-1-11 2-1-15	16 73	78 80 81 95 98 121 129 132 166 181 191	215 221 223 229	234 236 241 247	267 269 279 317						
	I L S L S P M N M L F A V	Q F S L H N K L	Q L I N S								
	T										
	LMV + ADV (1 mo)	2-2-1									
	2-2-2										
	2-2-4										
	2-2-5										
	2-2-7										
	2-2-9										
	2-2-1 and 15										
	2-2-13										
	2-2-16										
LMV + ADV (3 mo)	2-3-1										
	2-3-2										
	2-3-6										
	2-3-7										
	2-3-8, 12 and 15										
	2-3-11										
	2-3-16										

Antiviral agent (sampling time)	Number of clones	Amino acid sequence at RT position																																								
		37 47	75 91	163	189	210	230	237	241	257																																
		F	A	B	C	D	E																																			
		H N V P G V L F L A F L M	S E V Q L Q L P N C R L P A H																																							
ADV (18 mo)	GQ872210	36	38	40	55	85	84	99	104	142	146	151	155	178	180	181	183	199	204	221	223	230	233	236	251	256	263	266	267	269	271	276	277	279	287	289	294	310	320	337		
	3-1-1	S	Q	Q	L																Y	Y	A				D	D	K	K	K	P	K	K	K	K	K	K	K	N		
	3-1-3	S	Q	Q																	Y	Y	A				D	D	K	K											N	
	3-1-4	S	Q	Q																	Y	Y	A				D	D	K	K												N
	3-1-5	S	Q	Q																	Y	Y	A				D	D	K	K												N
	3-1-6	S	Q	Q																	Y	Y	A				D	D	K	K												N
	3-1-7 and 10	S	Q	Q																	Y	Y	A				D	D	K	K												N
	3-1-8	S	Q	Q																	Y	Y	A				D	D	K	K												N
	3-1-9	S	Q	Q																	Y	Y	A				D	D	K	K												N
LMV + ADV (4 mo)	3-2-1	S	Q	Q	A															Y	Y	A				D	D	K	K												N	
	3-2-2	S	Q	Q																Y	Y	A				D	D	K	K													N
	3-2-3	S	Q	Q																Y	Y	A				D	D	K	K													N
	3-2-4	S	Q	Q																Y	Y	A				D	D	K	K													N
	3-2-5	S	Q	Q																Y	Y	A				D	D	K	K													N
	3-2-6, 19 and 22	D	S	Q																Y	Y	A				D	D	K	K													N
	3-2-21	S	Q	Q																Y	Y	A				D	D	K	K													N
	3-2-23	S	Q	Q																Y	Y	A				D	D	K	K													N
	3-2-24	D	S	Q																Y	Y	A				D	D	K	K													N
	3-2-26	S	Q	Q																Y	Y	A				D	D	K	K													N
LMV + ADV (10 mo)	3-3-2	D	S	Q															Y	Y	A				D	D	K	K													N	
	3-3-5	S	Q	Q															Y	Y	A				D	D	K	K														N
	3-3-6	S	Q	Q															Y	Y	A				D	D	K	K														N
	3-3-7	S	Q	Q															Y	Y	A				D	D	K	K														N
	3-3-8	S	Q	Q															Y	Y	A				D	D	K	K														N
	3-3-10	D	S	Q	D														Y	Y	A				D	D	K	K														N
	3-3-11	S	Q	Q															Y	Y	A				D	D	K	K														N
3-3-14	D	S	Q															Y	Y	A				D	D	K	K														N	

D	Antiviral agent (sampling time)	Number of clones	Amino acid sequence at RT position																											
			31	36	42	54	62	65	80	103	122	127	129	131	180	181	204	216	221	228	232	234	236	267	271	275	317	332	333	
			F	A	B	C	D	E																						
ADV (13 mo)	GG872210		D	N	L	T	A	N	L	V	I	G	M	D	L	A	M	H	F	L	G	H	N	Q	Q	K	S	C	K	
	4-1-2 and 4											H	L										L	L	L	A	R	N		
	4-1-5											H	L										L	L	L	A	R	N		
	4-1-6											H	L										L	L	L	A	R	N		
	4-1-8											H	L										L	L	L	A	R	N		
	4-1-10											H	L										L	L	L	A	R	N		
	4-1-11											H	L										L	L	L	A	R	N		
	4-1-14											H	L										L	L	L	A	R	N		
	4-1-19											H	L										L	L	L	A	R	N		
LMV + ADV (8 mo)	4-2-3										I	N	Y	Q	H	M	I						L	L	L	A	R	N		
	4-2-4										I	N	Y	Q	H	V							L	L	L	A	R	N		
	4-2-5										I	N	Y	Q	H	T							L	L	L	A	R	N		
	4-2-7										I	N	Y	Q	H	T							L	L	L	A	R	N		
	4-2-18										I	N	Y	Q	H	T							L	L	L	A	R	N		
	4-2-22										I	N	Y	Q	H	T							L	L	L	A	R	N		
	4-3-2											V	N	Y	Q	H	V						L	L	L	A	R	N		
LMV + ADV (13 mo)	4-3-3										I	N	Y	Q	H	L	G						L	L	L	A	R	N		
	4-3-4										I	N	Y	Q	H	L							L	L	L	A	R	N		
	4-3-6										I	N	Y	Q	H	L							L	L	L	A	R	N		
	4-3-8 and 9										I	N	Y	Q	H	L							L	L	L	A	R	N		
	4-3-10										I	N	Y	Q	H	L							L	L	L	A	R	N		
	4-3-11										I	N	Y	Q	H	L	M	I					L	L	L	A	R	N		

Figure 1 Evolution of antiviral mutations in hepatitis B virus polymerase based on the clonal analysis of serial samples in patients. A: Patient 1; B: Patient 2; C: Patient 3; D: Patient 4. The boxes and capital letters denote the domains of the hepatitis B virus polymerase gene. ADV: Adefovir; LMV: Lamivudine; RT: Reverse transcriptase.

with an LMV-resistant mutation in South Korea and has been reported to be cost-effective so far^[3,33]. However, LMV-ADV sequential monotherapy has led to the frequent development of ADV resistance^[4,5,14]. Combination therapy has been a practical treatment modality for patients who had an ADV-resistant mutation after LMV-ADV sequential monotherapy because TDF was not available. However, to date, the best treatment option for those with ADV resistance has not been identified.

In the present study, we observed the emergence of mutants harboring the rtA181V/T mutation in patients who were treated with LMV during ADV monotherapy^[4].

The emergence of the rtA181V/T mutant was associated with non-response or viral breakthrough during LMV-ADV sequential monotherapy and LMV-ADV combination therapy. We performed the RFMP assay to detect mutations resistant to antiviral agents. This assay enables better quantitative detection of mixed populations, but the result revealed the possibility of the presence of substitutions at other sites in the HBV reverse transcriptase. To analyze the complete sequence, we sequenced all of the clones.

In the present study, clonal analyses during LMV-ADV combination therapy revealed the maintenance of an rtA181V/T mutant that emerged during ADV

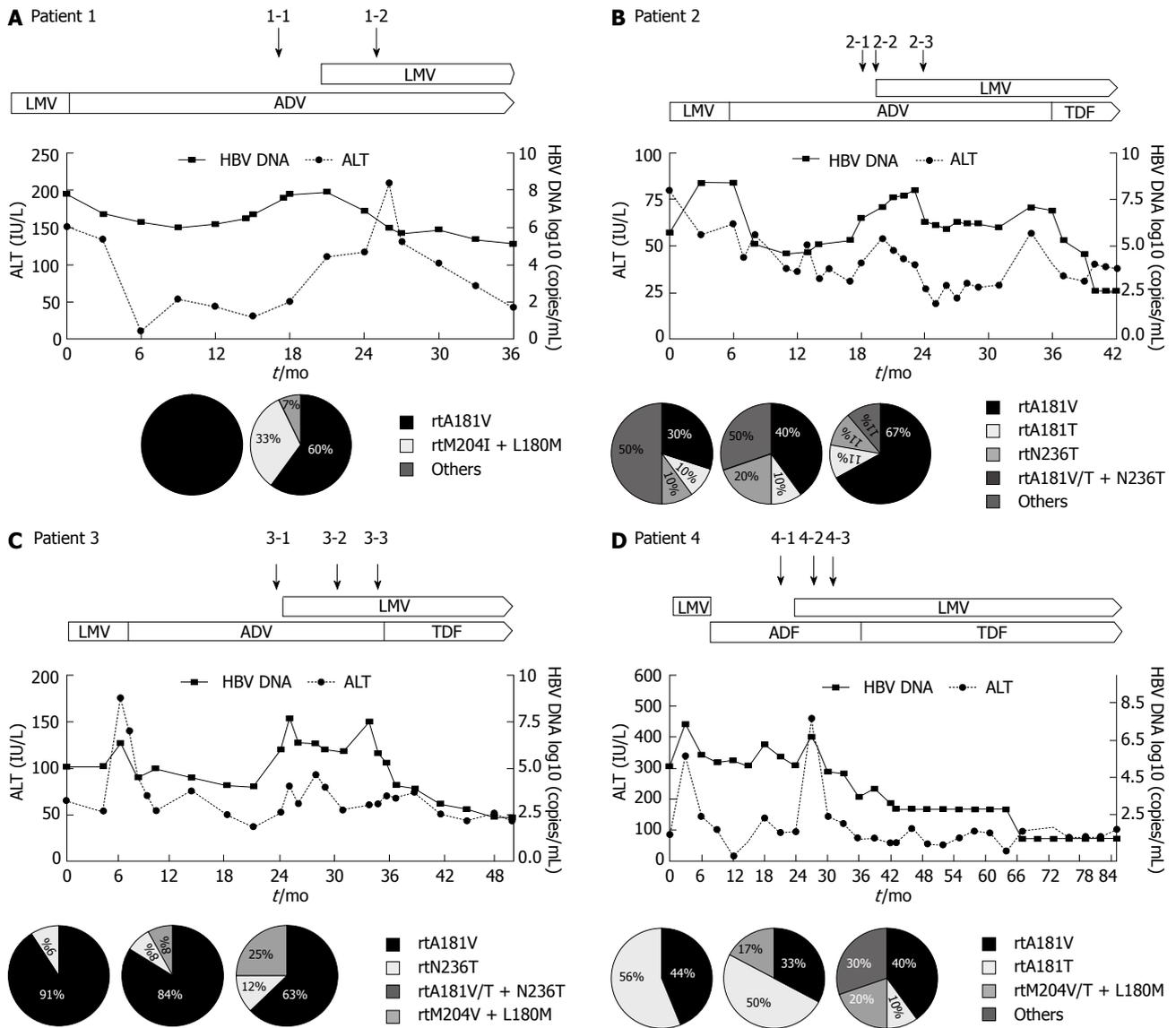


Figure 2 Clinical course and evolution of antiviral mutations in four patients. The arrows and numbers denote the time of the sampling of the serum and the sample number. ADV: Adefovir; ALT: Alanine aminotransferase; LMV: Lamivudine; TDF: Tenofovir; HBV: Hepatitis B virus.

monotherapy. The LMV-resistant mutations rtM204I/V and/or rtL180M reemerged during the LMV-ADV combination therapy. These results showed that LMV-ADV sequential monotherapy may induce the risk of the emergence of ADV-resistant strains in patients who were treated with LMV. Our observation also showed that add-on LMV therapy for ADV resistance might not suppress the pre-existing ADV-resistant mutation that emerged during the sequential treatment with ADV monotherapy for LMV resistance. This result was consistent with the study reported by Villet *et al.*^[24], who showed that a single amino acid change at position rt181 might induce cross-resistance to LMV and ADV.

This study demonstrated that a poor response to LMV-ADV combination therapy did not always occur due to the co-localization of each drug resistance mutation in the same genome. Antiviral agents with a low or modest antiviral potency and low genetic barrier of resistance, such as LMV or ADV, may strengthen viral fitness while not

being strong enough to suppress the drug mutation, and the combination of stronger drugs, such as ETV and TDF, with a high antiviral potency and high genetic barrier of resistance, would be more effective in the treatment of non-responders to LMV-ADV sequential monotherapy. Viral fitness, defined as the ability to produce infectious progeny in a defined environment, was proposed to be one of the most important factors in the process of selecting resistant mutants^[34-36]. We recently reported that TDF plus LMV is a useful therapeutic option for patients with resistance or non-response to ADV, particularly in patients with cirrhosis or who had previously received sequential LMV-ADV monotherapy. TDF-containing therapy was shown to result in a rapid viral load reduction, with HBV becoming undetectable in ADV-resistant patients. At three months, a viral load reduced to a level of 4 log₁₀ copies/mL was achieved in all six patients (100%), and fell to below the limit of detection in three patients (50%)^[37].

Our study has some limitations: the small number

of patients and the variations in the initial HBV DNA levels. These limitations may make establishing this combination as an optimal rescue therapy for patients with ADV-resistant HBV difficult. To verify the efficacy and safety of this regimen, further large cohort studies are warranted.

In conclusion, sequential LMV and ADV monotherapy may favor the emergence of HBV variants with cross-resistance to analogs of different chemical classes. The emergence of rtA181V/T with or without rtM204V/I resulted in viral breakthrough during ADV monotherapy and LMV-ADV combination treatment, as well as a poor response to ADV. Therefore, LMV-ADV combination therapy is not effective in patients with ADV-resistant mutations after LMV-ADV sequential monotherapy. This result may be attributed to an ADV mutation, rtA181V/T, which is responsible for cross-resistance to LMV and ADV. Therefore, careful monitoring and more effective antiviral agents should be considered in chronic hepatitis B patients with multiple resistance mutations.

COMMENTS

Background

The treatment of chronic hepatitis B has been improved by introducing nucleos(t)ide analogs such as lamivudine, adefovir, entecavir, telbivudine, and tenofovir. However, drug-resistant hepatitis B virus (HBV) mutants frequently arise during treatment with nucleos(t)ide analogs. Adefovir resistance occurs more frequently in the second-line treatment of lamivudine-resistant patients than in treatment-naïve patients.

Research frontiers

Little is known about clonal analysis in patients who successively received lamivudine monotherapy, adefovir monotherapy, and lamivudine-adefovir combination therapy. This research hotspot is to demonstrate HBV polymerase gene mutations during antiviral therapy using lamivudine-adefovir sequential monotherapy followed by lamivudine-adefovir combination therapy.

Innovations and breakthroughs

According to a recently reported meta-analysis, the therapeutic potential of the combination of adefovir with lamivudine is beneficial for treating lamivudine-resistant chronic hepatitis B patients with adefovir-associated mutations. However, lamivudine-adefovir sequential monotherapy has led to the frequent development of adefovir resistance. In the present study, clonal analyses during lamivudine-adefovir combination therapy revealed the maintenance of an rtA181V/T mutant that emerged during adefovir monotherapy. The lamivudine-resistant mutations rtM204I/V and/or rtL180M reemerged during lamivudine-adefovir combination therapy.

Applications

The study results suggest that add-on lamivudine therapy with adefovir for adefovir resistance may not suppress the pre-existing adefovir-resistant mutation that develops during the lamivudine-adefovir sequential monotherapy.

Peer review

Combination therapy of lamivudine and adefovir has been widely used in lamivudine-resistant chronic hepatitis B. However, a molecular mechanism is not clearly known by which non-response to combination therapy occurs. This is an interesting study to demonstrate the clonal evolution of HBV drug-resistant mutations during sequential and combination therapy in lamivudine-resistant chronic hepatitis B.

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Human epidermal growth factor receptor-2 in oesophageal cancers: An observational study

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Abstract

AIM: To determine the incidence of human epidermal growth factor receptor 2 (HER2) over expression in oesophageal cancers.

METHODS: A retrospective study, of one hundred consecutive cases of endoscopic histological samples of oesophageal cancers from a single British cancer network were included. Cancer cases were diagnosed between April 2007 and June 2010. HER2 over expression was assessed using immunohistochemistry, those that scored "0" and "+1" were considered "negative" for HER2; those that scored "+3" were considered "Positive". Cases that were scored "+2" on immunohistochemistry further went on to have *HER2* gene analysis using the Ventana HER brightfield dual-colour *in situ* hybridisations (HER B DISH) assay and either came back to be positive or negative for HER2 over expression. Overall survival was measured from date of histological diagnosis until date of death. 93% of the cases were followed up till five years or death, and all were followed up till two years. Cases of gastro-oesophageal junctional tumours were excluded.

RESULTS: The median age of our sample was 66 years (range: 38-91 years). Eighty one were male and 19 female. Ninety-one of the cases were adenocarcinoma of the oesophagus and the rest were cases of squamous cell carcinoma. The anatomical distribution of the tumours was; upper oesophagus 2, middle oesophagus 11, and 87 were in the lower oesophagus. Operative resection was completed in 15 cases; seven cases had attempted surgical resections, i.e., open and close, 33 patients received definitive chemo-radiation and 52 had palliative treatment. Twenty-five of the cancers showed evidence of HER2 over expression, all were adenocarcinomas. Of the 25 cases that showed evidence of HER2 over expression, 21 (84%) were located in the lower third of the oesophagus. On staging, 24 out of the 25 HER2 positive cases were at stage 3 or more (13 at stage 3 and 11 at stage 4), For HER2 negative cases 37 were at stage 3 and 32 were staged as stage 4. Seventeen out of twenty five cases (68%) with HER2 over expression received palliative therapy, in comparison to thirty five out of seventy five (46.7%) in tumours not expressing HER2. No significant difference in overall survival was demonstrated between patients whose cancers showed evidence of HER2 over expression and those who did not; median overall survival for HER2 positive tumours was 15 mo (95%CI, 11-19 mo) compared to 13 mo (95%CI, 9-17 mo) for HER2 negative ones. Two years cumulative survival for cases with HER2 over expression was 33.7% compared to 31.6% in cases without HER2 over expression ($P = 0.576$). Only cancer's stage significantly affected overall survival on both univariate and multivariable analysis ($P = 0.034$ and $P = 0.009$ respectively). None of the patients included in this study received Trastuzumab.

CONCLUSION: Twenty-seven point five percent of oesophageal adenocarcinomas showed evidence of HER2 over expression. Routine testing for human HER2 in oesophageal adenocarcinomas can have significant implication on treatments offered to patients that may

potentially affect their prognosis.

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Key words: Oesophageal adenocarcinoma; Human epidermal growth factor receptor 2; Immunohistochemistry; Dual-colour *in situ* hybridisations; Trastuzumab

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INTRODUCTION

Gastro-oesophageal (GO) cancer is the fifth most common malignancy (and fourth most common cause of cancer death) in the United Kingdom, affecting around 13 500 people each year^[1]. Despite all recent advances in the diagnosis and management of these tumours their prognosis remains poor. Worldwide, gastric cancer is the second most common cause of cancer death^[2]; Oesophageal cancer has an overall five year survival of only 8%, and in view of the relatively high rate of chemotherapy resistance in oesophageal adenocarcinomas^[3,4], additional treatment options are desperately needed.

Human epidermal growth factor receptor 2 (HER2) is the second member of the cell membrane surface bound receptor tyrosine kinase family; this family is made up of four glycoproteins^[5]. Trastuzumab (Herceptin[®]) is a humanized monoclonal antibody targeting HER2^[6]; more evidence is mounting to support its use in upper gastrointestinal tract malignancies.

The ToGA trial studied the benefits of adding Trastuzumab to chemotherapy in patients with inoperable locally advanced, recurrent or metastatic adenocarcinoma of the stomach or GO junction^[7]. Addition of Trastuzumab to chemotherapy significantly improved overall survival. Previous studies to the ToGA trial demonstrated over expression of HER2 in gastric cancers^[8,9]. However, compared to gastric cancer, comprehensive data about HER2 expression in oesophageal adenocarcinomas are relatively scarce^[10].

In view of these facts, the HER2 status for oesophageal tumours was studied in a consecutive series of one hundred oesophageal cancer biopsy specimens obtained at endoscopy between April 2007 and June 2010 aiming to determine the incidence of HER2 in oesophageal cancer. Secondary end points were to assess how HER2 over expression reflects on patients' overall survival, tumours' location and what treatments patients received.

MATERIALS AND METHODS

HER2 assays were performed by an independent, validated and audited laboratory. A Ventana pathway HER2/neu 4B5 assay was utilised. Immunohistochemistry (IHC) scoring criteria were those described by Hofmann *et al.*^[11]. Samples were scored as 0, +1, +2 and +3. 0 and +1 are considered negative +3 is positive. Patients with +2 results underwent *HER2* gene analysis using the Ventana HER [brightfield dual-colour *in situ* hybridisations (HER B DISH)] assay and either shown to be positive or negative for HER2 over expression.

One hundred consecutive cases of oesophageal cancer were identified between April 2007 and June 2010 from a prospectively maintained British regional upper gastrointestinal tract cancer network database. Biopsies obtained at index endoscopy were analysed for HER2 status. All patients were followed up for two years or until death, 93% were followed up till five years or death. Death certification was obtained from the Office for National Statistics. Cases of GO junctional tumours were excluded.

Statistical analysis

Overall survival was measured from date of histological diagnosis until date of death. Methods appropriate for parametric data were used. Cumulative survival was calculated according to the life-table method of Kaplan and Meier, and differences in survival between groups of patients were analyzed with the log rank test. Correlation was assessed using the Pearson χ^2 test; $P < 0.05$ was considered a statistically significance value. Data analysis was performed using SPSS version 18.0 (Chicago, United States).

RESULTS

The median age of our sample was 66 ± 10.51 years (range: 38-91 years). Eighty-one were male and 19 female. The anatomical distribution of the tumours was; upper oesophagus 2, middle oesophagus 11, and 87 were in the lower oesophagus. Ninety-one of the cases were adenocarcinoma of the oesophagus and the rest were cases of squamous cell carcinoma. Operative resection was completed in 15 cases; eleven cases had an Ivor-Lewis oesophagectomy, three cases had Transhiatal oesophagectomy and one case underwent a three stage oesophagectomy. Seven cases had attempted surgical resections, i.e., open and close. In cases not suitable for surgery, 33 patients received definitive chemo-radiation and 52 had palliative treatment.

In this group of one hundred patients, 25 cases showed evidence of HER2 over expression. On IHC, 28 cases were scored as "0", 38 cases were scored as "+1" and 18 cases were scored as "+3". Of the 16 cases that were scored as "+2" and further underwent analysis using bright field DISH, seven were found positive. All tumours expressing HER2 were adenocarcinomas.

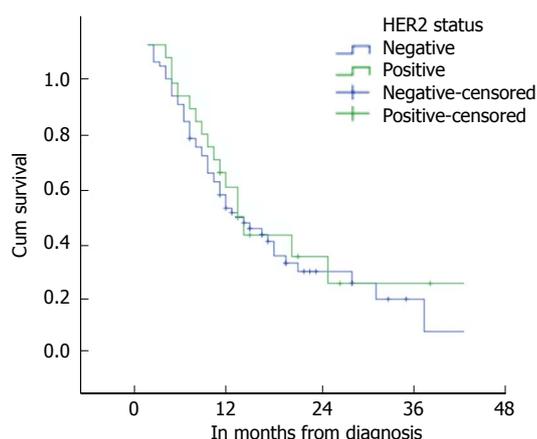


Figure 1 Kaplan-Meier survival curves for human epidermal growth factor receptor 2 positive vs negative groups. HER2: Human epidermal growth factor receptor 2.

None of the oesophageal squamous cell carcinoma cases showed evidence of HER2 over expression.

Of the 25 cases that showed evidence of HER2 over expression, 21 (84%) were located in the lower third of the oesophagus, four (16%) were in the middle third and none were in the upper third.

On staging, 24 out of the 25 HER2 positive cases were at stage 3 or more (13 at stage 3 and 11 at stage 4); one case was a stage 2 and none were a stage 1. For HER2 negative cases 37 and 32 were at stage 3 or 4 respectively, four cases were a stage 2 and two were a stage 1.

No significant difference in overall survival was demonstrated between patients whose cancers showed evidence of HER2 over expression and those who did not: Median overall survival for HER2 positive tumours was 15 mo (95%CI, 11-19 mo) compared to 13 mo (95%CI, 9-17 mo) for HER2 negative ones. This difference was not significant, both on univariate analysis ($P = 0.576$) and multivariable analysis ($P = 0.419$) that included patient's age, cancer stage, cancer location and HER2 status. On the other hand, cancers' stage significantly affected overall survival on both univariate and multivariable analysis ($P = 0.034$ and $P = 0.009$ respectively). Two years cumulative survival for cases with HER2 over expression was 33.7% compared to 31.6% in cases without HER2 over expression ($P = 0.576$). Figure 1 demonstrates Kaplan-Meier survival curves for the two groups.

Only two of the fifteen cases who had disease amenable to surgical resection showed HER2 over expression ($P = 0.208$). Both cases underwent Ivor-Lewis oesophagectomy. Definitive chemo-radiation was given to 33 patients, only six (18.2%) of them expressed HER2. Seventeen out of twenty five cases (68%) with HER2 over expression received palliative therapy, in comparison to thirty five out of seventy five (46.7%) in tumours not expressing HER2 ($P = 0.064$). None of the patients included in this study received Trastuzumab.

DISCUSSION

In our study population, 27.5% of oesophageal adeno-

carcinomas cases in a British regional cancer network demonstrated evidence of HER2 over expression, this rate is in line with previously published series from other countries^[12-14], and is as high, if not higher, than that found in other types of cancer.

In this study, HER2 positive oesophageal adenocarcinomas accounted for a small portion of cases which were suitable to have surgery or definitive chemo-radiation, 13.3% and 18.2% respectively; on the other hand, nearly two thirds of tumours expressing HER2 received palliative treatment (68%). These results may indicate a more aggressive behaviour for oesophageal adenocarcinomas expressing HER2, an observation expressed by other authors^[15].

In this series, HER2 overexpression did not affect overall survival; a recent series by Langer *et al*^[10] suggested otherwise, with tumours overexpressing HER2 having a shorter survival. Similar discrepancy has been described before and the literature holds conflicting data about the prognostic significance of HER2 overexpression^[16-19]. In our sample overall survival was measured from the time of histological diagnosis until time of death. This is inherently inaccurate in that it takes no account of the patient's onset and duration of symptoms nor the biological behaviour of the individual tumour. In Langer *et al*^[20] series, unlike ours, they used two different tissue microarrays for the assessment of HER2 status; with cores from different regions in the microarrays being assessed false-negative results due to intratumoral heterogeneity^[21,22] may have been eliminated. Moreover, for the determination of HER2 status, they used different *in situ* hybridisation methods, i.e., fluorescence *in situ* hybridisation, 3D fluorescence *in situ* hybridisation or bright field double *in situ* hybridisation, which eliminates false-negative results even further.

In this series none of the nine cases of oesophageal squamous cell carcinoma expressed HER2, there is, however, a growing body of evidence showing HER2 to be abnormally expressed in oesophageal squamous cell carcinomas and is associated with poor prognosis. Reported rates of HER2 overexpression in oesophageal squamous cell carcinoma can be as high as that found in oesophageal adenocarcinomas; researchers had described 26%-64% of oesophageal squamous cell carcinomas to test positive to HER2^[23,24]. HER2 over expression has been reported to be associated with extra mucosal tumour invasion and poor response to chemo radiation in oesophageal squamous cell carcinomas^[25,26].

Traditional phase III studies to ascertain the potential role of Trastuzumab in the treatment of oesophageal adenocarcinomas has proven difficult. A power calculation based on our results ($\alpha = 0.05$; 80% power) suggests a retrospective study of 180 patients would be required to clarify the situation. Having said that, a recent Phase I / II study by Safran *et al*^[27] exploring the use of Trastuzumab alongside chemo-radiotherapy in the treatment of oesophageal adenocarcinoma had initially aimed to recruit 25 patients; however, and after 42 mo, they only managed to enrol 19. Safran *et al*^[27] work had concluded

that adding Trastuzumab to chemo-radiotherapy regimens is safe and not associated with increased toxicity.

In the United Kingdom, The standard of care for patients with HER2 positive breast cancer is treatment with Trastuzumab^[28,29]. Also, the National Institute of Clinical Excellence guideline recommends Trastuzumab in combination with cisplatin and capecitabine or 5-fluorouracil in patients with metastatic adenocarcinoma of the stomach or GO junction expressing HER2. There is, however, no British guideline about the use Trastuzumab with HER2 positive oesophageal adenocarcinoma.

So, to summarise, this study demonstrates that in this British population of patients with oesophageal adenocarcinoma, 27.5% of them are HER2 positive. This is at least as high as that found in breast^[30] and probably higher than that found in gastric or GO junctional tumours. HER2 status in patients with oesophageal adenocarcinoma should be routinely assayed and patients should be offered treatment with Trastuzumab if their tumours showed evidence of HER2 over expression. New guidelines should be implemented in the United Kingdom in line with those issued in other countries to offer patients with oesophageal cancers a treatment that can potentially prolong their survival. More work is needed to look at HER2 status in patients with squamous carcinoma of the oesophagus.

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COMMENTS

Background

Over the last 20 years there has been a steady increase in the annual incidence of gastro-oesophageal (GO) cancers in the Western hemisphere. With that came a lot of research that brought developments to various aspects to these cancers diagnosis and treatment; never the less, these cancers continue to have a poor prognosis and new treatment modalities are desperately needed.

Research frontiers

Molecular targeted therapy offers a new frontier and hope to patients with GO cancers, especially since these cancers had shown resistance to conventional treatment modalities; i.e., chemotherapy and radiotherapy.

Innovations and breakthroughs

Molecular targeted therapy is becoming the standard of care for patients with suitable cancers of the oesophagus in many countries. In the United Kingdom, however, there are still no guidelines to recommend such therapy to British patients. This study demonstrates that the incidence of the target molecule for these new drugs in cases of oesophageal cancer in the United Kingdom is as high as that reported in other countries. British patients whose cancers express this molecule should be offered these new drugs.

Applications

Adding molecular targeted therapy to conventional treatments in patients with cancers of the oesophagus can potentially prolong their survival and improve their prognosis.

Peer review

The authors describe the results of human epidermal growth factor receptor 2

(HER2) assessment in esophageal cancers. They report that 27% of esophageal adenocarcinomas overexpressed HER2. The study is relatively straightforward and confirms existing knowledge on the subject.

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Effectiveness of *Saccharomyces boulardii* in a rat model of colitis

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Abstract

AIM: To investigate the effects of *Saccharomyces boulardii* (*S. boulardii*) in an experimental rat model of trinitrobenzene sulfonic acid (TNBS)-induced colitis.

METHODS: Thirty-two Wistar albino female rats were categorized into five groups. On the first day of the study, 50 mg TNBS was administered *via* a rectal catheter in order to induce colitis in all rats, except those in the control group. For 14 d, the rats were fed a standard diet, without the administration of any additional

supplements to either the control or TNBS groups, in addition to 1 mg/kg per day *S. boulardii* to the *S. boulardii* group, 1 mg/kg per day methyl prednisolone (MP) to the MP group. The animals in the *S. boulardii* + MP group were coadministered these doses of *S. boulardii* and MP. During the study, weight loss, stool consistency, and the presence of obvious blood in the stool were evaluated, and the disease activity index (DAI) for colitis was recorded. The intestines were examined and colitis was macro- and microscopically scored. The serum and tissue levels of tumor necrosis factor- α (TNF- α) and nitric oxide (NO) were determined, and fungemia was evaluated in the blood samples.

RESULTS: The mean DAI scores for the MP and *S. boulardii* + MP groups was significantly lower than the TNBS group (3.69 ± 0.61 vs 4.46 ± 0.34 , $P = 0.018$ and 3.77 ± 0.73 vs 4.46 ± 0.34 , $P = 0.025$, respectively). While no significant differences between the TNBS and the *S. boulardii* or MP groups could be determined in terms of serum NO levels, the level of serum NO in the *S. boulardii* + MP group was significantly higher than in the TNBS and *S. boulardii* groups (8.12 ± 4.25 $\mu\text{mol/L}$ vs 3.18 ± 1.19 $\mu\text{mol/L}$, $P = 0.013$; 8.12 ± 4.25 $\mu\text{mol/L}$ vs 3.47 ± 1.66 $\mu\text{mol/L}$, $P = 0.012$, respectively). The tissue NO levels in the *S. boulardii*, MP and *S. boulardii* + MP groups were significantly lower than the TNBS group (16.62 ± 2.27 $\mu\text{mol/L}$ vs 29.72 ± 6.10 $\mu\text{mol/L}$, $P = 0.002$; 14.66 ± 5.18 $\mu\text{mol/L}$ vs 29.72 ± 6.10 $\mu\text{mol/L}$, $P = 0.003$; 11.95 ± 2.34 $\mu\text{mol/L}$ vs 29.72 ± 6.10 $\mu\text{mol/L}$, $P = 0.002$, respectively). The tissue NO levels in the *S. boulardii*, MP and *S. boulardii* + MP groups were similar. The mean serum and tissue TNF- α levels were determined to be 12.97 ± 18.90 pg/mL and 21.75 ± 15.04 pg/mL in the control group, 18.25 ± 15.44 pg/mL and 25.27 ± 11.95 pg/mL in the TNBS group, 20.59 ± 16.15 pg/mL and 24.39 ± 13.06 pg/mL in the *S. boulardii* group, 9.05 ± 5.13 pg/mL and 24.46 ± 10.85 pg/mL in the MP group, and 13.95 ± 10.17 pg/mL and 24.26 ± 10.37 pg/mL in the *S. boulardii* + MP group.

Significant differences in terms of the levels of serum and tissue TNF- α and the macroscopic and microscopic scores were not found between the groups. *S. boulardii* fungemia was not observed in any of the rats. However, *Candida* fungemia was detected in one rat (14%) in the TNBS group, two rats (28%) in the *S. boulardii* group, three rats (50%) in the MP group, and three rats (42%) in *S. boulardii* + MP group.

CONCLUSION: *S. boulardii* does not demonstrate considerable effects on the DAI, pathological scores, or cytokine levels but does decrease the tissue NO levels.

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Key words: *Saccharomyces boulardii*; Rat; Trinitrobenzene sulfonic acid; Tumor necrosis factor- α ; Nitric oxide; Fungemia

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INTRODUCTION

It is currently speculated that inflammatory bowel disease (IBD) develops in genetically prone individuals as a result of defective immune responses against enteric bacterial flora antigens. Antibiotics and immunomodulatory therapies are believed to play an important role in the treatment of this disease^[1].

Probiotics are live microorganisms that positively affect health when ingested. *Saccharomyces boulardii* (*S. boulardii*) is a thermophilic nonpathogenic yeast that is selectively used to treat antibiotic-associated and traveler's diarrhea^[2]. The main mechanisms of action of *S. boulardii* include antimicrobial activities, trophic effects upon the intestinal mucosa, and the modification of the host-signaling pathways that are involved in inflammatory and noninflammatory intestinal diseases. It has been shown that *S. boulardii* inhibits the production of proinflammatory cytokines by inhibiting the main regulators of inflammation, such as nuclear factor κ B and mitogen-activated protein kinases, which play crucial roles in the pathogenesis of IBD^[3,4]. *S. boulardii* is believed to effectively treat IBD because of its antimicrobial activities and its regulatory effects on enteric flora and the immune system^[5]. In a recent study^[6], it was shown that treating human colon epithelial cells with *S. boulardii* increases the

expression of peroxisome proliferator-activated receptor-c and the secretion of inhibits interleukin-8 (IL-8). In the same study, it was demonstrated that *S. boulardii* decreases intestinal inflammation by reducing the mucosal expression of proinflammatory cytokines in rats with trinitrobenzene sulfonic acid (TNBS)-induced colitis. This earlier study demonstrates that colonic inflammation can be reduced by *S. boulardii* through the regulation of inflammatory gene expression.

Nitric oxide (NO) is an important messenger that is involved in vein permeability and tissue damage. It is possible that in patients with active ulcerative colitis (UC) and Crohn's disease (CD), the inducible activity of NO synthase is elevated in inflamed mucosal epithelial cells. Increased NO levels indicate inflammation and, in turn, the intensity of the disease. Variations in tissue NO levels are important indicators of progression and recovery from IBD. There is also evidence that *S. boulardii* inhibits inducible nitric oxide synthase^[7].

In the current literature, there are studies suggesting that *S. boulardii* causes fungemia, particularly in immunosuppressed and intensive care patients^[8]. However, controlled studies cannot be used to investigate the effectiveness of *S. boulardii* against IBD, although promising results have been reported in a few studies^[9,10].

The primary aim of our present study was to investigate the effects of *S. boulardii* on colonic inflammation and the disease activity index (DAI) in a rat model of TNBS-induced colitis. The secondary purpose of our current analyses was to investigate the risk of fungemia resulting from treatment with *S. boulardii* alone or in combination with corticosteroids.

MATERIALS AND METHODS

Animals

Approval was obtained from the animal ethics council of Dokuz Eylul University Medical Faculty (DEUTF). The DEUTF Hospital Experimental Research Laboratory provided 32 female Wistar albino rats weighing 200-250 g for use in this study which were divided into five groups. The control group included only four rats that were not treated with TNBS. All other groups consisted of seven rats (Table 1).

The rats were maintained in a room at a temperature of 23 ± 2 °C under a 12-h light/dark cycle at the DEUTF Experimental Animal Laboratory. Prior to and during the study, the rats were fed a standard diet (Yemta; Taris Ltd. Şti., Izmir, Turkey), and their weights were monitored daily. The rats were allowed water *ad libitum*.

Induction of colitis

After 24 h of fasting, 0.5 mL physiological serum was intracolonicly administered to the rats in the control group *via* a cannula that was placed 8 cm proximal to the anus using a rectally inserted flexible polypropylene catheter. To induce colitis in the other groups, the rats were intracolonicly treated with 0.5 mL of 100 mg/mL

Group	n	Application (day 1)	Application (days 1-14)
Control	4	Physiological serum	Physiological serum
TNBS	7	TNBS	No treatment
<i>S. boulardii</i>	7	TNBS	<i>S. boulardii</i>
MP	7	TNBS	MP
<i>S. boulardii</i> + MP	7	TNBS	<i>S. boulardii</i> + MP

TNBS: Trinitrobenzene sulfonic acid; *S. boulardii*: *Saccharomyces boulardii*; MP: Methyl prednisolone.

TNBS that was dissolved in 50% ethanol and administered *via* a cannula. Prior to catheter insertion, short-term sedation was provided *via* ether anesthesia. After TNBS administration, no rats developed perforation or exitus due to the formation of ulcerations in the colon.

Experimental design

As shown in the Table 1, 32 Wistar albino female rats were divided into five groups. The rats in the control group ($n = 4$) were not treated with TNBS after the intracolonic administration of physiological serum (*via* a cannula placed 8 cm proximal to the anus using a rectally inserted polypropylene catheter, similar to the administration of TNBS). After the administration of TNBS, the rats in the TNBS group ($n = 7$) were not treated. *S. boulardii* (Reflor; Biocodex laboratories, Gentilly, France) was prepared in its lyophilized form (282.5 mg/sachet with a biological activity of 5×10^9 viable cells) by the manufacturer. *S. boulardii* (1 mg/kg per day) was suspended in distilled water and added to the water supply of the rats in the *S. boulardii* group ($n = 7$) in the morning and evening starting on day 0. The rats in methyl prednisolone (MP) group ($n = 7$) were treated with MP (Prednol; Mustafa Nevzat, Istanbul, Turkey) at a dosage of 1 mg/kg per day, while the rats in the *S. boulardii* + MP group ($n = 7$) were treated with both *S. boulardii* and MP at the previously defined dosages using the previously discussed techniques.

Disease activity index

TNBS-induced colitis was scored according to the DAI proposed by Murthy *et al*^[11] (Table 2). Scoring was calculated according to body weight loss (as a percentage), differences in stool consistency, and the occurrence of rectal bleeding. Fecal occult blood testing (FOBT) of stool samples (Hemoccult II; Beckman Coulter Inc., Fullerton, CA, United States) was used to detect obscure bleeding.

Pathological examination

After 14 d, blood was drawn from the abdominal aorta under ether anesthesia following 24 h of fasting, and then the rats were sacrificed due to hypovolemia. Decapitation was performed after tissue samples were collected for pathological examination. The abdominal cavity was opened *via* a midline incision, and the whole small and large intestines were harvested from the pylorus to the rectum. The intestinal lumen was washed with physi-

Score	Weight loss (%)	Stool consistency	Rectal bleeding
0	-	Normal	-
1	1-5	Loose stool	Occult blood in stool
2	5-10	Loose stool	Occult blood in stool
3	10-20	Loose stool	Occult blood in stool
4	> 20	Watery stool	Obvious blood in stool

¹Data are reported by Murthy *et al*^[11].

ological serum containing phosphate buffer (PBS), and the intestinal materials collected from the opened lumen was fixed in formaldehyde. A pathologist who was blind to the groups conducted the pathological examinations of the intestinal samples.

The scoring method defined by Wallace *et al*^[12] was used to evaluate damage due to colonic inflammation. Fixed intestinal tissue samples were microscopically examined ($5 \times$ magnification) and scored from 0-10 according to various inflammation markers, such as the diameters of any developing ulcers, thickening of the intestinal wall, and hyperemia. While intestinal tissues without any evidence of lesions were scored as 0, intestinal tissues with serious ulcerations were scored as 10. Subsequently, histological sections, including the peripheral normal mucosa, were prepared from gross ulcerative lesions. Approximately 1-cm sections were obtained from the intestines and transported on ice to the Department of Clinical Microbiology (DEUTF, Izmir, Turkey) for homogenization before fixation in formaldehyde. The tissues were fixed in formaldehyde, embedded in paraffin, and stained with hematoxylin and eosin. For the microscopic evaluation, we employed the defined scoring system described by Ameho *et al*^[13].

Tissue homogenization

Prior to sacrifice, blood samples were drawn from the vena cava under ether anesthesia, and the serum was separated by centrifugation and stored at -70°C until use. Homogenization of the intestinal tissues was performed in accordance with current methods^[14]. The intestinal tissues were first homogenized in an ice-cold buffer [0.1 mol/L potassium phosphate (pH 7.5) and 20 mmol/L ethylene diamine tetra acetic acid (EDTA), 1:10 w/v] using a mechanical homogenizer (Potter B. Braun; Gemini, Apeldoorn, The Netherlands) and then in an ultrasonic homogenizer on ice. The resulting lysates were centrifuged at 14 000 rpm for 10 min followed by an additional spin at 14 000 rpm for 20 min. The proteins were purified using zinc sulfate (300 g/L) at a 1:20 ratio, and the final sample concentration of 15 g/L was obtained *via* centrifugation. The final products were centrifuged at 4°C for 20 min at 2000 rpm, and 100- μL samples were subsequently prepared for evaluation of cytokine and NO levels.

NO analysis

The 100- μL intestinal tissue lysates and serum samples

were mixed with an equal volume of 100 μL of Griess reagent (Ingredient A: 0.1% naphthalene diamine dihydrochloride at a final concentration of 5 mmol/L; Ingredient B: 1% sulfanilamide at a final concentration of 5 mmol/L in orthophosphoric acid) in a 96-well microtiter plate (Maxisorb Immunoplate; NUNC, Roskilde Denmark). After incubation for 10 min at room temperature, the absorbance at 540 nm was measured using a microplate reader (Reader Model 230S; Organon Teknika, Boxtel, The Netherlands). For each measurement, 2-fold increases in sodium nitrite in PBS, from 0-128 mmol/L, were used to generate a standard curve^[15].

Tumor necrosis factor- α analysis

Serum and tissue tumor necrosis factor (TNF)- α levels were measured using a commercially available rat-specific enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (Invitrogen; Life Technologies, Camarillo, CA, United States).

Fungal isolation and identification

Blood samples were cultured in Petri dishes containing the following culture media: Sabouraud dextrose agar (BD Difco; Becton, Dickinson, NJ, United States) supplemented with 100 mg/L chloramphenicol and selective CHROMagar Candida (CHROMagar Candida; CHROMagar, Paris, France). Following incubation at 30 °C for 72 h, the yeast colonies were visually quantified, if they existed. A specimen from each colony variant was placed in a tube containing Sabouraud dextrose agar for storage and identification. Identification was based on the results of filament production tests; the production of the germinative tube, ascospores, urease, and phenoloxidase; and zymogram, auxanogram, and growth rates at different incubation temperatures, as recommended by Boekhout *et al.*^[16,17].

Statistical analysis

Statistical analyses were conducted using SPSS software for windows 11.0 (SPSS, Chicago, IL). Data are expressed as the mean \pm SD. Quantitative data, such as the macroscopic and microscopic scores and NO and TNF- α levels, were compared between groups using the Kruskal-Wallis and Mann-Whitney *U* tests. Differences between the mean were considered statistically significant when $P < 0.05$.

RESULTS

Clinical evaluation

We did not observe diarrhea or bloody stool in the four rats in the control group during the course of this study but these effects were observed in 27 rats starting on day 1, plus one rat on day 2, following the administration of TNBS. In the TNBS and *S. boulardii* groups, a loose stool was noted on day 7 and never returned to normal and diarrhea persisted for a total of 14 d over the course of this study. In the MP group, a loose stool was observed

on day 3 and had returned to normal by day 9. However, in the *S. boulardii* + MP group, the stool returned to normal by day 10 after becoming loose on day 2. The MP and *S. boulardii* + MP groups demonstrated 9 and 10 d of diarrhea, respectively (Figure 1A).

While defecation in the TNBS and *S. boulardii* groups macroscopically continued for an average of 9 and 7 d, respectively, in the following days the FOBT results were positive until day 14. In the MP group, bloody defecation was observed for an average of three days, while for the following six days the FOBT results were positive even without clearly noting bloody defecation. In addition, the blood in the stool was, on average, not observed until day 9. In the *S. boulardii* + MP group, bloody stool was observed for an average of two days, and the FOBT results were positive for seven days; however, there was no traceable blood in the stool samples after day 9 (Figure 1B).

The rats in the control group gained an average of 4.3 g in weight, those in the TNBS and *S. boulardii* groups gained an average of 11.9 and 2.4 g, respectively, and the rats in the MP and *S. boulardii* + MP groups lost 3 and 5.9 g, respectively. No significant differences in body weight changes could be determined between the *S. boulardii*, MP, and *S. boulardii* + MP groups. There were significant differences in body weight changes for the MP and *S. boulardii* + MP groups compared with the TNBS group ($P = 0.01$ and 0.02 , respectively) (Table 3).

Based on the scoring system that was previously suggested by Murthy *et al.*^[11], DAI scores were 1 ± 0 for the control group, 4.46 ± 0.34 for the TNBS group, 4.07 ± 0.77 for the *S. boulardii* group, 3.69 ± 0.61 for the MP group, and 3.77 ± 0.73 for the *S. boulardii* + MP group. The DAI scores of the MP and *S. boulardii* + MP groups were significantly lower compared with the TNBS group ($P = 0.018$ and 0.025 , respectively). Regarding the other groups, no significant differences in DAI could be determined (Figure 1C).

Pathological evaluation

Macroscopic ulceration was not observed in the control group, although inflammation was observed at the microscopic level. Upon macroscopic examination, a 14-mm ulcer in the intestinal tissue was observed in one rat in the TNBS group, while 3, 2, and 3 larger ulcers (> 2 cm) were observed in the *S. boulardii*, MP, and *S. boulardii* + MP groups, respectively. After macroscopic and microscopic scoring, of all the groups that were induced to form colitis, the lowest score was observed in the TNBS group. When the treated groups were examined, the *S. boulardii* group demonstrated the lowest scores, although significant differences were not found between the MP and *S. boulardii* + MP groups (Figure 1D).

Serum and tissue NO levels

The mean serum NO levels in the control, TNBS, *S. boulardii*, MP, and *S. boulardii* + MP groups were determined to be 5.92 ± 3.65 $\mu\text{mol/L}$, 3.18 ± 1.19 $\mu\text{mol/L}$, 3.47 ± 1.66 $\mu\text{mol/L}$, 8.22 ± 6.28 $\mu\text{mol/L}$, and 8.12 ± 4.25

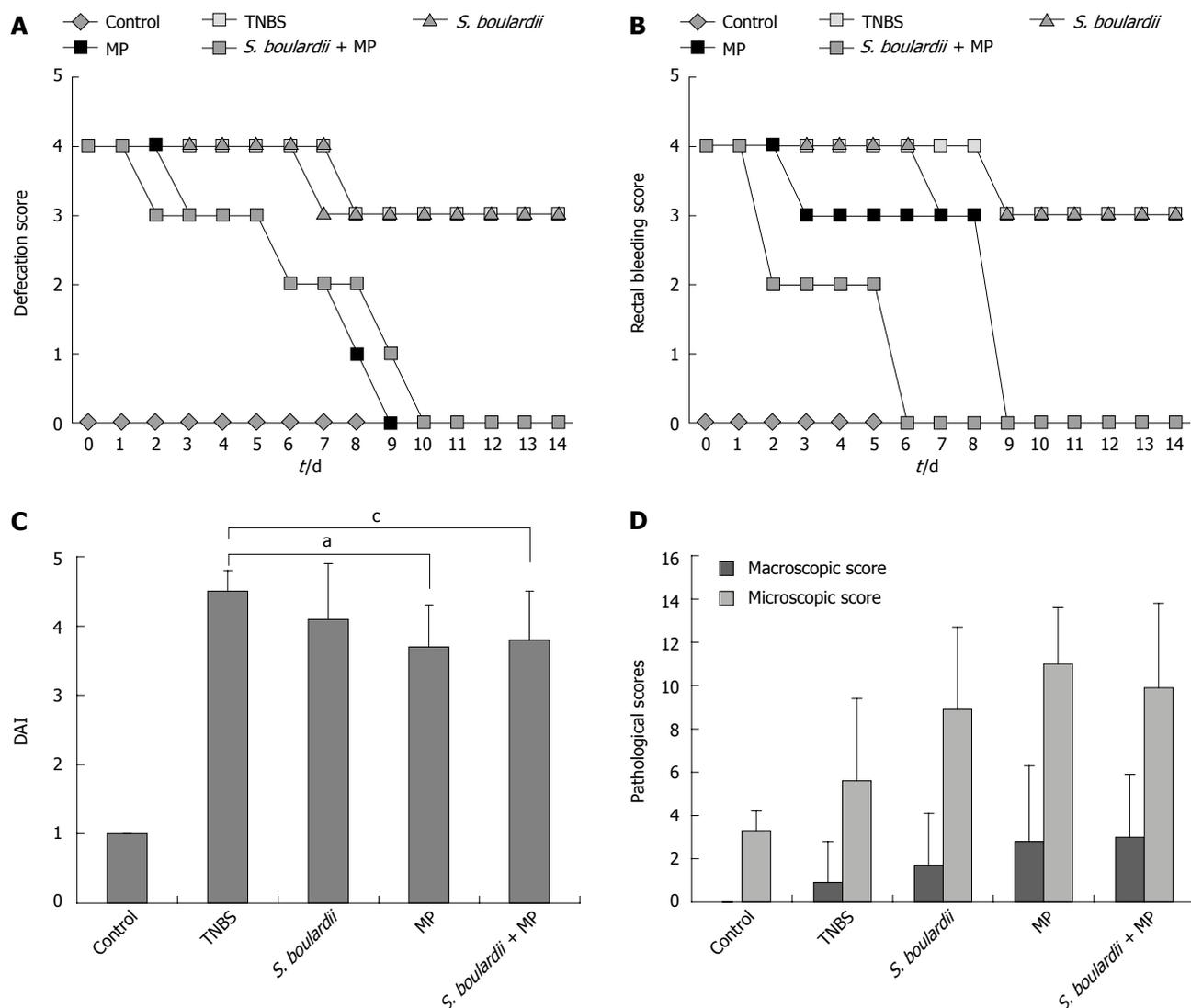


Figure 1 Daily defecation trends, rectal bleeding scores, disease activity index analyses, macro- and microscopic pathological scores of the experimental groups. A: Daily defecation trends; B: Rectal bleeding scores; C: Disease activity index analyses; The results are shown as the mean ± SD. ^a*P* = 0.018 between trinitrobenzene sulfonic acid (TNBS) and methyl prednisolone (MP) group; ^b*P* = 0.025 between TNBS and *Saccharomyces boulardii* (*S. boulardii*) and TNBS group; D: Macro- and microscopic pathological scores. The results are shown as the mean ± SD. DAI: Disease activity index.

μmol/L, respectively. The lowest serum NO levels were observed in the TNBS and *S. boulardii* groups. The serum NO levels in *S. boulardii* + MP group were higher than those in the TNBS and *S. boulardii* groups (*P* = 0.013 and 0.012, respectively).

The mean tissue NO levels in the control, TNBS, *S. boulardii*, MP, and *S. boulardii* + MP groups were determined to be 7.95 ± 0.50 μmol/L, 29.72 ± 6.10 μmol/L, 16.62 ± 2.27 μmol/L, 14.66 ± 5.18 μmol/L, and 11.95 ± 2.34 μmol/L, respectively. The highest tissue NO level was observed in the TNBS group. The tissue NO levels in the *S. boulardii*, MP, and *S. boulardii* + MP groups were significantly lower in comparison with the TNBS group (*P* = 0.002, 0.003 and 0.002, respectively). However, the tissue NO levels in the *S. boulardii*, MP, and *S. boulardii* + MP groups were similar (Figure 2A).

Serum and tissue TNF-α levels

The mean serum TNF-α levels were determined to be

12.97 ± 18.90 pg/mL in the control group, 18.25 ± 15.44 pg/mL in TNBS group, 20.59 ± 16.15 pg/mL in *S. boulardii* group, 9.05 ± 5.13 pg/mL in MP group, and 13.95 ± 10.17 pg/mL in *S. boulardii* + MP group. The mean tissue TNF-α levels were determined to be 21.75 ± 15.04 pg/mL in the control group, 25.27 ± 11.95 pg/mL in TNBS group, 24.39 ± 13.06 pg/mL in *S. boulardii* group, 24.46 ± 10.85 pg/mL in MP group, and 24.26 ± 10.37 pg/mL in *S. boulardii* + MP group. The serum and tissue TNF-α levels were comparable between groups (Figure 2B).

Detection and identification of fungal pathogens

Fungemia was not observed in the control group but was detected in 1 of 7 rats (14%) in the TNBS group, 2 of 7 rats (28%) in the *S. boulardii* group, 3 of 6 rats (50%) in the MP group (1 rat in the MP group was excluded because sufficient blood samples could not be obtained), and 3 of 7 rats (42%) in the *S. boulardii* + MP group. The

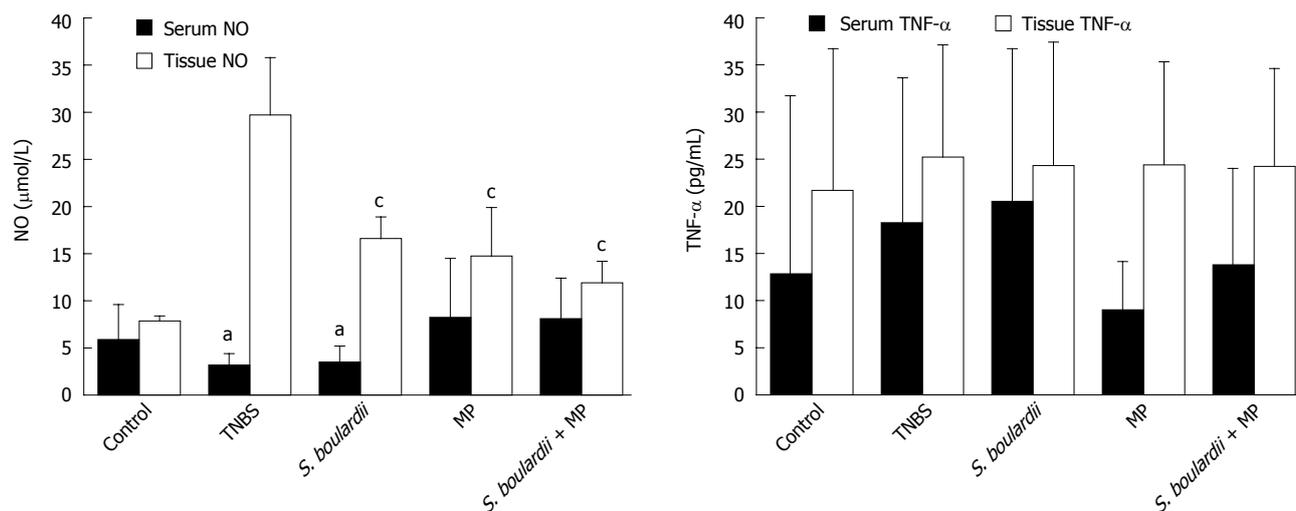


Figure 2 Serum and tissue nitric oxide and tumor necrosis factor- α levels of the experimental groups. A: Serum and tissue nitric oxide levels; B: Serum and tissue tumor necrosis factor (TNF)- α levels. The results are shown as the mean \pm SD. ^a $P < 0.05$ vs *Saccharomyces boulardii* (*S. boulardii*) + methyl prednisolone (MP); ^c $P < 0.05$ vs trinitrobenzene sulfonic acid (TNBS). NO: Nitric oxide.

Table 3 Changes in body weight in the experimental groups

Groups	Baseline weight	Final weight	Difference in body weight
Control	214.0 \pm 18.4	218.25 \pm 19.6	4.3
TNBS	218.1 \pm 18.6	230.0 \pm 17.9	+ 11.9 ^{a,b}
<i>S. boulardii</i>	195.6 \pm 9.1	198.0 \pm 20.2	+ 2.4
MP	199.3 \pm 9.1	196.33 \pm 9.1	- 3.0
<i>S. boulardii</i> + MP	197.3 \pm 10.7	191.43 \pm 9.1	- 5.9

The results are shown as the mean \pm SD. ^a $P = 0.02$ vs *Saccharomyces boulardii* (*S. boulardii*) + methyl prednisolone (MP); ^b $P = 0.01$ vs MP. TNBS: Trinitrobenzene sulfonic acid.

fungemia was determined to be *Candida* fungi other than *C. albicans* using an identification process as explained in materials and methods section. *S. boulardii* fungemia was not identified in any of the rats.

DISCUSSION

It is thought that IBD develops in genetically prone individuals as a result of defective immune responses against the antigens of enteric bacterial flora. Antibiotics and immunomodulatory therapies are believed to be important for the treatment of these diseases^[1]. *S. boulardii* is a probiotic yeast, and it is known that probiotics inhibit pathogenic invasion and also demonstrate regulatory effects on the enteric flora and immune system^[2-5]. Although there are several published studies on probiotic bacterial therapies in experimental animals with induced IBD, there are only a few studies on *S. boulardii*. In this study, the effectiveness of *S. boulardii*, DAI, macro- and microscopic pathological ulcer scores, proinflammatory cytokine levels (i.e., serum and tissue levels of TNF- α), and the serum and tissue levels of lipid peroxidation products (i.e., NO) were evaluated.

In our study, the DAI in the groups that were admin-

istered individual MP or MP with *S. boulardii* were evaluated at significantly lower levels compared with the TNBS group. Meaningful differences in the DAI scores of the *S. boulardii* group were not determined, although the DAI score of the *S. boulardii* group was lower than that of the TNBS group. As expected, these results show that the treatment of colitis using MP effectively improves the symptoms that are observed in rat models of induced colitis. However, the DAI scores were comparable between the MP and *S. boulardii* + MP groups, suggesting that the application of MP in combination with *S. boulardii* might not be more effective than the individual application of MP. In a recent study^[18], *Lactobacillus* and *Bifidobacterium* were used to prevent colitis in rats using dextran sodium sulfate, and DAI scores in the probiotic-treated group were lower than the control group. However there are no other reported studies that have investigated the effects of *S. boulardii* on DAI in an experimental model of colitis.

There are a few clinical studies on the application of *S. boulardii* for the treatment of UC and CD patients. Guslandi *et al*^[9] conducted a study using 32 CD patients who were medically treated and had been in remission for 3 mo. For 6 mo, a group was administered only mesalamine (3 g/d) and another group was administered mesalamine (2 g/d) and *S. boulardii* (1 g/d). After 6 mo, 10 patients in the mesalamine-treated group and 15 patients from the mesalamine + *S. boulardii*-treated group were still in remission ($P < 0.04$). The same researchers conducted another pilot study^[10] on 25 patients with UC of mild/moderate intensity who could not be treated with corticosteroids. After 3 mo of mesalamine (3 g/d) treatment, the patients were additionally supplemented with *S. boulardii* (250 mg administered 3 \times per day) for 4 wk. At the end of treatment, 17 patients (68%) were in remission and had demonstrated an obvious reduction in their clinical activity scores ($P < 0.05$). However, in both studies, *S.*

boulardii was not used alone and a control group was not evaluated.

In our study, macroscopic and microscopic pathological scoring was performed to evaluate the effectiveness of *S. boulardii* against colonic inflammation. Statistically significant differences were not observed between the treated and untreated groups that were induced to form colitis. In addition, no improvement was noted in the colon mucosa following the administration of individual applications of MP and *S. boulardii* or the dual application of MP and *S. boulardii*. However, the use of probiotics, such as *Lactobacillus* and VSL#3 (contains one strain of *streptococcus thermophilus*, three strains of *bifidobacterium* and four strains of *lactobacillus*), for the treatment TNBS-induced colitis in rat models have demonstrated significant improvements in macroscopic and microscopic scores^[19,20]. Surprisingly, in our study, the macroscopic scores in the *S. boulardii*, MP, and *S. boulardii* + MP groups were higher, although they were not significantly different when compared with the TNBS group. In the MP and *S. boulardii* + MP groups, the macroscopic and microscopic pathology scores were higher than in the TNBS group, although these groups also demonstrated significantly lower DAI scores. Thus, these results suggest that the clinical responses are not consistent with the histopathological results. The higher macroscopic and microscopic pathology scores in the treatment groups compared with the TNBS group can be explained by delayed effects in ulcer improvement due to fungal colonization in the gastrointestinal (GI) tract. In our study, non-*Candida albicans* fungemia was detected at considerable frequencies in the treated groups. In a study^[21] on inhibiting *Candida* translocation in the GI tract using probiotics, a group of patients with UC and rats with acetic acid-induced stomach ulcers were included. It was shown that *Candida* colonies formed in these groups, which was accompanied by the delayed recovery of stomach ulcers and the persistence of both of gastric ulcers and UC symptoms. An increase in cytokine expression, especially TNF- α and IL-1 levels, was detected in the rats that were inoculated with *Candida*.

IBD is an immunosuppressive disease caused by a defective intestinal mucosal barrier that can be brought on by applied treatments. Thus, during the course of the disease, insidious infections, such as cytomegalovirus and *C. albicans*, can develop. There are reported cases of the development of fungemia caused by *Candida* species, such as *C. parapsilosis*, *C. albicans*, and *Saccharomyces cerevisiae*, in UC patients. *S. boulardii* fungemia was reported in a 33-year-old male patient who was diagnosed with IBD, underwent intestinal surgery, and was in the intensive care unit^[22]. In our study, fungemia due to *S. boulardii* did not develop in any of the groups. Accordingly, in the colitis rat model, an increase in the risk of developing *S. boulardii* fungemia was not determined upon the application of *S. boulardii* alone or in conjunction with MP. In this study, while fungemia was not observed in the control group, non-*C. albicans* was observed in the TNBS, *S.*

boulardii, MP, *S. boulardii* + MP groups with frequencies of 14%, 28%, 50%, and 48%, respectively. This result is not consistent with previously published reports on the inhibition of *Candida* translocation in the gastrointestinal tract due to the use of probiotics in immunosuppressed rats^[21,23].

In this study, the serum NO level in the group treated with *S. boulardii* + MP was high compared with the TNBS and *S. boulardii* groups. In addition, serum NO levels were comparable between the other groups. However, tissue NO levels in all 3 treatment groups were statistically and significantly lower in comparison with the TNBS group. As observed, the serum and tissue NO levels were inconsistent. However, it is known that the serum NO level is affected by systemic events, and tissue NO levels are more reliable. As a result, based on the results of the TNBS group, tissue NO levels are found to be low in all treatment groups. These results suggest that use of *S. boulardii* and MP alone or in combination can reduce the intensity of inflammation and damage to the colitis mucosa. It was also revealed that the addition of *S. boulardii* to MP treatment does not yield a synergistic effect because the tissue NO levels of the MP and *S. boulardii* + MP groups were similar. In another study^[7], *S. boulardii* treatment reportedly affected NO levels in a rat diarrhea model that was induced by castor oil. In that study, *S. boulardii* was a successful diarrhea treatment that inhibited inducible NO synthase activity. In addition, other probiotics, especially *Lactobacillus* that has been used in induced colitis models, have been reported to reduce the tissue NO level by inhibiting inducible NO synthase activities^[24].

TNF- α is produced by CD4 + T lymphocytes that are assembled around inflamed mucosa. TNF- α is a strong chemokine that functions in pathological inflammatory signal transduction by directing the migration of neutrophils to inflamed mucosa. Therefore, serum and tissue TNF- α levels are mainly used to evaluate the intensity of inflammation. In many studies conducted using *Lactobacillus* in TNBS-induced colitis models, the tissue TNF- α levels in the groups that were administered *Lactobacillus* were significantly reduced compared with the control group^[25,26]. It has been demonstrated that the *Lactobacillus* species used in those studies reduces the number of CD4 + T cells in inflamed mucosa, thus reducing TNF- α production. In addition to these effects, *Lactobacillus* increases the production of anti-inflammatory IL-10 by shifting the T helper₁ (Th₁) cellular immune response towards Th₂ and Th₃. By changing the TNF- α /IL-10 ratio, the intensity of inflammation can be reduced^[26]. In a recent study^[6], it was demonstrated that *S. boulardii* decreases intestinal inflammation by reducing the mucosal expression of proinflammatory cytokines in rats with TNBS-induced colitis. In our study, the serum and tissue TNF- α levels were similar in all groups. These results can be explained by non-*Candida albicans* fungemia, which can cause an increase in cytokine expression^[21]. While there are an insufficient number of studies conducted on *S. boulardii*, *S.*

boulardii is believed to be involved in anti-inflammatory effects by affecting various inflammatory mechanisms^[5-7].

In conclusion, this study establishes that *S. boulardii* does not improve DAI or colonic inflammation in rats with TNBS-induced colitis and does not reduce serum or tissue TNF- α levels. The only significant effect of *S. boulardii* is reducing tissue NO levels. *S. boulardii*-based fungemia was not detected in any of the rats included in this study.

COMMENTS

Background

It is thought that inflammatory bowel disease (IBD) develops in genetically prone individuals as a result of a defective immune response against the antigens of enteric bacterial flora. Antibiotics and immunomodulatory therapies play an important role in the treatment of these diseases. *Saccharomyces boulardii* (*S. boulardii*) is a probiotic yeast, and it is known that probiotics inhibit pathogenic invasion and demonstrate regulatory effects on the enteric flora and immune system. Although there are several published studies on the use of probiotic bacterial therapy in experimental animals with induced IBD, there are a few studies on the involvement of *S. boulardii*.

Research frontiers

The present study shows that *S. boulardii* is a probiotic agent that demonstrates no effects on the disease activity index (DAI), serum and tissue tumor necrosis factor- α (TNF- α) levels, or pathologic findings in a rat model of trinitrobenzene sulfonic acid (TNBS)-induced colitis. However *S. boulardii* may reduce tissue nitric oxide (NO) levels, which is an important messenger involved in vein permeability and tissue damage. *S. boulardii*-based fungemia was not detected.

Innovations and breakthroughs

There are a few clinical studies on the efficacy of *S. boulardii* for treating IBD. Based on the findings of these published studies, *S. boulardii* appears to be promising. In a recent study, it was shown that treating human colon epithelial cells with *S. boulardii* increases the expression of peroxisome proliferator-activated receptor-c and inhibits the secretion of IL-8. In the same study, it was demonstrated that *S. boulardii* decreases intestinal inflammation by reducing the mucosal expression of proinflammatory cytokines in rats with TNBS-induced colitis. However the effects of *S. boulardii* on DAI and NO were not evaluated. The present study was conducted to investigate the effects of *S. boulardii* on DAI, pathological scores, TNF- α , and NO. Additionally, the risk of fungemia, which could result from treatment with *S. boulardii* alone or in combination with corticosteroids, was also evaluated.

Applications

The present study shows that *S. boulardii* does not improve DAI or colonic inflammation in rats with TNBS-induced colitis or reduce TNF- α levels. These results suggest that *S. boulardii* may not be an effective treatment for patients with IBD. In contrast, the limited number of studies conducted on this issue have reported some promising results. Therefore, further studies are needed in order to draw a firm conclusion.

Terminology

Crohn's disease and ulcerative colitis, both of which are referred to as IBD, are chronic inflammatory disorders of the gastrointestinal tract that have characteristic clinical, pathological, endoscopic, and radiological features. TNBS-induced colitis is well-established in various animal models of mucosal inflammation that have been used for over 2 decades for the study of IBD pathogenesis and in preclinical studies. Probiotics are live microorganisms that positively affect health when ingested. *S. boulardii* is a live yeast that is extensively used as a probiotic.

Peer review

This study examine the impact of *S. boulardii* on TNBS colitis. This is an excellent experimental study that evaluated the effects of *S. boulardii* on clinical activity scores, TNF- α levels, serum and tissue NO levels, and macroscopic and microscopic pathological scores in a rat model of TNBS-induced colitis.

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Nutritional support teams increase percutaneous endoscopic gastrostomy uptake in motor neuron disease

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Abstract

AIM: To examine factors influencing percutaneous endoscopic gastrostomy (PEG) uptake and outcomes in motor neuron disease (MND) in a tertiary care centre.

METHODS: Case notes from all patients with a confirmed diagnosis of MND who had attended the clinic at the Repatriation General Hospital between January 2007 and January 2011 and who had since died, were audited. Data were extracted for demographics (age and gender), disease characteristics (date of onset, bulbar or peripheral predominance, complications), date and nature of discussion of gastrostomy insertion, nutritional status [weight measurements, body mass index (BMI)], date of gastrostomy insertion and subsequent progress (duration of survival) and quality of life (QoL) [Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised (ALSFERS-R)]. In addition, the type of clinician initiating

the discussion regarding gastrostomy was recorded as Nutritional Support Team (involved in providing nutrition input viz Gastroenterologist, Speech Pathologist, Dietitian) and other (involved in non-nutritional aspects of patient care). Factors affecting placement and outcomes including length of survival, change in weight and QoL were determined.

RESULTS: Case records were available for all 86 patients (49 men, mean age at diagnosis 66.4 years). Thirty-eight patients had bulbar symptoms and 48 had peripheral disease as their presenting feature. Sixty-six patients reported dysphagia. Thirty-one patients had undergone gastrostomy insertion. The major indications for PEG placement were dysphagia and weight loss. Nine patients required immediate full feeding, whereas 17 patients initially used the gastrostomy to supplement oral intake, 4 for medication administration and 1 for hydration. Initially the PEG regime met $73\% \pm 31\%$ of the estimated total energy requirements, increasing to $87\% \pm 32\%$ prior to death. There was stabilization of weight in patients undergoing gastrostomy [BMI at 3 mo (22.6 ± 2.2 kg/m²) and 6 mo (22.5 ± 2.0 kg/m²) after PEG placement compared to weight at the time of the procedure (22.5 ± 3.0 kg/m²)]. However, weight loss recurred in the terminal stages of the illness. There was a strong trend for longer survival from diagnosis among MND in PEG recipients with limb onset presentation compared to similar patients who did not undergo the procedure ($P = 0.063$). Initial discussions regarding PEG insertion occurred earlier after diagnosis when seen by nutrition support team (NST) clinicians compared to other clinicians. (5.4 ± 7.0 mo vs 11.9 ± 13.4 mo, $P = 0.028$). There was a significant increase in PEG uptake (56% vs 24% , $P = 0.011$) if PEG discussions were initiated by the NST staff compared to other clinicians. There was no change in the ALSFRS-R score in patients who underwent PEG (pre 34.1 ± 8.6 vs post 34.8 ± 7.4), although in non-PEG recipients there was a non-significant fall in this score (33.7 ± 7.9 vs 31.6 ± 8.8). Four patients died within one month of the procedure,

4 developed bacterial site infection requiring antibiotics and 1 required endoscopic therapy for gastric bleeding. Less serious complications attributed to the procedure included persistent gastrostomy site discomfort, poor appetite, altered bowel function and bloating.

CONCLUSION: Initial discussion with NST clinicians increases PEG uptake in MND. Gastrostomy stabilizes patient weight but weight loss recurs with advancing disease.

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Key words: Motor neuron disease; Multidisciplinary management; Nutrition support team; Percutaneous endoscopic gastrostomy; Survival

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INTRODUCTION

In patients with motor neuron disease (MND), the management of dysphagia either at diagnosis or developing during disease progression remains a major clinical issue^[1-3]. Provision of adequate nutrition is important in these patients as malnutrition and dehydration can increase the rate of disease progression and adversely upset quality of life (QoL)^[1]. Although percutaneous endoscopic gastrostomy (PEG) insertion is an important strategy in optimizing nutritional management, the effect of this intervention on survival remains controversial with studies reporting both benefit^[2] and lack of effectiveness^[3,4]. The reasons for these discrepancies are unclear and may relate, in part, to the timing of PEG insertion in different patient subgroups^[4,5]. Weight gain or weight maintenance has been reported as another positive outcome of PEG, but the duration of the effect is also uncertain^[2,6]. Although a number of indications, including weight loss and choking have been proposed for PEG insertion in MND^[6-8], published data indicate that the procedure is performed in less than 50% of patients, who fulfill these criteria^[1]. In part, this may reflect delays in PEG insertion until there is a major clinical deterioration in swallowing^[7], although limited data show subclinical abnormalities in pharyngeal function often occur earlier^[9]. Thus, it is possible that some patients who might benefit from supplemental nutrition may not receive this at the appropriate time^[7].

A number of strategies have been proposed to in-

crease the use of PEG including nutrition education and early discussion of alternative feeding routes^[10,11]. It has also been suggested that involvement of multidisciplinary teams may have a role and potentially improve the length of survival^[11-13].

The aim of the current study was to examine the factors associated with the uptake and the outcomes of PEG placement in patients with MND being managed in a tertiary referral centre.

MATERIALS AND METHODS

A retrospective case note audit was conducted at the Repatriation General Hospital (RGH), a 270-bed university affiliated teaching metropolitan hospital. The study was approved by the Southern Adelaide Clinical Human Research Ethics Committee. To preserve patient anonymity, a unique study ID was assigned to each patient, and personal details were kept separate from the research data.

Patients were included in this study if they had attended RGH for management of MND, and had died between January 2007 and January 2011. All diagnoses of MND were confirmed by one or two neurologists based on El Escorial criteria after appropriate clinical examinations and investigations. Patient characteristics including gender, age, dates of MND onset and diagnosis, presentation features of MND, and complications (the presence of respiratory insufficiency, dysphagia and dementia) were recorded.

The type of MND was classified as either bulbar or limb disease according to symptoms at presentation. Patients with both bulbar and limb symptoms were classified as having bulbar disease.

To assess nutrition status, weights at four times were determined: usual (prior to diagnosis of MND), at diagnosis, at assessment (when the dietetic service was involved), and at death. In patients undergoing gastrostomy, the weight at PEG insertion, and 3 and 6 mo post-procedure were also recorded. Body mass index (BMI) and percentage weight loss (PWL) were also calculated over time.

To assess the role of different clinical groups in PEG management in MND, the type of clinician initiating the discussion of PEG, dates of initial PEG discussion and PEG insertion, and reasons for accepting or declining the procedure, together with the rationale for PEG were recorded. Clinicians with expertise in PEG insertion and management (dietitian, gastroenterologist or speech pathologist) were considered as a nutritional support team (NST). Other clinicians involved in MND management were recorded individually (e.g, palliative care physicians, neurologists, specialist nurses, sleep registered nurses, rheumatologists and general physicians) and grouped as other.

To evaluate the effect of PEG placement on QoL, the results from the Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised (ALSF-RS-R)^[14] were obtained from the Palliative Care MND clinic at the RGH. Scores were extracted from the records at the closest time prior

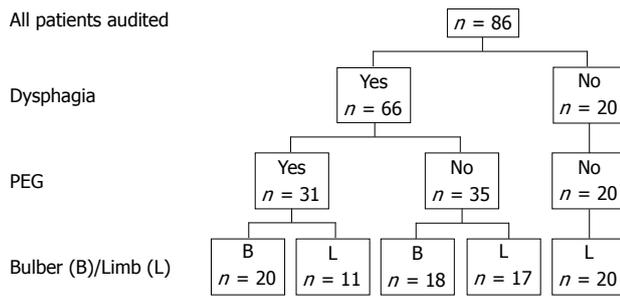


Figure 1 Patient numbers according to dysphagia, percutaneous endoscopic gastrostomy insertion and type of motor neuron disease. PEG: Percutaneous endoscopic gastrostomy.

to (≤ 1 to 5 mo) (Q1) and after (≤ 1 to 5 mo) (Q2) PEG insertion in these patients. For non-PEG patients, ALSFRS-R assessments at “correlated” time points Q1’ and Q2’, corresponding to the mean interval before and after PEG insertion were used for comparison.

The outcomes in terms of formula delivery and level of nutrition support as well as any complications of PEG placement were also recorded.

Extracted data were entered into a spreadsheet and analysed using Predictive Analytics Software Statistics Version 18.0.3 for Windows (PASW, formerly SPSS, SPSS Inc., 2010, Chicago, IL, United States). Patient characteristics were recorded as mean \pm SD and counts (percentages). Categorical data were compared by χ^2 tests. Continuous data between groups and within groups were compared by independent-sample *T* tests and paired-samples *T* tests, respectively. $P \leq 0.05$ was considered statistically significant in all analyses.

RESULTS

Case records were available for all 86 patients with MND who attended the hospital and met the selection criteria. Demographic and disease characteristics are shown in Table 1. The breakdown of the number of patients according to the type of MND, presence of dysphagia and PEG insertion are shown in Figure 1.

In total, 31 subjects underwent PEG placement using a pull technique with either a 20 Fr ($n = 28$) or an 18 Fr device. All of these patients had dysphagia. Patients with bulbar presentation (20/38, 53%), were more likely to have PEG inserted than those presenting with limb symptoms (11/48, 23%, $P = 0.004$).

PEG placement was discussed with 75.6% (65/86) patients, 92.1%, with bulbar (35/38) and 62.5% with limb onset (30/48). The NST initiated these discussions in 36 patients and other clinicians in 29 patients. There was a significant increase in the percentage of patients undergoing PEG when the initial discussions were held with the NST (56% *vs* 24%, $P < 0.02$, Table 2). In addition, these patients had longer time from symptom onset to diagnosis, and a shorter time from diagnosis to PEG. There was no difference in the time from the initial discussion to the

Table 1 Summary of patient demographic and disease characteristics characteristics of subjects (mean \pm SD) or n (%)

Males	49 (57)
Type of MND	
Bulbar as predominant symptom	38 (44)
Limb as predominant symptom	48 (56)
Age at symptom onset (yr)	65.0 \pm 13.7
Age at diagnosis (yr)	66.4 \pm 13.0
Survival after PEG placement (mo)	11.3 \pm 10.1
Living arrangements	
Alone	13 (15)
With spouse	50 (58)
With other family member	10 (12)
Care facility	7 (8)
Not recorded	6 (7)
Complications during illness	
Dysphagia	66 (77)
Respiratory insufficiency	47 (55)
Dementia	5 (6)
Symptom onset to PEG placement (mo)	20.3 \pm 8.0
Diagnosis to PEG placement (mo)	10.8 \pm 8.3

MND: Motor neuron disease; PEG: Percutaneous endoscopic gastrostomy.

procedure between the groups.

The major reason for having a PEG inserted was dysphagia ($n = 26$), with severe weight loss ($n = 4$) and prophyllaxis ($n = 1$) also recorded. The reasons for PEG insertion not proceeding after discussion ($n = 38$) were patient preference ($n = 17$), death before PEG placement [$n = 9$, (4 discussed with NST, 5 with other)] and lack of medical fitness for procedure ($n = 1$). In 11 patients no reason for refusal was recorded. In patients who died before the PEG could be undertaken, 4 instances were due to advanced disease and respiratory failure preventing the safe performance of the procedure. Two of these were initially discussed with NST and 2 with other clinicians. Of the other 5 patients (2 NST, 3 other) the patients initially agreed to the gastrostomy but then failed to proceed with the procedure.

Regardless of the presentation (i.e., bulbar or limb), there was a trend towards longer survival in MND patients with dysphagia after PEG insertion (21.6 \pm 15.6 mo), compared to patients who had dysphagia, but did not undergo the procedure (16.8 \pm 11.0 mo, $P = 0.089$). This increased length of survival reflected the effect in patients with limb onset MND who developed dysphagia (PEG: 26.4 \pm 20.4 mo *vs* non-PEG: 14.4 \pm 10.8 mo, $P = 0.063$) (Figure 2) as in bulbar MND patients there was no difference in survival with or without PEG (19.2 \pm 12.0 mo *vs* 18.0 \pm 10.8 mo, $P = 0.656$).

PEG insertion was accompanied by stabilization of patient weight 3 and 6 mo after placement. In contrast, patients who did not undergo PEG placement had ongoing weight loss. However, further weight loss occurred in all patients as MND progressed (Table 3). Thus, the mean BMI for PEG patients decreased significantly from usual weight to diagnosis ($P = 0.003$), diagnosis to assessment for PEG ($P = 0.001$) and from this assessment to death ($P = 0.023$). There was a trend for PEG to be offered to patients who

Table 2 Relationship between the uptake of percutaneous endoscopic gastrostomy and clinician initiating discussion

	Nutritional support team	Other clinicians	P value
Initial PEG discussions, <i>n</i>	36	29	
Patients undergoing PEG insertion after initial discussion, <i>n</i>	20 (56%, 20/36)	7 (24%, 7/29)	0.011
MND symptom onset to MND diagnosis, mo	12.8 (± 7.5), <i>n</i> = 26	8.4 (± 3.9), <i>n</i> = 17	0.017
MND diagnosis to initial PEG discussion, mo	5.4 (± 7.0), <i>n</i> = 20	11.9 (± 13.4), <i>n</i> = 6	0.028
Initial PEG discussion to placement, mo	3.5 (± 3.3), <i>n</i> = 20	4.4 (± 3.2), <i>n</i> = 6	0.572
PEG placement to death, mo	12.9 (± 11.1), <i>n</i> = 20	8.8 (± 10.1), <i>n</i> = 6	0.422
MND symptom onset to death, mo	30.0 (± 13.2), <i>n</i> = 26	31.2 (± 13.2), <i>n</i> = 17	0.740
MND diagnosis to death, mo	18.0 (± 15.6), <i>n</i> = 36	21.6 (± 15.6), <i>n</i> = 27	0.393

MND: Motor neuron disease; PEG: Percutaneous endoscopic gastrostomy.

Table 3 Comparison of weight assessments for patients with motor neuron disease undergoing percutaneous endoscopic gastrostomy

Time points	<i>n</i> ¹	Body mass index (mean ± SD) kg/m ²
Usual	20	26.6 ± 4.3
Diagnosis	23	25.3 ± 4.0
Assessment	25	23.8 ± 3.6
PEG insertion	25	22.5 ± 3.0
3 mo post-PEG	16	22.6 ± 2.2
6 mo post-PEG	13	22.5 ± 2.0
Death	16	21.2 ± 2.2
		Percentage of weight loss (mean ± SD)
Usual to diagnosis	22	5.8 ± 7.5
Diagnosis to assessment	26	5.9 ± 7.0
Assessment to PEG insertion	28	5.9 ± 5.9
PEG insertion to 3 mo after	18	-0.1 ± 6.0 ²
3 to 6 mo post-PEG	13	1.4 ± 5.1
6 mo post-PEG to death	12	2.4 ± 4.9

¹Some data unavailable due to patients being unable to be weighed. Usual-pre-MND diagnosis; Diagnosis-at diagnosis of MND; Assessment-when dietetic service was involved pre-PEG insertion; ²Percentage of weight gain. MND: Motor neuron disease; PEG: Percutaneous endoscopic gastrostomy.

had greater weight loss from diagnosis to assessment (PWL of PEG patients: 5.9% ± 7.0% *vs* PWL of non-PEG patients with dysphagia: 2.3% ± 6.5%, *P* = 0.076).

Initially, 17 patients used PEG to supplement oral intake; nine required immediate full feeding; four used PEG for medication administration and one for hydration. Eight patients were nil by mouth. Initially the feeding regime met 73% ± 31% of the estimated total energy requirements and this increased to 87% ± 32% prior to death.

Fifty-nine patients (68.6%) had at least one assessment with the ALSFRS-R performed during their illness. There was no change in the ALSFRS-R score in patients who underwent PEG (34.1 ± 8.6, *n* = 19 at Q1 and 34.8 ± 7.4, *n* = 17 at Q2). Interestingly, in patients who did not undergo PEG placement, there was a non-significant fall in the ALSFRS-R score from 33.7 ± 7.9 (*n* = 31) at Q1¹ to 31.6 ± 8.8 (*n* = 18) at Q2². There was no significant difference between Q1 and Q1¹ (*P* = 0.886), or Q2 and Q2² (*P* = 0.252).

Possible adverse effects related to the procedure were

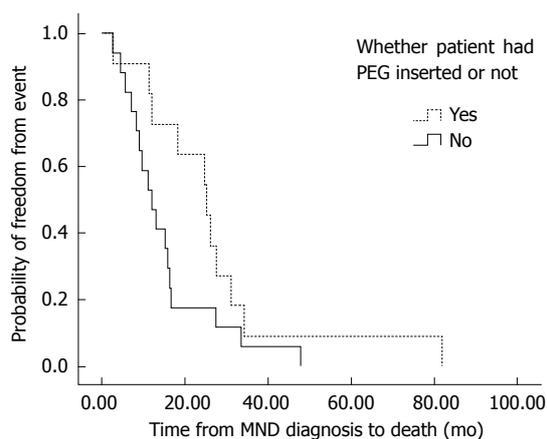


Figure 2 Kaplan-Meier plots of survival probabilities after percutaneous endoscopic gastrostomy placement among patients with limb onset motor neuron disease who developed dysphagia. MND: Motor neuron disease; PEG: Percutaneous endoscopic gastrostomy.

recorded in 19 patients after PEG insertion. These included pain at the site of the gastrostomy at follow up (*n* = 9), poor appetite (*n* = 4), bacterial site infection requiring antibiotics (*n* = 4), death within 30 d (*n* = 4, respiratory failure), nausea (*n* = 3), constipation (*n* = 2), diarrhea (*n* = 2), vomiting (*n* = 2), bloating (*n* = 1) and gastric hemorrhage (*n* = 1).

DISCUSSION

The new findings from this study are that initial discussions of PEG insertion by a NST significantly increases the uptake of PEG by patients with MND, compared to when discussions were initiated by other clinicians. There was also a trend for increased survival for patients with limb onset disease who had a PEG, but not for patients with bulbar onset disease. Consistent with previous studies, PEG placement initially arrested weight loss, although with disease progression patients again lost weight prior to death. Data on the QoL were incomplete but no decrease was seen in the ratings in patients undergoing PEG placement.

Previous reports suggest that the uptake of PEG is approximately 20%^[1], although up to 80% of MND patients developed dysphagia at some point in their illness.

The reasons for this are unclear, but may reflect lack of enthusiasm by both patient and clinicians about a procedure whose value remains uncertain. Also, patients may lack sufficient information about the procedure and its implications. Additionally in some patients, respiratory dysfunction may present a contraindication to gastrostomy by preventing safe performance of the procedure^[9].

The current report extends previous data showing that involvement of multidisciplinary teams in the care of patients with MND enhances the uptake of interventions, including gastrostomy^[10,12,15,16]. In this study, possible PEG placement was discussed with the majority of patients regardless of the presence of dysphagia or the type of presentation of MND. However, the initiation of discussion by clinicians with a background in nutrition significantly increased the patients' uptake of PEG insertion. It is possible that clinicians familiar with MND are more aware of the importance of nutrition adequacy in MND management and clinicians who are closely involved in management of PEG insertions are able to better allay patients' concerns. Furthermore, the NST raised the topic significantly earlier than other clinicians after diagnosis, which potentially allowed more time for the patients to adjust to the concept and undergo PEG insertion before their overall condition deteriorated. The time from PEG discussion to PEG insertion was similar between these two clinician groups suggesting that patients were not simply referred to the NST when nutrition was inadequate. Overall survival of patients undergoing PEG placement was similar irrespective of their referring clinician, suggesting that there was no difference in the patients with whom the procedure was discussed to explain the difference in uptake.

The effect of PEG feeding on survival in patients with MND has been controversial. It is possible that differences in reported outcomes may reflect the different subtypes of MND undergoing the procedure as well as the timing of the procedure^[2,6,9]. In the current study, there was a strong trend for prolonged survival after PEG, but only in those patients with limb onset disease. The reasons for this are unclear. An early study^[2] suggested that PEG prolonged survival significantly in MND patients with bulbar symptoms, including those in whom dysphagia developed at any stage. However, in this study the symptoms at presentation (i.e., bulbar or limb) were not defined^[2]. Another case control study^[5] found no significant survival benefit of the use of PEG, but PEG was associated with prolonged survival in their whole study cohort and among those with bulbar onset; the reasons for this discrepancy are unclear but may reflect an older patient profile in the current study. Forbes *et al.*^[3] found no survival difference, and in their study limb onset patients fared worse than bulbar onset patients, although the survival in this study was shorter than in the present study. Strong *et al.*^[4] found that gastrojejunostomy was associated with shorter survival in either bulbar onset

or limb onset patients; however, the control group in this study contained patients who did not require PEG placement and therefore might have had better nutrition status initially.

Maintaining a patient's weight is a possible advantage of PEG in nutrition management in MND, but the duration of this effect is unclear. In the present study, the patients' weight stabilized for six months after PEG placement, but with disease progression, patients again lost weight. Weight gain at three months post-PEG has been previously described^[5,6], but only one report^[2] has described ongoing weight gain over 12 mo post-gastrostomy. In that study, the patients' baseline BMI prior to PEG insertion was lower (19.7 kg/m²) than in the present study (22.5 kg/m²) and this may explain the discrepancy.

The benefits of PEG in prolonging survival and maintaining weight did not reach statistical significance in this study, and may have reflected a bias in patient selection, since patients who underwent PEG placement had more severe weight loss. Several studies have concluded that lower PWL prior to PEG placement is associated with longer survival^[5]. This suggests that early recognition of weight loss may be important to optimize timing of the procedure^[7]. Consequently PWL has been recommended as the best indicator of malnutrition and for the referral for PEG, rather than BMI^[5,12,16]. In the United Kingdom, using $\geq 10\%$ PWL from baseline has been preferred as an indicator for PEG insertion instead of BMI in MND patients^[17]. An Irish review suggested that PEG was warranted when 5%-10% of weight loss was observed in MND patients^[18], and Chiò *et al.*^[5] found that patients with PWL $\geq 10\%$ fared worse after PEG placement. Thus, the optimal timing remains uncertain^[1,9]. In the current study, an additional interesting finding was that, consistent with other reports^[18], patients experienced rapid weight loss prior to death even with PEG nutrition. The reasons for this are unclear. Although overall PEG feeding did not achieve 100% of the feeding goals (reflecting the desire to provide supplemental feeding/hydration in some patients, and voluntary restriction in others in the terminal phase of their illness) overall nutrition intake appeared adequate. It is possible that loss of muscle mass with disease progression^[19] and increased energy requirement due to respiratory insufficiency^[18] may be important contributory factors.

A further potential benefit from PEG insertion in MND patients has been the potential to improve QoL^[6], but this has not been systematically assessed^[1,2,3,17]. In the current study, ALSFRS-R scores were maintained after the gastrostomy PEG. However, interpretation of the data is limited, in part because of difficulties in obtaining comparable data between PEG and non-PEG patients. In addition, the comparison of QoL is likely to be affected by the severity of dysphagia before PEG placement which in itself contributes to the probability

of the procedure^[8]. Also, adverse effects associated with gastrostomy placement are likely to reduce any positive effects^[1]. Although weight loss, pain and poor appetite have been identified as indicators of decreased QoL^[1,6], their relationship to PEG placement is difficult to assess.

The retrospective nature of the current study means that some caution is required in the interpretation of the data. Thus, although data from all patients with MND seen at the hospital were included in the study, it is impossible to exclude biases due to patient referral. However, the demographics of the patient group are similar to those reported previously. Moreover, as the hospital provides state-wide palliative care for the majority of patients with MND, data are likely to be representative of Australian patients with the condition. Although nutritional advice provided by services external to the hospital was not obtained, it seems unlikely that this impacted significantly on patient care.

In conclusion, initial discussion about PEG placement with a nutrition support team increased the uptake of PEG in patients with MND. Gastrostomy insertion was associated with a strong trend towards longer survival of patients with dysphagia who had limb onset but not bulbar disease. In patients undergoing PEG placement there was initial stabilization of weight, but with disease progression patient weight again decreased.

COMMENTS

Background

Although the importance of percutaneous endoscopic gastrostomy (PEG) feeding to facilitate nutrition support and reduce sarcopenia (potentially decreasing the rate of disease progression) is well recognised in patients with motor neuron disease (MND), only a minority of suitable patients undergo the procedure. The reasons for this are likely to be multifactorial, but may include the availability of information about the intervention. Multidisciplinary management of MND is associated with better uptake, but the reasons for this are uncertain.

Research frontiers

Currently only supportive therapy such as enteral nutrition is available for MND, but who should receive this and when is unclear. Increased knowledge on which sub-groups of patients are assisted by PEG feeding and the timing of the gastrostomy insertion allows patients and clinicians to make more informed decisions to optimize the benefits.

Innovations and breakthroughs

Patients who received counseling from a Nutrition Support Professional were more likely to undergo the procedure. Interestingly the patients with peripheral onset of disease had a trend to longer survival consistent with the concept that muscle mass is preserved by adequate nutrition.

Applications

The findings support the use of multidisciplinary teams in this disease and also provide a possible rationale for the findings of better survival in units where this is undertaken.

Terminology

MND is a progressive neurological condition of unknown aetiology characterized by relentless progression to respiratory failure.

Peer review

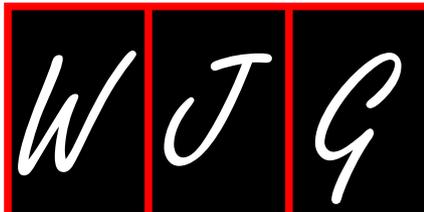
Well written manuscript (retrospective study) regarding PEG tube utilization in patients with MND. It provides some new insight to early PEG placement in patients with malnutrition and MND.

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Clinicopathological features of minute pharyngeal lesions diagnosed by narrow-band imaging endoscopy and biopsy

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Abstract

AIM: To evaluate the utility of magnified narrow-band imaging (NBI) endoscopy for diagnosing and treating minute pharyngeal neoplasia.

METHODS: Magnified NBI gastrointestinal examinations were performed by the first author. A magnification hood was attached to the tip of the endoscope for quick focusing. Most of the examinations were performed under sedation. Magnified NBI examinations were performed for all of the pharyngeal lesions that had noticeable brownish areas under unmagnified NBI observation, and an intrapapillary capillary loop (IPCL) classification was made. A total of 93 consecutive pharyngeal lesions were diagnosed as IPCL type IV and were suspected to represent dysplasia. Sixty-two lesions of approximately 1 mm in diameter were biopsied in the clinic, and 17 lesions with larger diameters were resected by endoscopic submucosal dissection (ESD) at the Hiroshima University Hospital. In addition to the histological diagnoses, the lesion diameters were microscopically measured in 45 of the 62 biopsies. Thirty-

four of the 62 biopsied patients received endoscopic follow up.

RESULTS: Minute pharyngeal lesions were diagnosed in 93 of approximately 3000 patients receiving magnified NBI examinations at the clinic. Of the 93 patients with IPCL type IV lesions, 80 were men, and 13 were women. Fifty-six were drinkers, and 57 were smokers. Two had esophageal cancer. Twenty-one lesions were located on the posterior hypopharyngeal wall, and 72 lesions were located on the posterior oropharyngeal wall. All 93 lesions were flat and showed similar findings in the magnified and unmagnified NBI examinations. Although almost all of the IPCL type IV lesions showed faint redness when examined under white light, it was difficult to diagnose the lesions using only this technique because the contrast was weaker than that achieved in the NBI examinations. Of the 93 lesions, only 3 had diameters greater than 2.1 mm. Sixty-two lesions of approximately 1 mm were biopsied in the clinic, whereas 17 larger lesions were treated by ESD at the Hiroshima University Hospital. Of the 79 pharyngeal lesions that were biopsied or resected by ESD, 5 were histologically diagnosed as high-grade dysplasia, 39 were diagnosed as low-grade dysplasia, and 39 were determined to be non-dysplastic lesions. There were no cancerous lesions. Histologically, abnormal cell size variations and increased nuclear size were observed in all of the high-grade dysplasia lesions, while the incidence of these findings in the low-grade dysplasia lesions was low. Of the 62 biopsied lesions, 45 were microscopically measurable. The measured diameters ranged from 0.1 to 2.0 mm. The dysplasia ratios increased with the diameters. A follow-up endoscopic examination of the 34 biopsied patients found the rate of complete resection by biopsy to be 79%. The largest lesion in which complete resection was expected was a low-grade dysplasia of 1.9 mm in diameter.

CONCLUSION: Minute pharyngeal lesions suspected to be dysplasia that are identified by NBI magnifying

endoscopy should be biopsied to determine the diagnosis and further treatment.

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Key words: Biopsy; Minute pharyngeal lesions; Narrow-band imaging

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Kumamoto T, Sentani K, Oka S, Tanaka S, Yasui W. Clinicopathological features of minute pharyngeal lesions diagnosed by narrow-band imaging endoscopy and biopsy. *World J Gastroenterol* 2012; 18(44): 6468-6474 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i44/6468.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i44.6468>

INTRODUCTION

Magnified narrow-band imaging (NBI) endoscopy is reportedly useful for early pharyngeal^[1-5] and esophageal cancer diagnosis^[6-10]. Magnified NBI is useful both for detecting suspicious changes and for further diagnostic purposes, such as determining whether a lesion is suspected of being dysplasia or cancer^[11,12]. We routinely examined upper gastrointestinal tracts using magnified NBI for one and a half years. When the unmagnified NBI detected a brownish area in the pharynx, a magnified NBI examination was used to aid the intrapapillary capillary loop (IPCL) classification^[12-14]. If IPCL type IV or V was observed, dysplasia or cancer was suspected, and treatment was considered. Although endoscopic submucosal dissection (ESD) leads to complete resection, ESD is an inpatient procedure that requires intubation anesthesia. For smaller (approximately 1 mm) lesions, therefore, our first choice is to biopsy the lesion for histological diagnosis while performing a complete resection. There have been no previous reports on the utility of biopsying minute pharyngeal lesions. This article reports our histological diagnosis, treatment, and follow-up findings from minute pharyngeal dysplasia biopsies.

MATERIALS AND METHODS

Patients

Most patients over 30 years old who require gastrointestinal endoscopy at the clinic are examined by magnified NBI. From August 2008 to March 2010, a total of 93 consecutive patients with IPCL type IV pharyngeal lesions, as determined by magnified NBI, were enrolled in this study (Table 1). The patients included 80 men aged 39-87 years (mean age, 66.9 years) and 13 women aged

43-84 years (mean age, 67.1 years). Drinking and smoking habits were assessed for 86 patients. Lesions of approximately 1 mm in diameter were found in 62 patients and were biopsied at the clinic, and the 17 patients with larger lesions were endoscopically resected by ESD at the Hiroshima University Hospital. Detailed histological assessments were performed for 62 of the biopsies. Thirty-four of the 62 biopsied patients received follow up endoscopy.

Instruments

The following instruments were used in this study: a magnifying endoscope that was capable of × 80 magnification (GIF H260Z; Olympus Optical Co. Ltd, Tokyo, Japan), a standard videoendoscopy system (EVIS LUCERA; Olympus), and an NBI system (Olympus).

Endoscopic examination

All of the endoscopic examinations were performed by the first author (Kumamoto T). A magnification hood (MB-46, Olympus) was attached to the tip of the endoscope. Intravenous access and pulse oximetry monitoring were established prior to the examination. Most of the examinations were performed under intravenous pethidine hydrochloride (17.5-70 mg) and midazolam (0.5-4 mg) sedation. The pharynx was mainly observed by NBI from the beginning of the examination. The pharynx was examined in the following order: uvula, posterior oropharyngeal wall, epiglottis, posterior hypopharyngeal wall, and pyriform sinus. The pharynx observation time was approximately 1 min. Magnified NBI was used for all pharynx lesions with noticeable brownish areas in the NBI examination, and an IPCL classification was performed. The IPCL classification followed the criteria of Dr. Inoue H^[12,13]. According to these criteria, a lesion must meet three of the following four characteristics to be classified as IPCL type IV: dilatation, tortuous running, caliber changes, and different shapes in each IPCL^[12-14]. In the 93 IPCL type IV lesions, smaller lesions of approximately 1 mm in diameter were biopsied with disposable biopsy forceps (FB-210K, Olympus). To avoid post-biopsy bleeding, all anticoagulants were discontinued from 3 d before to 3 d after the biopsy. The biopsy patients remained in the clinic for 2-3 h, including a 1 h post-sedation recovery time. Annual magnified NBI examinations were recommended to all of the biopsy patients, and 34 of 62 biopsy patients received follow up. To reduce inter-observer variation, the results of the NBI and magnified NBI examinations were independently evaluated by 2 endoscopy examiners (Kumamoto T and Oka S). When the evaluations differed, a consensus decision was achieved by reviewing the magnified NBI images.

Histological methods and criteria for pathological diagnosis

The biopsy specimens were extended and fixed to a styrene foam plate by fine acupuncture needles. All of the specimens were fixed in 10% formalin and embedded in

Table 1 Patient characteristics (n = 93)

Characteristics	No.
Age, yr (range)	67 (39-87)
Sex, men/women	80/13
Alcohol consumption	
Yes	56 (54 men, 2 women)
No	30 (22 men, 8 women)
Smoker	
Yes	57 (56 men, 1 woman)
No	29 (20 men, 9 women)
Esophageal cancer history	
Yes	2
No	91
Other cancer history	
Yes	9 (8 gastric cancer, 1 colon cancer)
No	84
Location	
Hypopharynx	21 (17 men, 4 women)
Oropharynx	72 (63 men, 9 women)
Biopsy cases	62
High-grade dysplasia	3
Low-grade dysplasia	25
Non-dysplasia	34
ESD cases	17
High-grade dysplasia	2
Low-grade dysplasia	14
Non-dysplasia	1

ESD: Endoscopic submucosal dissection.

paraffin wax. The tissue specimens were cut into 3- μ m thick sections, and all of the sections received routine pathological diagnoses. The pathological parameters of each lesion were independently evaluated by two pathologists (Sentani K, Yasui W) and used for further analyses. The dysplasia diagnoses followed the criteria proposed by the World Health Organization^[15]. In this study, dysplasia was classified as low-grade (mild or moderate dysplasia) or high-grade (severe dysplasia). These criteria were based on the architectural and cytological abnormalities. In addition to these abnormalities, IPCL changes were considered^[16]. As shown in Table 2, the histological diagnoses were based on the IPCL changes and on architectural and cytological atypia. The IPCL changes were defined as upward extension, dilatation and branching, and diameter expansion. Architectural atypia was determined by a proliferative cell distribution and the tumor front, and cytological atypia was assessed by cell size, nuclear arrangement and nuclear size. The lesion diameters were measured under light microscopy using the built-in measurement system of the light microscope, which measured to an accuracy of 0.1 mm.

RESULTS

Minute pharyngeal lesions were diagnosed in 93 of approximately 3000 patients who were examined by magnified NBI. The clinicopathological characteristics of the patients are shown in Table 1. Of the 93 patients, 80 were men, and 13 were women. Fifty-six patients were drinkers, 57 were smokers, and 2 had esophageal cancer. Other

Table 2 Histological characteristics of the biopsied pharyngeal lesions n (%)

	Non-D	LGD	HGD
Number	34	25	3
IPCL			
Upward extension	26 (76)	24 (96)	3 (100)
Dilatation and branching	5 (15)	21 (84)	3 (100)
Diameter expansion	0 (0)	8 (25)	1 (33)
Architectural atypia			
Proliferative cell distribution			
$\geq 2/3$	0 (0)	0 (0)	3 (100)
$< 2/3$	34 (100)	25 (100)	0 (0)
Tumor front	0 (0)	23 (92)	3 (100)
Cytological atypia			
Abnormal variation in cell size	0 (0)	2 (8)	3 (100)
Abnormal nuclear arrangement	0 (0)	23 (92)	3 (100)
Increased nuclear size			
High	0 (0)	0 (0)	3 (100)
Absent or low	34 (100)	25 (100)	0 (0)

Non-D: Non-dysplastic; LGD: Low-grade dysplasia; HGD: High-grade dysplasia; IPCL: Intra-papillary capillary loop.

cancers included gastric cancer in 8 patients and colon cancer in 1 patient. Twenty-one lesions were located on the posterior hypopharyngeal wall, and 72 lesions were found on the posterior oropharyngeal wall.

All 93 lesions were flat and showed similar findings in the magnified and unmagnified NBI examinations (Figures 1B, C; 2A, B). Most of the IPCL type IV lesions were identifiable immediately after the magnified NBI diagnosis by a faint redness under white light (Figure 1A). However, it was difficult to diagnose IPCL type IV lesions by this characteristic before an NBI examination because the contrast is weaker than that of NBI (Figure 1A, B). Of the 93 lesions, only 3 were greater than 2.1 mm in diameter, and the remaining lesions were less than 2.0 mm in diameter. Sixty-two lesions of approximately 1 mm in diameter were biopsied for histological diagnoses at the clinic, and 17 larger lesions were treated by ESD at the Hiroshima University Hospital. There were no complications, such as bleeding or pharyngeal pain, during or after the ESD.

The histological diagnoses of the 79 lesions resected by biopsy or ESD included 5 cases of high-grade dysplasia, 39 cases of low-grade dysplasia, and 35 non-dysplastic lesions. The 35 non-dysplastic cases consisted of 19 inflamed (pharyngitis) and 16 normal lesions. A lesion diagnosed as high-grade dysplasia is shown in Figure 1, and an example of low-grade dysplasia is shown in Figure 2. There were no cancerous lesions. The histological features of each type of dysplasia are shown in Table 2. In high-grade dysplasia, the polarity of the nucleus was lost, and the nuclear density was markedly increased throughout the intraepithelial layer, although the superficial portion of the epithelium was mature (Figure 1E). The microvascular irregularities were more severe than those seen in low-grade dysplasia. Basal cell palisading was observed in the low-grade dysplasia lesions; however, proliferative cells with enlarged nuclei that proliferated

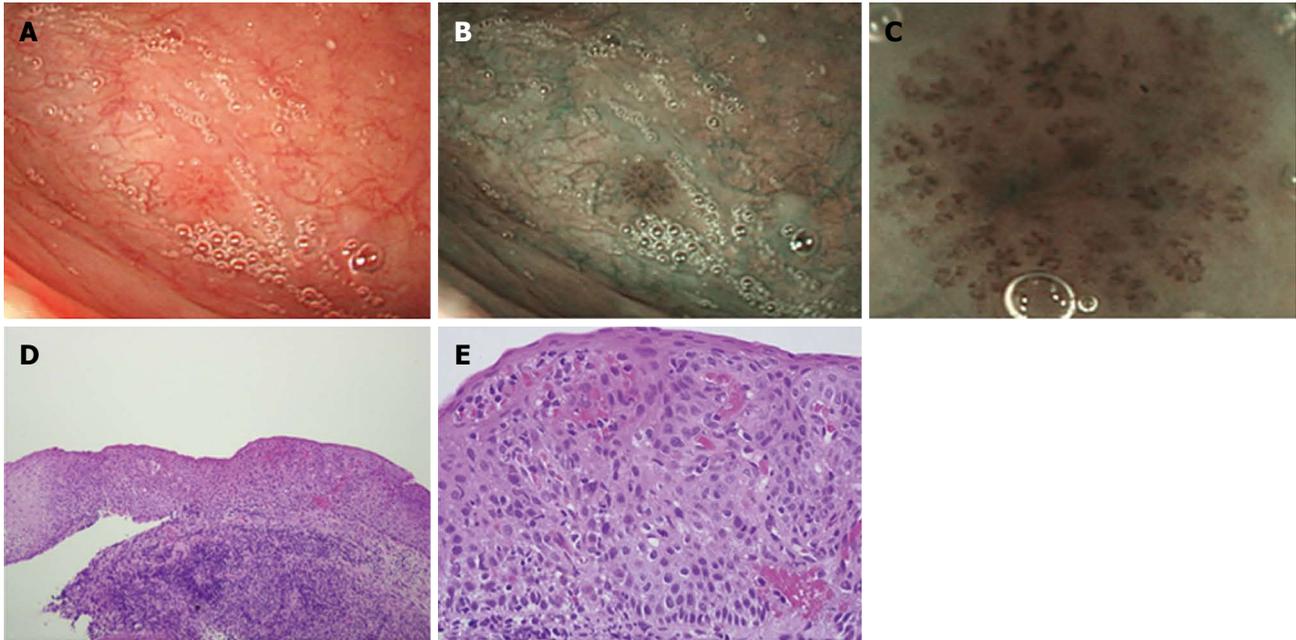


Figure 1 Narrow-band imaging view and histological image of high-grade dysplasia. A: An endoscopic photograph showing an oropharynx with high-grade dysplasia. The slightly reddish colored mucosa is the dysplastic lesion; B: The narrow-band imaging (NBI) corresponding to A, showing a well-demarcated brownish area; C: The magnified NBI view, showing an intra-papillary capillary loop type IV pattern. Irregular morphological changes in the superficial microvessels can be observed in the brownish area; D: Low-power magnification of the biopsied specimen, showing tumor front formation and complete epithelial layer invasion. The diameter of the lesion was 1.1 mm [hematoxylin and eosin (HE), original $\times 100$]; E: Histologically, the lesion showed abnormal cell size variation and increased nuclear size; it was diagnosed as high-grade dysplasia (HE, original $\times 400$).

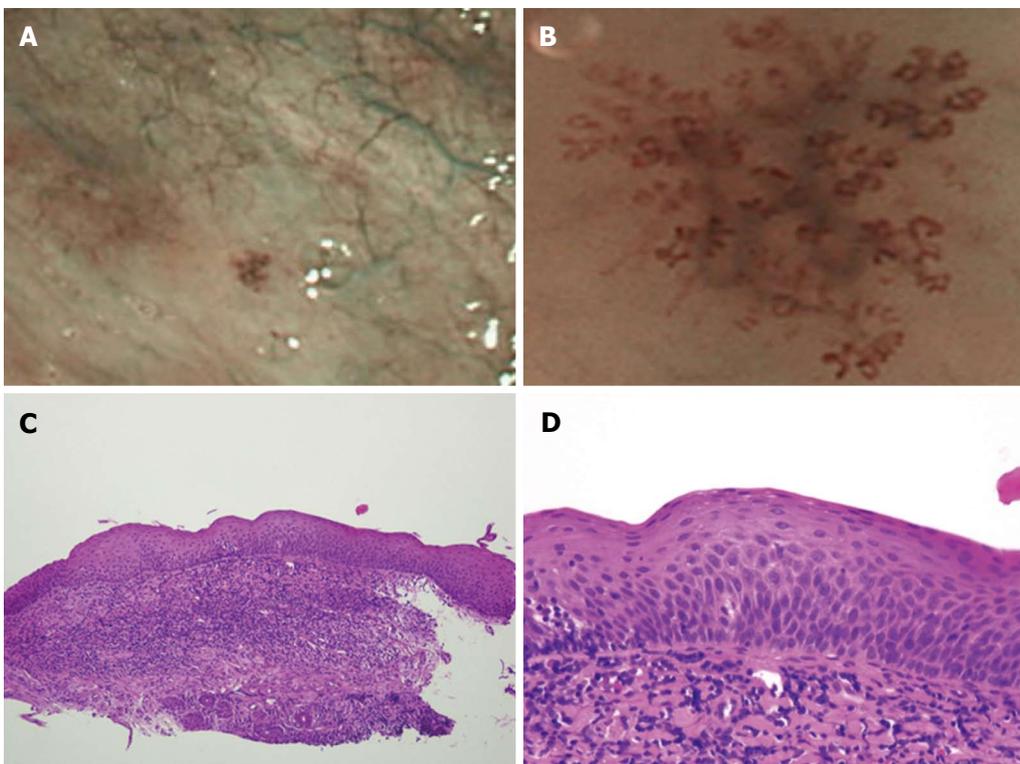


Figure 2 Narrow-band imaging view and histological image of low-grade dysplasia. A: Narrow-band imaging (NBI) showing the oropharynx with a well-demarcated brownish area; B: A magnified NBI magnifying view showing an intra-papillary capillary loop type IV pattern; C: Low power magnification of the biopsied specimen [hematoxylin and eosin (HE), original $\times 100$]; D: Histologically, the lesion showed minimal cell size variation and was diagnosed as low-grade dysplasia (HE, original $\times 400$).

Table 3 Diameters of the biopsied lesions *n* (%)

Diameter (mm)	<i>n</i>	Non-D	LGD	HGD
0.1-0.2	3	3 (100)	0 (0)	0 (0)
0.3-0.4	5	3 (60)	2 (40)	0 (0)
0.5-0.6	5	4 (80)	1 (20)	0 (0)
0.7-0.8	10	6 (60)	4 (40)	0 (0)
0.9-1.0	7	2 (29)	4 (57)	1 (14)
1.1-1.2	9	1 (11)	7 (78)	1 (11)
1.3-1.4	2	0 (0)	1 (50)	1 (50)
1.5-1.8	1	0 (0)	1 (100)	0 (0)
1.9-2.0	3	0 (0)	3 (100)	0 (0)

Non-D: Non-dysplasia; LGD: Low-grade dysplasia; HGD: High-grade dysplasia.

in a lamellar pattern were limited to the lower two-thirds of the epithelial layer (Figure 2D). IPCL abnormalities such as upward extension, dilatation, and diameter expansion were clearly recognized. In the high-grade dysplasia lesions, abnormal variations in cell size and increased nuclear size were observed in all of the lesions, whereas the incidence of these findings in the low-grade dysplasia lesions was low (Figures 1E, 2D). In the non-dysplastic lesions, such as inflamed squamous epithelium, intercellular edema and intraepithelial inflammatory cells were recognizable.

Of the 62 biopsied lesions, 45 were measurable (Table 3). The measured diameters were 0.1-2.0 mm (average, 1.12 mm); 30 lesions were 0.1-1.0 mm in diameter, and 15 lesions were 1.1-2.0 mm in diameter. The distribution of the lesion diameters and the neoplasia ratios are shown in Table 3. The dysplasia ratios (low-grade or high-grade) were 0% less than 0.2 mm, 20%-40% from 0.3 to 0.8 mm, 71% from 0.9 to 1.0 mm, 89% from 1.1 to 1.2 mm, and 100% from 1.3 to 2.0 mm. The ratio increased as the diameter increased. The diameters of the 3 high-grade lesions were 1.0, 1.1, and 1.3 mm.

Twenty-seven of the 34 biopsy patients who received endoscopic follow up (79%) had no lesions at the biopsy site in their NBI examinations, and complete resection from the biopsy was expected in these patients. The interval from the first diagnosis to the follow-up ranged from 4-22 mo (mean, 12.5 mo). The diameters of the lesions in the 3 incomplete resection cases were 0.7 mm, 1.1 mm, and 1.1 mm. Sixteen of the 34 cases were of high- or low-grade dysplasia, and 13 of these 16 cases (81%) had no lesions at the biopsy site. Complete resection from the biopsy was expected in these cases. The largest lesion for which complete resection was expected was 1.9 mm in diameter and was classified as low-grade dysplasia.

DISCUSSION

Magnified NBI endoscopy is preferable for diagnosing minute pharyngeal lesions^[17-21]. The current practice at our clinic is to observe the pharynx and esophagus by NBI from the beginning of the examination. A distal magnification attachment on the endoscope tip is effective

for quick focusing. The typical time for a magnified NBI examination from the pharynx to the duodenum was 10-15 min. The routine pharynx examination time was approximately 1 min.

During the period from September 2008 to March 2010, 93 patients with minute pharyngeal IPCL type IV lesions of approximately 1 mm in diameter were diagnosed at our clinic by magnified NBI endoscopy. During this period, we performed approximately 3000 routine examinations of the upper gastrointestinal tract using magnified NBI endoscopy. Thus, the frequency of such lesions was approximately 3%. Although it is well known that male drinkers and smokers aged over 50 are at high risk for pharyngeal carcinoma^[22], we also observed several cases of low-grade dysplasia in women, and the youngest case was a 39-year-old man. Therefore, we routinely examine almost all men and women over 30 with magnified NBI endoscopy. In the present study, 2 patients had esophageal cancer^[23-26], 8 had gastric cancer, and one had colon cancer. A history of other cancers may also be considered a risk factor.

All 93 lesions were intraepithelial flat lesions. Because the magnified NBI endoscopy findings were similar and showed no clear differences among high-grade dysplasia, low-grade dysplasia and non-dysplastic lesions, the 93 lesions appeared to have similar characteristics. If the lesions had been followed for a longer period, transitions from low-grade to high-grade dysplasia might have occurred. There is controversy over whether such lesions should be followed, biopsied, or treated. Because the histological diagnosis for 3 biopsied cases and 2 ESD cases was high-grade dysplasia, at least 5 lesions (all of which were in men) were considered to be precancerous. Although there were no cancerous lesions in this series^[18], routinely using magnified NBI endoscopy seemed effective for diagnosing pharyngeal precancerous lesions early and showed that IPCL type IV lesions should be biopsied or resected by ESD. Because esophageal IPCL type IV lesions are thought to represent high-grade dysplasia, treatment is recommended^[13]. However, treating smaller esophageal or pharyngeal IPCL type IV lesions (approximately 1 mm in diameter) is still controversial. Whether they should be treated or followed remains to be determined. Because the smaller pharyngeal IPCL type IV lesions in our study contained some precancerous lesions, we recommend biopsy and treatment over follow up.

A survey of the diameters of the biopsied lesions indicated an increase in the dysplasia ratio as the diameter increased, and all lesions over 1.3 mm were found to be dysplastic. Our clinic sees typical outpatients with varied gastrointestinal symptoms or abnormal gastric X-ray findings, and the diameter distribution we observed appears to be close to the natural distribution of such lesions. The diameter of the 3 high-grade lesions ranged from 1.0 to 1.3 mm; therefore, it would appear that lesions greater than 1.0 mm in diameter should be resected by biopsy or ESD. Furthermore, because low-grade lesions as small as 0.3 mm in diameter were observed, it would be better to

resect all lesions less than 1.0 mm in diameter. The diameter distribution appears to show the natural progression from low-grade to high-grade dysplasia.

A follow-up study of the biopsied cases revealed that the resection rate by biopsy alone was 79%. The diameter of the largest resected lesion was 1.9 mm. Although annual endoscopic follow-up must be continued, biopsy may lead to complete resection in some cases and can be performed as a first-line therapy. Single-use disposable biopsy forceps were used to obtain a biopsy specimen as large and as deep as possible for complete resection. For the biopsy, the plane of the opened biopsy forceps should be horizontal, which can be achieved by rotating the handle. Precise, lesion-centered biopsies of 1 mm lesions are difficult^[13] and require high levels of concentration and cooperation between the examiner and the assisting medical staff. Recently, we have biopsied these lesions prior to inserting the endoscope into the esophagus because if we try to biopsy during the final withdrawal stage, the observations may be disturbed by secreted mucus and minute bleeding. Biopsying the pharynx appeared to be safe providing all anticoagulant drugs were discontinued from 3 d before to 3 d after biopsy. We experienced no complications (such as bleeding) after the biopsies.

Compared to the esophagus, the pharynx is sensitive to being touched by an endoscope. Therefore, almost all of the magnified NBI magnifying examinations were performed under sedation. The hypopharynx is particularly sensitive. Without sedation, the pharyngeal reflex causes responsive artificial bleeding, particularly when touched by the distal attachment during examination, and NBI observation becomes difficult due to the brownish color change in the entire endoscopic field. In our experience, magnified NBI endoscopy can be routinely performed with the patient under sedation. However, because deep sedation may cause respiratory depression, sedation should be used cautiously with an intravenous drip, and with oxygen and the counteracting effect of flumazenil readily available. In high-risk patients, such as those with respiratory or heart disease, for safety, we removed the distal attachment on the scope tip or changed the scope to a smaller diameter non-magnifying scope (GIF H260, Olympus).

In conclusion, magnified NBI endoscopy appears to be preferable for diagnosing pharyngeal neoplasia. Biopsy was useful for the diagnosis and treatment of minute pharyngeal neoplasia.

COMMENTS

Background

It has been difficult to diagnose an early-stage pharyngeal carcinoma. Narrow-band imaging (NBI) has enabled more accurate diagnosis and increased the detection rate of superficial pharyngeal carcinomas. Magnified NBI endoscopy is quite effective for diagnosing early-stage pharyngeal and esophageal carcinoma, that is, carcinomas in the squamous cell regions. The intrapapillary capillary loop (IPCL) classification for magnified NBI endoscopy is applicable only to the squamous cell regions, that is, the pharynx and the esophagus. Magnified NBI endoscopy for the stomach and the colon is evaluated by different diagnos-

tic classifications.

Research frontiers

Magnified NBI endoscopy made it possible to diagnose minute pharyngeal lesions with diameters of approximately 1 mm. It includes two steps. First, using NBI endoscopy without magnification, the authors detected suspicious changes as brownish areas. Second, using NBI magnifying of the brownish areas, the authors were able to diagnose whether the changes were suspicious of dysplasia or cancer or not according to the IPCL classification. IPCL type IV or V shows the possibility of dysplasia or cancer, whereas IPCL type I neglects the possibility.

Innovations and breakthroughs

For larger pharyngeal IPCL type IV lesions over 10 mm diameter, endoscopic submucosal dissection (ESD) is recommended. However, for smaller lesions of approximately 1 mm diameter, it remains to be determined whether they should be resected by ESD or followed up. Studies on the biopsy of such lesions are few. The authors concluded that biopsy can be the first-line procedure for such lesions not only for diagnosis but also for treatment.

Applications

The NBI system and magnifying endoscope are necessary to begin magnified NBI endoscopy. As the pharynx is sensitive, magnified NBI endoscopy of the pharynx should be performed under sedation. For safety, sedation should be carried out cautiously. As several biopsied minute pharyngeal lesions were high grade dysplasia, minute pharyngeal lesions should be biopsied for diagnosis. Furthermore, follow-up study of the biopsied lesions showed that even complete resection was expected by biopsy.

Terminology

NBI: NBI is a new image-enhanced optical technology that uses narrow band NBI filters; IPCL: IPCL is the microvascular tumor vessel classification system used for NBI magnifying endoscopy.

Peer review

Recently it has been reported that NBI can be useful in the early detection of superficial pharyngeal cancer. To determine the criteria of endoscopic treatment in patients with pharyngeal cancer and dysplasia, the resected specimen and follow up data are required. It is a good idea to investigate the usefulness of NBI magnifying endoscopy in the pharynx because biopsy in the pharynx is difficult.

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Rome III survey of irritable bowel syndrome among ethnic Malays

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Abstract

AIM: To survey irritable bowel syndrome (IBS) using Rome III criteria among Malays from the north-eastern region of Peninsular Malaysia.

METHODS: A previously validated Malay language Rome III IBS diagnostic questionnaire was used in the current study. A prospective sample of 232 Malay subjects (80% power) was initially screened. Using a stratified random sampling strategy, a total of 221 Malay subjects (112 subjects in a "full time job" and 109 subjects in "no full time job") were recruited. Subjects were visitors (friends and relatives) within the hospital compound and were representative of the local com-

munity. Red flags and psychosocial alarm symptoms were also assessed in the current study using previously translated and validated questionnaires. Subjects with IBS were sub-typed into constipation-predominant, diarrhea-predominant, mixed type and un-subtyped. Univariable and multivariable analyses were used to test for association between socioeconomic factors and presence of red flags and psychosocial alarm features among the Malays with IBS.

RESULTS: IBS was present in 10.9% (24/221), red flags in 22.2% (49/221) and psychosocial alarm features in 9.0% (20/221). Red flags were more commonly reported in subjects with IBS (83.3%) than psychosocial alarm features (20.8%, $P < 0.001$). Subjects with IBS were older (mean age 41.4 years vs 36.9 years, $P = 0.08$), but no difference in gender was noted ($P = 0.4$). Using univariable analysis, IBS was significantly associated with a tertiary education, high individual income above RM1000, married status, ex-smoker and the presence of red flags (all $P < 0.05$). In multiple logistic regression analysis, only the presence of red flags was significantly associated with IBS (odds ratio: 0.02, 95%CI: 0.004-0.1, $P < 0.001$). The commonest IBS sub-type was mixed type (58.3%), followed by constipation-predominant (20.8%), diarrhea-predominant (16.7%) and un-subtyped (4.2%). Four of 13 Malay females (30.8%) with IBS also had menstrual pain. Most subjects with IBS had at least one red flag (70.8%), 12.5% had two red flags and 16.7% with no red flags. The commonest red flag was a bowel habit change in subjects > 50 years old and this was reported by 16.7% of subjects with IBS.

CONCLUSION: Using the Rome III criteria, IBS was common among ethnic Malays from the north-eastern region of Peninsular Malaysia.

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Key words: Irritable bowel syndrome; Malays; Prevalence; Rome III criteria; Malaysia

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INTRODUCTION

In the West, irritable bowel syndrome (IBS) is a major gastroenterological condition seen in daily clinical practice, with a prevalence of 10%-20%^[1-5]. The prevalence of IBS in the East is reportedly lower. Previous studies from Thailand and Singapore reported a prevalence of less than 5%^[6,7]. Recent studies from Hong Kong, Beijing and India also reported a similar low prevalence, although the rate may differ depending on the criteria used^[8-11].

One of the earlier studies from Malaysia reported a prevalence of 15.8% among a group of young multi-ethnic medical students using Rome I criteria in central Peninsular Malaysia^[12]. A later study by Rajendra *et al.*^[13] reported a rate of 15.5% using Rome II criteria in a mixed urban and rural multi-ethnic population in the north-western region of Peninsular Malaysia. The prevalence rates of IBS reported among the ethnic Malays (12.4%-15.8%) from both studies were higher than that reported by a recent study from Singapore (6.8%-10.3%)^[14].

The north-eastern region of Peninsular Malaysia is more economically deprived and less developed compared with western and central parts of Peninsular Malaysia, and most of its population consists of ethnic Malays. We aimed to assess the prevalence of IBS among the Malay population in this region recruited within a hospital setting using a validated Malay language Rome III IBS Diagnostic Questionnaire. In addition, we also aimed to assess and compare the rates of red flags (alarm symptoms which may suggest the presence of organic diseases), psychosocial alarm features and other socioeconomic markers in IBS *vs* non-IBS in this population.

MATERIALS AND METHODS

Subjects

This was a prospective cross-sectional survey involving a native Malay population in the state of Kelantan situated in the north-eastern region of Peninsular Malaysia. The city of Kota Bharu is the state capital of Kelantan

with an estimated population of 570 000, and 90% of its population consists of ethnic Malays^[15]. The university hospital of Universiti Sains Malaysia (USM), located in the heart of Kota Bharu, is the largest tertiary hospital serving the whole state of Kelantan. As the region is less developed compared to the west coast of Peninsular Malaysia, private practice is scarce and all layers of the Malay community (from the poor to the rich) tend to seek healthcare at the hospital.

Volunteers consisting of visitors (friends and relatives of patients) within the hospital compound were identified with the assistance of an independent research assistant who was blinded to the study but was trained to assess for inclusion of volunteers based on a set of predefined criteria. A random sampling strategy (stratified according to occupational status; volunteers with no full time job *vs* volunteers with a full time job) was then employed by the investigator. All subjects were recruited from different families in order to avoid ambiguity. Subjects of different ethnic backgrounds, past medical, surgical or psychiatric backgrounds, pregnancy and physical disabilities which did not allow them to complete the questionnaire or attend the interview were excluded from the study.

A total of 232 subjects were screened with a response rate of 95.2%. The sample size was calculated for the Malay population only and was based on a prevalence rate of 15%^[12], an alpha level of 0.05 and a power of 80%. There were 221 Malay subjects (109 with “no full time job” and 112 with a “full time job”) included in the study to answer the printed questionnaire booklet and/or face-to-face interview with a trained interviewer after giving informed consent. There were two subjects with no full time job whose responses were not complete or inadequate for analysis and were therefore excluded from the final analysis.

Malay language Rome III IBS diagnostic questionnaire

The Rome III IBS diagnostic questionnaire was translated into the Malay language and validated according to guidelines set by the Rome foundation. The Rome III IBS diagnostic questionnaire contains 10 items with answers on an ordinal scale and individual frequency thresholds for each question. The 10 items include questions on symptom criteria (items 4 to 8), a question to exclude pain of gynecological origin (item 2), questions on time between transient and chronic gut symptoms (items 1 and 3), and questions to sub-type IBS according to stool form (items 9 and 10). Briefly, the Rome III symptom criteria for IBS included the following: (1) recurrent abdominal pain or discomfort at least 3 d a month in the last 3 mo with two or more of the following: improvement with defecation; onset associated with a change in frequency of defecation; onset associated with a change in form (appearance) of stool; and (2) the above criterion fulfilled for the last 3 mo with symptom onset at least 6 mo before diagnosis. The Malay language Rome III IBS diagnostic questionnaire has been reported to have an intra-class correlation coefficient (ICC) of 0.996 and a good discriminant valid-

Table 1 Univariable and multivariable analysis of irritable bowel syndrome and non-irritable bowel syndrome among ethnic Malays in the north-eastern region of Peninsular Malaysia using Rome III criteria *n* (%)

Parameters	IBS (<i>n</i> = 24)	No IBS (<i>n</i> = 197)	Crude OR (95%CI)	Adjusted OR ¹ (95%CI)	<i>P</i> value ¹
Age (yr, mean ± SD)	41.42 ± 13.5	36.87 ± 15.9	1.02 (-11.2, 2.13)	1.07 (0.99, 1.16)	0.08
Sex					
Male	11 (45.8)	93 (47.2)	0.95 (0.40, 2.21)	2.32 (0.39, 13.87)	0.36
Female	13 (54.2)	104 (52.8)	1.00	1.00	
Education level					
Primary education	1 (4.2)	40 (20.3)	0.08 (0.01, 0.61) ²	0.15 (0.01, 2.70)	0.20
Secondary education	7 (29.2)	107 (54.3)	0.20 (0.08, 0.53) ²	0.27 (0.05, 1.40)	0.12
Tertiary education	16 (66.7)	50 (25.4)	1.00	1.00	
Occupational status					
Not working	9 (37.5)	100 (50.8)	0.58 (0.24, 1.39)	1.36 (0.20, 9.04)	0.75
Work full-time	15 (62.5)	97 (49.2)	1.00	1.00	
Individual income					
Low income (≤ RM1000)	6 (25.0)	130 (66.0)	0.17 (0.06, 0.45) ²	0.77 (0.1, 6.14)	0.80
High income (> RM1000)	18 (75.0)	67 (34.0)	1.00	1.00	
Marital status					
Single or divorced	4 (16.7)	74 (37.6)	0.33 (0.11, 1.01) ²	0.64 (0.06, 6.42)	0.71
Married	20 (83.3)	123 (62.4)	1.00	1.00	
Number of children in family ¹	3.58 (2.3)	2.79 (2.9)	1.09 (0.95, 1.25)	0.87 (0.58, 1.32)	0.51
Smoking status					
Non-smoker	15 (62.5)	150 (76.1)	1.00	1.00	
Current smoker	2 (8.3)	31 (15.7)	0.64 (0.14, 2.97)	1.09 (0.18, 10.19)	0.94
Ex-smoker	7 (29.2)	16 (8.1)	4.37 (1.56, 12.31) ²	1.92 (0.19, 19.52)	0.58
Red flag symptoms					
Symptoms absent	4 (16.7)	168 (85.3)	0.03 (0.01, 0.11) ²	0.02 (0.004, 0.10)	< 0.001
Symptoms present	20 (83.3)	29 (14.7)	1.00	1.00	
Psychosocial alarm					
None	19 (79.2)	182 (92.4)	0.10 (0.01, 1.74)	0.19 (0.005, 7.64)	0.38
Non-serious problems	4 (16.7)	14 (7.1)	0.29 (0.01, 5.67)	0.20 (0.004, 10.37)	0.42
Serious problems	1 (4.2)	1 (0.5)	1.00	1.00	

¹Adjusted for all parameters listed in Table 1, *P* < 0.05 significant; ²Significant on univariate analysis with *P* < 0.05. OR: Odds ratio; RM: Ringgit Malaysia; IBS: Irritable bowel syndrome.

ity (*P* < 0.001)^[16].

We have included red flags and psychosocial alarm questionnaires in the current study since red flags and psychological symptoms have been commonly reported from previous studies but their association with IBS is unknown^[12,17]. Briefly, the red flag questionnaire included a history in the past 3 mo of fever, weight loss, cancer in family members, blood mixed with stool, anemia and change in bowel habit after age 50. The psychosocial alarm questionnaire had seven items designed to identify those patients with psychological “problems” which can be “serious” or “non-serious”. The ICCs for the Malay language Red Flags and Psychosocial Alarm Questionnaires has been reported to be 0.962 and 0.994, respectively^[16].

Only one trained interviewer was involved to maintain consistency, and the interviewer was experienced with the Rome III questionnaires and Rome III diagnostic criteria for IBS. In addition, we incorporated additional questions in the survey including age, gender, educational level, individual income, marital status, number of children in the family, smoking status and presence of menstrual pain in females. Subjects with IBS were sub-typed based on Rome III supportive symptoms into the following categories: IBS-C, constipation-predominant; IBS-D, diarrhea-predominant; IBS-M, mixed type and IBS-U, un-subtyped.

The study was approved by the Human Ethics Committee of USM.

Statistical analysis

All data are presented as frequency and percentage unless otherwise stated. Univariable logistic regression was used to test the association of independent variables (including age, sex, education level, occupational status, individual’s income level, marital status, number of children in the family, smoking status, red flags and psychosocial alarm features) with presence or absence of IBS, and reported as crude odds ratios (ORs) and 95% CIs. The same variables listed above were adjusted using multivariable logistic regression analysis and reported as adjusted ORs, 95% CI and *P* value. All analyses were carried out using SPSS version 18.0 (SPSS Inc, Chicago, IL, United States).

RESULTS

Among the 221 surveyed Malay subjects, 10.9% (24/221) had features of IBS according to the Rome III criteria. The mean age of recruited subjects was 37.7 years (15.7 years) and although subjects with IBS were slightly older (mean age 41.4 years) this was not statistically significant (*P* = 0.08). There was no difference in the prevalence rate of IBS between genders among Malay subjects (*P* =

Table 2 Comparison of prevalence of irritable bowel syndrome across populations in Malaysia and Singapore

Studies	Region	Sample size (n)	Diagnostic criteria	Population type	Reported prevalence of IBS
Tan <i>et al</i> ^[12]	Klang Valley, Central Peninsular Malaysia	533	Rome I	Multi-ethnic; young; medical students Malays 278 (52.2%), Chinese 179 (33.6%), Indian 48 (8.6%)	Overall 84 (15.8%); Malays 15.8%, Chinese 16.2%, Indian 15.2%
Rajendra <i>et al</i> ^[13]	North-western Peninsular Malaysia	949	Rome II	Multi-ethnic; mean age 33.6 yr; mixed urban and rural; Malays 314 (33.1%)	Overall 148 (15.5%); Malays 12.4%, Chinese 17.5%, Indians 16.8%
Current study	North-eastern Peninsular Malaysia	221	Rome III	Malays only; mean age 37.7 yr; mixed urban and rural	Malays 24/221 (10.9%)
Gwee <i>et al</i> ^[14]	Singapore	2276	Manning, Rome I, II	Multi-ethnic; mean age 40 yr; urban; Malays 263 (11.6%)	Overall: Manning 11%, Rome I 10.4%, Rome II 8.6%; Malays: Manning 9.9%, Rome I 10.3%, Rome II 6.8%

IBS: Irritable bowel syndrome.

0.4) (Table 1). Red flag symptoms were present in 83.3% (20/24) of subjects with IBS and 22.2% (49/221) of the total subjects recruited ($P < 0.001$) (Table 1). Psychosocial alarm features were present in 20.8 (5/24) of subjects with IBS and 9.0% (20/221) of the total subjects recruited ($P = 0.4$) (Table 1).

With univariable analysis, tertiary education, high individual income ($> RM1000$), married status, ex-smoker and presence of red flag symptoms were significantly associated with IBS among Malays (Table 1). With multiple logistic regression analysis, only the presence of red flags remained significantly associated with IBS (OR: 0.02, 95%CI: 0.004-0.1, $P < 0.001$).

The commonest IBS sub-type was of mixed type, IBS-M (58.3%), followed by IBS-C (20.8%), IBS-D (16.7%), and IBS-U (4.2%). Four of 13 (30.8%) female subjects with IBS also complained of menstrual pain. Most subjects with IBS had at least one red flag (70.8%), 12.5% had 2 red flags and 16.7% had no red flags. A bowel habit change for those above 50 years old was reported in 16.7% of subjects with IBS.

DISCUSSION

In the current study, the reported IBS rate of 10.9% among ethnic Malays was very similar to the study from Singapore (prevalence of 10.3% using Rome I criteria)^[14] (Table 2). The reported IBS rate among ethnic Malays in the current study was relatively low compared with that in the study by Rajendra *et al*^[13] (rate of 12.4% using Rome II criteria) and Tan *et al*^[12] (rate of 15.8% using Rome I criteria) (Table 2).

These differences in prevalence rates between studies may be explained by differences in population demographic and socioeconomic backgrounds between different regions of Peninsular Malaysia. In the study by Tan *et al*^[12], the studied population consisted of highly educated and healthy young Malaysians (mean age 22 ± 1.8 years) in the Klang Valley, a well-developed economic region in the central belt of the Peninsula. It is known

that IBS is more common in a younger and more educated population^[9,18]. In the study by Rajendra *et al*^[13], the population from the north-western region of Peninsular Malaysia was a mix of urban and rural communities, was older (mean age 33.6 ± 13 years) and was recruited based on race-stratified disproportionate random sampling to ensure sufficient representation of all ethnic minorities. The economy in this region of Malaysia is less developed than the Klang Valley but is more developed than the north-eastern region of Peninsular Malaysia. The older population and the mixture of a rural community might explain the lower prevalence of IBS reported in the study by Rajendra *et al*^[13] as compared with the study by Tan *et al*^[12]. Our current study recruited only ethnic Malays (mean age 37.7 ± 15.7 years) who were visitors (friends and relatives) within a hospital setting. To ensure a homogenous representation of the general population, a random stratified sampling strategy based on occupation was used. An older population, a lower socioeconomic background compared with the other regions of the Peninsula and less education may have explained the prevalence seen in our current study. The similarity in prevalence rates between the Malays in our study and the study from Singapore somewhat validates our findings.

The diagnostic Rome criteria used was different across different studies from Malaysia and this can partially influence the reported prevalence rates^[19]. The current study did not compare different Rome diagnostic criteria which is a limitation. The total sampling size was also different between studies but if the sample calculation on just the ethnic Malays was taken into account then it was not much different (221 subjects in the current study, 278 subjects in Tan *et al*^[12], 314 subjects in Rajendra *et al*^[13] and 263 subjects in Gwee *et al*^[14]).

There was no gender difference among the Malays with or without IBS in the current study. This was in contrast with the studies of Rajendra *et al*^[13] and Tan *et al*^[12] which reported a female preponderance in subjects with IBS. While the cause is unknown, it is possible that

in their studies, the women are more educated and have greater health-seeking behavior in the more developed west coast of Peninsular Malaysia. Alternatively, it could be an ethnic-specific phenomenon with more Chinese or Indian women reporting more IBS symptoms than the Malay women. Another possibility is that more women in their study may have misconstrued menstrual cramp as an IBS symptom. We have noticed that a third of the Malay women with IBS in the current study also complained of menstruation-related pain. It is also possible that males in our study seek more medical attention than the females, similar to what has been reported by hospital-based studies from India and Sri Lanka^[11,20,21].

The current study also included assessment for red flags and psychosocial alarm symptoms since they were commonly reported from previous studies but their association with IBS is unknown^[12,17]. Of all surveyed subjects, 9% (20/221) with red flag signs might have gastrointestinal symptoms from various diseases. While the question on the change in bowel habit is present in both the red flag questionnaire and the Rome III IBS diagnostic questionnaire, the question on age is not included in the IBS diagnostic questionnaire. We have noticed a significant percentage of subjects over 50 years old with symptoms of IBS who also reported a change in their bowel habit. This is important since elderly subjects above 50 years old have a higher risk for other organic diseases especially colorectal cancer.

Psychosocial alarm features were not commonly reported in the Malay population, either in the IBS or non-IBS sub-group, and if they were present, they were often dismissed as not being serious. This is in contrast with the Western populations where psychological disturbances were fairly common in those subjects with IBS especially in females^[22]. One of the reasons for this discrepancy is the questionnaire itself, which may not have identified the idioms of psychological distress in this population^[23]. Secondly, the socio-cultural stigmata attached to psychological and mental disturbance in this largely traditional Malay population may have resulted in the under-reporting of symptoms. Finally, high levels of family support, religious beliefs and other unknown cultural factors may have protected them against psychological distress^[24].

The commonest sub-type of IBS reported in the current study was of mixed type followed by the constipation type which was similar to the study from Singapore^[14]. This distribution was however different from the study by Rajendra *et al.*^[13] where constipation-predominant was the commonest subtype followed by the mixed type and diarrheal type. Many subjects with mixed type IBS often have features of constipation rather than diarrhea, and therefore the distinction of mixed or constipation type needs further clarification on their symptoms. This is important since treatment targeted against serotonin receptors can have contrasting effects on constipation or diarrhea.

There were limitations worth noting in the current study. It could be argued that recruiting subjects within

the hospital compound might have led to sampling bias but this was unlikely. The recruited subjects involved all layers of the Malay community (from the poor to the rich) within a region of stable population dynamics and similar socio-cultural backgrounds. Furthermore, only native Malays were included in the current study and a proper stratified random sampling strategy was carried out. The sample size in the current study might have been relatively small but the study only involved ethnic Malays, and it was calculated with a good power. The current study did not report on results of investigations performed to rule out organic diseases in those subjects with red flags as this was not our research objective. However previous studies have shown that investigating these subjects very often has a low yield^[25,26]. The current study did not compare the prevalence using different types of diagnostic criteria (Rome I, II and Manning criteria) for IBS. However this is the first study on IBS among ethnic Malays using a locally translated and validated Rome III IBS diagnostic questionnaire.

In conclusion, using the Rome III criteria, IBS was found to be prevalent among ethnic Malays from the north-eastern region of Peninsular Malaysia.

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COMMENTS

Background

There is variability in prevalence rates of irritable bowel syndrome (IBS) among the ethnic Malays (6.8%-15.8%) across South-East Asia. Previous reported studies from Malaysia and Singapore involved heterogeneous populations and different diagnostic criteria.

Research frontiers

Using a validated Malay language Rome III diagnostic questionnaire, this study surveyed for IBS among ethnic Malays from the North-Eastern region of Peninsular Malaysia.

Innovations and breakthroughs

Using Rome III criteria, IBS was found to be common among ethnic Malays from a poorly developed region of Peninsular Malaysia. They were generally older (mean age 41.4 years) with no difference between genders. Red flags were common in this population and this was similar to other reported studies.

Applications

Exact reasons for higher prevalence rates of IBS among Malays are unknown and await further studies. Within the primary care setting, the validated questionnaire may be useful to identify this group of Malay subjects who often present with vague symptoms.

Terminology

The new Rome III criteria for IBS are symptom-based (abdominal pain or discomfort and onset associated with a change in frequency and appearance of stools) and time-based (symptoms for the last 3 mo with onset at least 6 mo previously). Red flags and psychosocial alarm questionnaires are independent from Rome III diagnostic questionnaires and are helpful to identify subjects "at risk" for organic and psychological disorders.

Peer review

Surveying IBS amongst ethnic Malays offers a somewhat different perspective showing firstly, a higher frequency compared to other South-East Asian studies, confirming other Malaysian reports and secondly, gender equivalence like stud-

ies from India.

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Novel biosensor-based microarray assay for detecting rs8099917 and rs12979860 genotypes

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Abstract

AIM: To evaluate a novel biosensor-based microarray (BBM) assay for detecting rs12979860 and rs8099917 genotypes.

METHODS: Four probes specific for rs8099917C/T or rs12979860G/T detection and three sets of quality control probes were designed, constructed and arrayed on an optical biosensor to develop a microarray assay. Two sets of primers were used in a one tube polymerase chain reaction (PCR) system to amplify two target fragments simultaneously. The biosensor microarray contained probes that had been sequenced to confirm that they included the rs8099917C/T or rs12979860G/T alleles of interest and could serve as the specific assay standards. In addition to rehybridization of four probes of known sequence, a total of 40 clinical samples collected from hepatitis C seropositive patients were also tested. The target fragments of all

40 samples were amplified in a 50 μ L PCR system. Ten μ L of each amplicon was tested by BBM assay, and another 40 μ L was used for sequencing. The agreement of the results obtained by the two methods was tested statistically using the kappa coefficient. The sensitivity of the BBM assay was evaluated using serial dilutions of ten clinical blood samples containing 10^3 - 10^4 white cells/ μ L.

RESULTS: As shown by polyacrylamide gel electrophoresis, two target segments of the interleukin 28B-associated polymorphisms (SNPs) were successfully amplified in the one-tube PCR system. The lengths of the two amplified fragments were consistent with the known length of the target sequences, 137 and 159 bps. After hybridization of the PCR amplicons with the probes located on the BBM array, the signals of each allele of both the rs8099917 SNPs and rs12979860 SNPs were observed simultaneously and were clearly visible by the unaided eye. The signals were distinct from each other, could be interpreted visually, and accurately recorded using an ordinary digital camera. To evaluate the specificity of the assay, both the plasmids and clinical samples were applied to the microarray. First, 30 PCR amplicons of the various SNP alleles were hybridized on the BBM microarray. Full agreement between plasmids and the BBM assay was observed, with 30/30 correct matches (100%). The kappa value for the BBM assay with plasmids was 1.00 ($P < 0.05$). For the 40 clinical blood samples, the BBM assay hybridization and direct sequencing results were compared for each amplicon. For patient blood samples, agreement was 28/28 for rs8099917T/T, 9/11 for rs8099917T/G, 1/1 for rs8099917G/G, 24/24 for rs12979860C/C, 11/14 for rs12979860C/T, and 2/2 for rs12979860T/T. Only five clinical samples of amplicon assay and direct sequencing results were discordant and heterozygotes: 2/11 rs8099917T/G and 3/14 rs12979860C/T. The agreement of outcomes between BBM assay and direct sequencing for the detection of rs8099917 and rs12979860 was

95% and 92.5%, respectively; and the corresponding kappa values were 0.88 and 0.85 (A kappa value > 0.75 was defined as substantial agreement). The BBM assay and sequencing had similar specificities for detection and identification of the two SNPs and their alleles. The sensitivity evaluation showed that the BBM assay could detect and identify SNP sequences present in blood samples containing as few as 10^2 white blood cells/ μ L.

CONCLUSION: This biosensor microarray assay was highly specific, sensitive, rapid and easy to perform. It is compatible with clinical practice for detection of rs8099917 and rs12979860.

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Key words: Biosensor-based microarray; Hepatitis C virus; rs8099917; rs12979860; Detection; Assay

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Li PY, Zhou XJ, Yao L, Fang XH, Ren JN, Song JW. Novel biosensor-based microarray assay for detecting rs8099917 and rs12979860 genotypes. *World J Gastroenterol* 2012; 18(44): 6481-6488 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i44/6481.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i44.6481>

INTRODUCTION

Hepatitis C virus (HCV) infection is a worldwide health problem. It is estimated that about 3% of the world population are infected with HCV, including more than 170 million with chronic infection, who are at risk of developing liver cirrhosis and/or liver cancer. Approximately 3 to 4 million become infected with HCV, and more than 350 000 people die from HCV-related liver diseases each year^[1]. Only about 30% patients with acute hepatitis C experience spontaneous clearance^[2,3], while the remaining 70% develop persistent chronic infection and gradually progress to liver cirrhosis and/or hepatocellular carcinoma^[4,5]. Chronic HCV infection that progresses to end-stage liver disease often requires a liver transplant, with high cost and extensive use of medical resources. Hepatocellular carcinoma is the seventh most common cancer worldwide and the third leading cause of cancer-related deaths. HCV thus has a very high morbidity and mortality, which result in a substantial burden on society^[5].

Since the discovery of HCV in 1989, some improvement in treatment have been gained with the application of routine weekly injections of pegylated interferon (IFN) plus daily administration of oral ribavirin (Rb)^[6]. Although triple therapy with IFN + Rb and boceprevir or telaprevir has produced a sustained virologic response

(SVR) of approximately 70%, this regimen still requires co-administration of IFN + Rb and has a high cost in most countries^[7]. At present, only 50% of patients infected with HCV genotype 1 achieved an SVR, defined as absence of HCV RNA six months after the cessation of therapy^[8,9]. Even in non-genotype 1 patients, who are reported to have better responses to the combination treatment, the SVR rate was about 75%^[10,11]. A number of viral and host factors may reduce adherence to the treatment. In addition to a limited treatment response to current combined therapy by some patients, treatment is often poorly tolerated due to various adverse reactions, including flu-like symptoms, severe fatigue, and neutropenia^[12]. Moreover, the inconvenience of a prolonged course of weekly injections of pegylated interferon and the high costs of the antiviral drugs make some patients give up the combined therapy. Additional research to advance HCV treatment regimens is needed to increase the patient adherence and response to therapy, thereby increasing SVR rates.

It is also puzzling why approximately 30% of patients with acute HCV infection experience spontaneous viral clearance, whereas the rest become chronically infected^[2,3]. Meanwhile, the global epidemiology of HCV infection shows that the different HCV genotypes clearly show geographic variation in their relative frequencies. Genotype 1, consisting of subtypes 1a and 1b, is the most prevalent genotype worldwide, with a higher prevalence of 1b in Europe and 1a in the United States^[11-13]. The estimated prevalence of people with detectable anti-HCV antibodies is highest in Africa, with an overall seroprevalence of 5.3%, and most common in genotype 4. In contrast, the infection rate of HCV is lower, and genotype 2 or 3 are more frequent in Asia^[11-13]. Furthermore, previous studies have revealed that the effectiveness of the combination treatment with peg-interferon and ribavirin differs among various ethnic populations^[14]. In the US, chronic HCV patients of European ancestry have a higher probability of being cured than patients of African ancestry^[15]. It is well documented that patients originating from East Asia are more likely to achieve SVR than patients from Europe^[16]. These observations suggest that host genetic variations play a critical role in patients infected with HCV.

Four recent genome-wide association studies (GWAS) independently identified several single nucleotide polymorphisms (SNPs) in the interleukin 28B (IL28B) locus. Variability at that locus is known to be associated with the successful treatment of HCV infection^[15,17-19]. Ge *et al*^[15] identified an rs12979860 SNP residing approximately 3 kilo-bases upstream of the *IL28B* gene as the variant most strikingly associated with SVR, and demonstrated that patients with the CC genotype had a higher SVR rate than those with the TT genotype. Suppiah *et al*^[17], Tanaka *et al*^[18], and Rauch *et al*^[19] found a strong association between another SNP, rs8099917 located 8 kilo-bases upstream of the *IL28B* gene, and SVR, which is in linkage disequilibrium with rs12979860. Tanaka *et al*^[18] also found

that the rs8099917 GG allele was the most significant host factor for predicting nonvirological response after adjusting for confounding factors. Their results suggest that Japanese patients with a minor GG allele may require new antiviral therapies to achieve SVRs.

These exciting discoveries show promise that detecting the *IL28B* genotype will help predict treatment response, thus guiding clinical decisions in the choice of treatment regimens for chronic HCV infection^[7]. Such discoveries may ultimately usher in the era of personalized treatment for HCV infection^[18,20].

An easily performed and specific method for the detection of *IL28B* genotypes is urgently needed to improve the currently available methods. Currently there are several assays for the detection of *IL28B* genotypes. However, due to technological limitations, these assays focus on either rs8099917 or rs12979860. Until now, there has been no assay for the simultaneous detection of rs8099917 or rs12979860. Furthermore, none of the available assays can be routinely applied in clinical practice. For example, direct sequencing, widely used in current research, is not suitable for clinical practice because of its complex technology, high cost, and time consumption.

The primary aim of this study was to develop a novel biosensor-based clinical microarray (BBM) assay stemming from our previous research to detect the *IL28B* genotypes containing rs12979860 and rs8099917 SNPs, and to evaluate the specificity and sensitivity of the test^[21,22].

MATERIALS AND METHODS

Plasmids and clinical samples

Plasmids (Invitrogen, Shanghai, China) confirmed by direct sequencing to contain both rs8099917 and rs12979860 SNPs, were used to construct the probes that were arrayed on the BBM; and plasmids diluted to 10⁴ copies/mL were used to determine the assay specificity. The two alleles of each SNP were tested separately. Blood samples obtained from HCV seropositive patients at the 5th Affiliated Hospital of Sun Yat-Sen University (Zhuhai, China) were used to evaluate the clinical sensitivity and specificity of the BBM assay. The study protocol was approved by the Ethics Committee of our hospital and all patients enrolled in this study provided written informed consent.

BBM preparation

Several probes (Invitrogen, California, United States), highly specific for rs8099917 or rs12979860 polymorphisms, plus positive and negative quality control probes, were designed and synthesized. All the probe sequences included in this study were from a public database (<http://www.ncbi.nlm.nih.gov/>) and were based on the known human genome in the region of the *IL28B* gene. After optimization, the probes specific for the SNPs as well as positive and negative control probes were arrayed on the BBM as shown in Figure 1. The positive control probes were used to monitor the assay process. The negative probes served to monitor contamination. The probe se-

quences are summarized in Table 1.

DNA extraction and polymerase chain reaction amplification

Human genomic DNA was extracted from 200 μ L of patient blood samples using 200 μ L commercially available DNA extraction buffer (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions. To determine the SNP genotypes, two sets of special primers were designed to amplify the rs8099917 and rs12979860 SNP fragments within the *IL28B* gene region (Table 1). The extracted DNA was amplified by polymerase chain reaction (PCR). The reaction mixture (25 μ L) contained 5 μ L DNA, 2.5 μ L of 10 μ mol/L buffer (Juntan, Shanghai, China), 0.5 μ L deoxynucleoside triphosphates (Roche, Basel, Switzerland), 1.5 μ L primers (Invitrogen, California, United States), 1.25 U hot Taq polymerase (Juntan, Shanghai, China), and 14.25 μ L high-performance liquid chromatography-grade water. Simultaneous amplification of two SNPs was carried out under the following conditions: an initial denaturation at 95 $^{\circ}$ C for 10 min, 40 cycles at a denaturation temperature of 94 $^{\circ}$ C for 30 s, annealing at 56 $^{\circ}$ C for 30 s, and extension at 72 $^{\circ}$ C for 30 s, with a final prolonged extension at 72 $^{\circ}$ C for 5 min. All PCR products were visualized on a 2% agarose gel or by polyacrylamide gel electrophoresis (PAGE) electrophoresis.

Detection of SNP genotype

All SNP amplicons were subjected to reverse hybridization and direct sequencing. A 10 μ L volume of PCR amplified product was heated at 99 $^{\circ}$ C for 5 min and denatured, with subsequent cooling for 5 min on ice. Then the amplified product was placed on the surface of the BBM and incubated at 50 $^{\circ}$ C for 60 min with a prepared hybridization reaction mixture. The BBM was eluted three times at 45 $^{\circ}$ C, incubated with anti-biotin horseradish peroxidase reagent at room temperature (10-35 $^{\circ}$ C) for 10 min, rinsed three times with a buffer wash, and incubated with tetramethylbenzidine for 2 min in the dark. Finally, the residues on the BBM were washed in 0.1 \times standard saline citrate and distilled water at room temperature so as to obtain a clear signal. The remaining amplified PCR product was sent for direct sequencing.

Assay specificity

Four synthetic plasmids, each including an SNP allele (rs12979860CC, rs12979860TT, rs8099917GG, and rs8099917TT) and 40 HCV-seropositive blood samples were used to validate the specificity of the assay to detect the two SNPs. Each successfully amplified product obtained from these plasmids was evaluated by the BBM assay, and the results thus obtained were compared with their known genotypes. For the clinical evaluation, amplicons obtained from patient blood samples were tested under the same conditions as the amplified plasmid products. The percent agreement of the BBM assay results with the results of direct sequencing was determined for

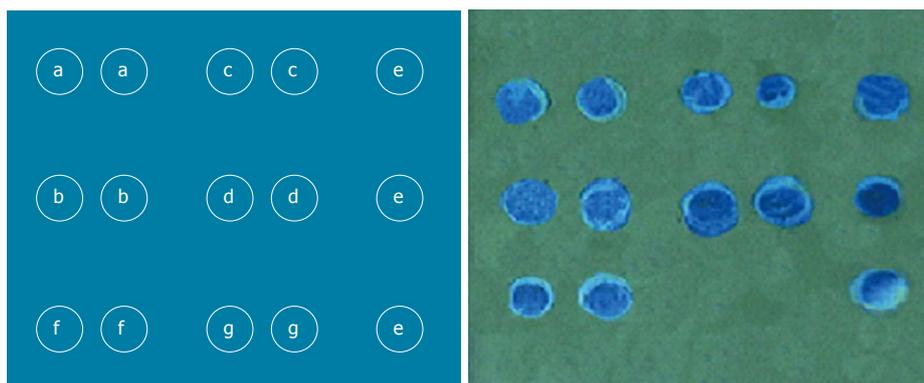


Figure 1 Designed interleukin 28B biosensor-based microarray and results of constructed plasmids detected by biosensor microarray assay. a: rs12979860C; b: rs12979860T; c: rs8099917 T; d: rs8099918 G; e: System control probes; f: Positive probes; g: Negative probes.

Table 1 Sequence of primers and probes	
Names	Sequences
IL28B primers forward (9860)	GTGCCCTGTCGTACTGAAC
IL28B primers reverse (9860)	CGCTGAGCACTGCCTGGGC
IL28B primers forward (9917)	ATTTGTCACGTTCCTCCT
IL28B primers reverse (9917)	GCCTAACTGATACGCTATAA
rs12979860 probe (C allele)	AAAAAAAAAAAAAGCTCCCCGAAGGCGGAACCAGGGTTGAAT
rs12979860 probe (T allele)	AAAAAAAAAAAAAGCTCCCCGAAGGCGTGAACCAGGGTTGAAT
rs8099917 probe (T allele)	AAAAAAAAAACTTTCGTGAGCAATTCACCCAAATTGGAA
rs8099917 probe (G allele)	AAAAAAAAAACTTTCGTGAGCAATGTCACCCAAATTGGAA

IL28B: Interleukin 28B.

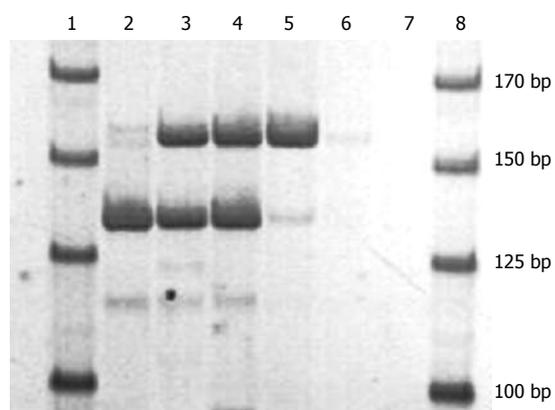


Figure 2 Polymerase chain reaction products visualized on polyacrylamide gel electrophoresis. Lanes 1 and 8: Marker; Lanes 2, 5, 6: A single fragment amplified rs12979860 or rs8099917 primers; Lanes 3 and 4: Two fragments amplified by one tube polymerase chain reaction system of rs12979860 and rs8099917; Lane 7: Negative control.

both the synthetic probes and the clinical samples.

Assay sensitivity and detection limit

To estimate the sensitivity and detection limit of the SNP genotype assay in clinical practice, ten blood samples were used. The sensitivity calculation was based the white blood cell (WBC) count in the original sample and the amount of DNA extracted from the WBC remaining in serial dilutions of each sample. The WBC counts were determined in each sample and then diluted to 10^1 - 10^3

cells/ μ L. The DNA was extracted from 1 μ L of each sample and was amplified. All samples with successful PCR amplification were evaluated for SNP genotype by the BBM assay.

Statistical analysis

The kappa coefficient was used to measure the agreement between direct sequencing and the BBM assay for the detection of SNP genotype. A kappa value > 0.75 was defined as substantial agreement, and a kappa > 0.95 as perfect agreement. A $P < 0.05$ was considered statistically significant.

RESULTS

Amplification of rs8099917 and rs12979860 fragments

Both fragments were amplified simultaneously in the one-tube system, and the amplicons were analyzed on PAGE electrophoresis (Figure 2). The length of the two amplified fragments was about 137 and 159 bp, being consistent with the length of the target fragments. The results indicated that these two target fragments could be amplified in a single tube PCR system.

Simultaneous detection of two SNPs

As shown in Figures 1 and 3, the signals on the chip appeared sufficiently distinct seen with the unaided eye and photographed with a normal digital camera. It was thus confirmed that no special equipment was needed to read

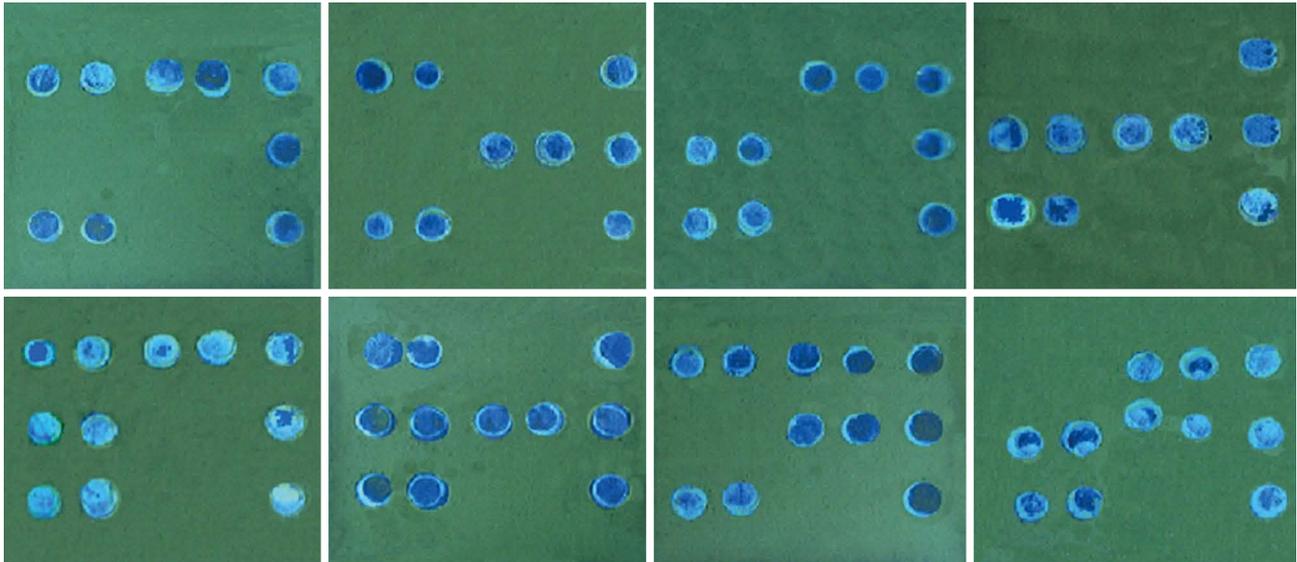


Figure 3 Patterns detected by biosensor microarray assay for rs8099917 and 12979860 genotypes.

Table 2 Concordance of biosensor-based microarray assay with given plasmids for rs12979860 and rs8099917

BBM assay	Plasmids with given genotype			Total
	CC	CT	TT	
rs12979860				
CC	10	0	0	10
CT	0	10	0	10
TT	0	10	10	10
Total	10	10	10	30
rs8099917				
TT	10	0	0	10
GT	0	10	0	10
GG	0	0	10	10
Total	10	10	10	30

Kappa = 1.00. BBM: Biosensor-based microarray.

the BBM assay results. Moreover, each allele of both rs8099917 rs12979860 was successfully detected at the same time. The BBM assay was able to simultaneously genotype the two SNPs.

Assay specificity

As shown in Figure 3, the positive signals were displayed clearly, and there were no signals from the sites containing the negative probes. There were no ambiguous signals on the chip background, which could have interfered with precise interpretation of the results. This suggested that the assay was specific for the detection of rs8099917 and rs12979860 polymorphisms.

To confirm the BBM assay specificity for detection of each allele, we tested all the possible genotypes using the available synthetic plasmids. As shown in Figure 3, the BBM assay clearly differentiated all the genotypes and haplotypes of the two SNPs. The signals of the alleles of each SNP were clearly differentiated from each other, with no visible evidence of cross-reaction at any SNP binding site. Data obtained using 30 plasmid amplicons

Table 3 Concordance of biosensor-based microarray assay with polymerase chain reaction sequencing of the blood samples for rs12979860 and rs8099917

BBM assay	Direct sequencing			Total
	CC	CT	TT	
rs129798601				
CC	24	0	0	24
CT	3	11	0	14
TT	0	0	2	2
Total	27	11	2	40
rs80999172				
TT	28	0	0	28
GT	2	9	0	11
GG	0	0	1	2
Total	30	9	1	40

¹Kappa = 0.88; ²Kappa = 0.85. BBM: Biosensor-based microarray.

showed the same results for all the known genotypes of rs12979860 or rs8099917 (Tables 2 and 3), and its agreement rate with the plasmids was up to 100% in our tests. The results showed that the BBM was an accurate assay for genotype determination of these two SNPs.

To validate the BBM assay for clinical application, the amplicons derived from the 40 blood samples taken from HCV-infected patients were examined by both direct sequencing and BBM. As shown in Tables 2 and 3, the percent of agreement of the BBM assay results and direct sequencing of rs8099917 and rs12979860 were 95% and 92.5%, respectively. The corresponding kappa values were 0.88 and 0.85, respectively, with a *P* value < 0.05. The BBM assay accurately detected the two SNPs in clinical samples. Discordance of the BBM assay and direct sequencing of PCR amplicons most often occurred with heterozygous clinical samples.

Assay sensitivity and detection limit

Ten clinical blood samples and a series of sequential dilu-

tions were used to validate the sensitivity and detection limit of BBM. Blood samples with WBC counts ranging from 10^1 to 10^3 cells/ μL were amplified with BBM primers, and visualized by PAGE electrophoresis. Amplicons with detectable human genomic DNA were assayed by reverse hybridization with the BBM. The hybridization signal was visualized clearly in the BBM assay in a total of 20 dilutions with WBC counts in the patient blood samples ranging from 10^2 to 10^3 cells/ μL .

DISCUSSION

The combined therapy of pegylated interferon plus ribavirin is currently the standard of care for chronic HCV infection around the world^[6]. Its administration in clinical practice has resulted in substantial progress in the treatment of chronic hepatitis C over the past decades. However, several persistent shortcomings of the combined therapy prevent some patients from completing the entire 48-wk course of treatment. Patient adherence is frequently compromised by an inability to tolerate adverse reactions or the many weeks of routine subcutaneous injections, and the high cost of the drugs^[8,9,12]. The limited efficacy of pegylated interferon/ribavirin therapy to produce SVR, which varies unpredictably from patient to patient, makes it difficult for health care providers to make an informed choice of treatment regimens^[18-20]. There is an urgent need of personalized therapy for chronic hepatitis C.

The fact that about 30% of patients achieve natural clearance following acute hepatitis C virus infection and ethnic differences in response to treatment suggests that host genetic variation plays a critical role in the drug response^[2,11,17,19,20]. Four recent studies have found that two SNPs, rs8099917 and rs12979860, located near the *IL28B* gene were highly associated with treatment response and spontaneous clearance following acute hepatitis C infection^[15,17-19]. Subsequent studies found that the two SNPs had an impact on the occurrence of various side effects of the combined therapy, treatment response and the HCV RNA genotype distribution of the infecting virus^[23,24]. Other studies have shown that *IL28B* polymorphisms were a significant, independent predictive factor regardless of HCV genotype or HCV RNA load. The determination of *IL28B* polymorphisms may assist in evaluating the likelihood of response to treatment with peg-interferon and ribavirin therapy in patients chronically infected with HCV, especially for genotype 1 patients^[25-28]. The recent significant findings regarding *IL28B* SNPs and the genotyping of HCV showed how personalizing treatment for HCV infection may be possible^[24,27]. The development of an accurate assay for the detection of rs8099917 and rs12979860 polymorphisms, and progress in HCV genotyping will help both clinicians and patients choose the treatment regimen and its anticipated duration^[22] and this may be an early step in the era of personalized therapy for chronic hepatitis C^[20,29,30].

Host genetic diversity is of great significance in mak-

ing an informed decision regarding the risk-benefit treatment and the likelihood of success for any individual treatment, so developing a simple, rapid and clinically available assay is an urgent demand. An assay that could accurately and rapidly detect the *IL28B* SNPs is a priority for clinical practitioners. Developing a novel and efficient assay for the detection of *IL28B* polymorphisms has been the focus of numerous researches.

Direct sequencing of PCR amplicons, restriction fragment length polymorphism (RFLP) and traditional microarrays are valuable research techniques. However, the technical demands of these assays make them unsuitable for routine diagnostic use in clinical practice. For example, direct sequencing is technologically complicated, time-consuming and needs special expertise^[28,29]. The RFLP is almost impossible to be used for the detection of all significant SNPs since it requires a specific cutting site and restriction enzyme for the particular site^[31]. Microarray assays widely used in the previous research, apart from being provided by specialized and high-cost facilities, can only detect either of the two SNPs separately^[19,21-23]. No microarray assay was available currently that can detect the two SNPs simultaneously. There is a real need for a highly sensitive and specific rapid assay for the detection of *IL28B* polymorphisms that is easy to perform and compatible with clinical practice. The BBM described in our report may be an ideal clinical assay.

As depicted in Figure 3, the positive signals on the chip were sufficiently distinct to be completely discriminated from negative results. The results with plasmid amplicons showed that all the SNP alleles could be detected successfully and that heterozygous and homozygous alleles could be distinguished. Of 40 plasmid amplicons employed to validate the specificity of the BBM assay, a complete agreement between the results of the BBM assay and known plasmid sequences was observed, confirming the high specificity of the BBM assay in determining the rs8099917 and rs12979860 genotypes.

The clinical blood samples successfully amplified by the BBM assay primers were sent for both direct sequencing and reverse hybridization with BBM. The BBM assay showed a high agreement with direct sequencing in detecting the rs8099917 and rs12979860 polymorphisms. The agreement rates were 95% and 92.5%, and the kappa values were 0.88 and 0.85 for rs8099917 polymorphisms and rs12979860, respectively. These data indicated that the BBM assay showed a good agreement with direct sequencing and that it would be a satisfactory novel assay when used for determination of the two SNPs. The main difference between the BBM results and direct sequencing was the detection of allele heterozygotes. The BBM assay was more sensitive in detecting heterozygous alleles, which was consistent with other reverse hybridization procedures such as line probe assay^[32,33]. In brief, we demonstrated that the novel BBM assay could successfully detect all genotypes of the two SNPs simultaneously, not only in the synthetic plasmids but also in the clinical samples used in this study.

To evaluate the sensitivity and detection limit of the BBM assay, serial dilutions of 10 clinical blood samples were tested. The amount of human DNA from white blood cells in 1 μL peripheral blood containing 10^2 WBC/ μL was sufficient for SNP detection by the BBM assay. The DNA extracted from all samples was successfully amplified, and the blood samples with a starting white blood cell count $> 10^2$ WBC/ μL hybridized with the probes arrayed on the BBM. As the white blood cell count of human peripheral blood is generally greater than 10^3 WBC/ μL , the detection limit of the BBM assay ensures that clinical blood samples would be tested accurately. This novel method thus met the sensitivity criteria for clinical diagnosis, i.e., determination of IL28B polymorphisms of HCV-infected patients.

The results indicated that the BBM assay has the advantages of simple and convenient operation as well as rapid detection. After a successful PCR amplification, it took only one hour to detect the IL28B polymorphisms and less than four hours before getting the final result. More important, the signal on the chip was clear enough to be interpreted even by the naked eye, which meant that only an ordinary digital camera was needed to record the results. The results also suggest that the BBM assay might also be used in developing countries having limited access to the latest laboratory facilities. The assay is also suitable for clinical diagnostic and research laboratories where large numbers of samples are tested on a daily basis. In short, the BBM assay is more likely to be accepted for the clinical detection of IL28B polymorphisms than the other existing methods^[21,22].

The BBM assay has some disadvantages to overcome in its future development. The patient population benefiting from the BBM assay is small, being limited to patients with chronic HCV infection, meanwhile, the assay is only capable of detecting the presence of known SNPs. With the identification of more SNPs associated with drug response, novel probes have to be designed and the BBM assay has to be constantly upgraded.

In conclusion, we have demonstrated that the BBM assay is simple, rapid, accurate, and suitable for clinical application. The assay is highly sensitive and specific, and can simultaneously perform the detection of rs8099917 and rs12979860 genotypes, thereby enabling further progress in the diagnosis and treatment of chronic HCV infection.

COMMENTS

Background

Antivirals are essential to the therapeutic management of hepatitis C virus (HCV)-infected patients. However, sustained virological response is not achieved in all patients who receive the standard combination therapy of once-weekly injections of pegylated interferon plus daily oral ribavirin or even in those treated with the triple therapy regimen. Recent research has characterized single nucleotide polymorphisms (SNPs) in the interleukin 28B (*IL28B*) gene as the most important host factor influencing the efficacy of HCV therapy. Many scientists believe that identifying each of the IL28B SNPs related to HCV treatment response will usher in a new era of HCV personalized therapy.

Research frontiers

Four recent genome-wide association studies (GWAS) independently identified several SNPs in the *IL28B* gene locus that are associated with an individual's ability to respond to therapy for HCV infection. Ge *et al.*^[15] characterized rs12979860 as the variant most strongly associated with SVR, and demonstrated that patients with the CC genotype have a higher SVR rate than those with the TT genotype. In contrast, studies demonstrated that rs8099917 has the strongest association with SVR. However, GWAS are capable of only testing SNPs in isolation and could not determine if interaction between these two SNPs, or more, can affect a patient's response to HCV therapy. Thus, it is urgent to develop an accurate and easy clinical assay to test the panel of IL28B SNPs in a patient and assess the effect of multiple SNPs on SVR.

Innovations and breakthroughs

This is the first report of an assay that is capable of defining the rs8099917 and rs12979860 SNPs using clinical samples. This innovative biosensor-based microarray (BBM) assay can identify the two SNPs in less than four hours. In addition, evidence is provided to show that the new assay can accurately identify all of the SNPs and gene alleles with plasmids. There was a good concordance between BBM-detected SNPs in clinical samples and direct sequencing results of PCR amplicons and plasmids. Finally, the excellent sensitivity and reproducibility of the BBM assay supports the clinical applicability of this new detection approach.

Applications

This study aimed to develop a clinically applicable assay to accurately and rapidly detect IL28B SNPs in patient samples. The results indicate that the BBM assay is simple, rapid, accurate, and highly sensitive and specific in detecting the rs8099917 and rs12979860 genotypes. Thus, this novel assay has promise for clinical application and may facilitate accurate and timely prognosis of HCV-infected patients so that the appropriate therapies may be initiated earlier.

Terminology

BBM is a novel SNP detection assay based on biosensor technology, its most obvious advantage is *in situ* amplification that facilitates easy interpretation of results; rs8099917 and rs12979860 are SNPs in the *IL28B* gene locus; SNP is a single nucleotide polymorphism.

Peer review

In this work, the authors describe a new BBM assay to rapidly detect two SNPs in the *IL28B* gene. The interest of the paper is its description of this rapid and inexpensive technique to detect IL28B SNPs in clinical samples. The results suggest that the BBM assay is a simple, rapid, sensitive, and highly specific method that may be applied clinically to detect rs8099917 and rs12979860 without any large-scale instrumentation.

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Risk clinicopathological factors for lymph node metastasis in poorly differentiated early gastric cancer and their impact on laparoscopic wedge resection

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Abstract

AIM: To investigate the predictive factors of lymph node metastasis (LNM) in poorly differentiated early gastric cancer (EGC), and enlarge the possibility of using laparoscopic wedge resection (LWR).

METHODS: We retrospectively analyzed 85 patients with poorly differentiated EGC who underwent surgical resection between January 1992 and December 2010. The association between the clinicopathological factors and the presence of LNM was retrospectively analyzed by univariate and multivariate logistic regression analyses. Odds ratios (OR) with 95%CI were calculated. We further examined the relationship between the positive number of the three significant predictive factors and

the LNM rate.

RESULTS: In the univariate analysis, tumor size ($P = 0.011$), depth of invasion ($P = 0.007$) and lymphatic vessel involvement ($P < 0.001$) were significantly associated with a higher rate of LNM. In the multivariate model, tumor size (OR = 7.125, 95%CI: 1.251-38.218, $P = 0.041$), depth of invasion (OR = 16.624, 95%CI: 1.571-82.134, $P = 0.036$) and lymphatic vessel involvement (OR = 39.112, 95%CI: 1.745-123.671, $P = 0.011$) were found to be independently risk clinicopathological factors for LNM. Of the 85 patients diagnosed with poorly differentiated EGC, 12 (14.1%) had LNM. The LNM rates were 5.7%, 42.9% and 57.1%, respectively in cases with one, two and three of the risk factors respectively in poorly differentiated EGC. There was no LNM in 29 patients without the three risk clinicopathological factors.

CONCLUSION: LWR alone may be sufficient treatment for intramucosal poorly differentiated EGC if the tumor is less than or equal to 2.0 cm in size, and when lymphatic vessel involvement is absent at postoperative histological examination.

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Key words: Poorly differentiated early gastric cancer; Early gastric cancer; Lymph node metastasis; Clinicopathological characteristics; Laparoscopic wedge resection

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INTRODUCTION

Local resection for early gastric cancer (EGC) was first reported by Kitaoka *et al*^[1] in 1984. Laparoscopic wedge resection (LWR) is a procedure based on local resection. This minimally invasive technique can be applied for the management of EGC without the risk of lymph node metastases (LNM)^[2-7]. The application of LWR has been limited to differentiated EGC because of the higher risk of lymph node metastases in undifferentiated EGC, compared to differentiated EGC^[8,9]. Therefore, gastrectomy with lymphadenectomy has been considered to be an essential treatment for patients with undifferentiated EGC. Undifferentiated carcinoma of gastric cancer includes poorly differentiated adenocarcinoma, signet ring cell carcinoma, and mucinous adenocarcinoma^[10]. However, almost all (96.6%) surgical cases of poorly differentiated EGC confined to the mucosa, have been found not to have LNM^[11], suggesting that gastrectomy with lymphadenectomy may be over-treatment for these cases.

Therefore, we carried out this retrospectively study to determine the clinicopathological factors that are predictive of LNM in poorly differentiated EGC. Furthermore, we established a simple criterion to expand the possibility of using LWR for the treatment of poorly differentiated EGC.

MATERIALS AND METHODS

Patients

Patients underwent a radical operation due to EGC in the Department of Oncology, Affiliated Xing Tai People's Hospital of Hebei Medical University, Xingtai, China between January 1992 and December 2010 were included in the screening for identification of cases with EGC in this retrospective study.

The inclusion criteria were: (1) lymph node dissection beyond limited (D1) dissection was performed; (2) the resected specimens and lymph nodes were pathologically analyzed, and poorly differentiated EGC was diagnosed, according to the Japanese Classification of Gastric Carcinoma (JCGC)^[10]; and (3) patient's medical records were available in the database.

During the 18 years, 85 patients (60 male, 25 female; mean age 52 years, range: 29-82 years) with histopathologically poorly differentiated tumor were identified to meet the inclusion criteria for further analysis in this study.

The study protocol was approved by the Ethics Com-

mittee of Hebei Medical University.

Dissection and classification of lymph nodes

Lymph nodes of each case were meticulously dissected from the enbloc specimens, and the classification of the dissected lymph nodes was determined by a surgeon after he/she who carefully reviewed the excised specimens based on the JCGC^[10]. Briefly, lymph nodes were classified into group 1 (perigastric lymph nodes) and groups 2 (lymph nodes along the left gastric artery, the common hepatic artery, and the splenic artery and around the celiac axis)^[10].

Assessment and classification of lymph node metastasis

Then, the resected lymph nodes were sectioned and stained with hematoxylin and eosin and examined by pathologists for metastasis and lymphatic vessel involvement (LVI).

Association between clinicopathological parameters and lymph node metastasis

Clinicopathological parameters that are covered in the JCGC^[10] were included in this study. They were the gender (male and female), age (< 60 years, ≥ 60 years), family medical history of gastric cancer, number of tumors (single or multitude), the location of the tumor (upper, middle, or lower of the stomach), tumor size (maximum dimension ≤ 2 cm, or > 2 cm), macroscopic type [protruded (type I), superficial elevated (type II a), flat (type II b), superficial depressed (type II c), or excavated (type III)], depth of invasion (mucosa, submucosa), lymphatic vessel involvement.

The associations between various clinicopathological factors and LNM were examined as described below.

Statistical analysis

All data were analyzed using SPSS 15.0 statistical software (Chicago, IL, United States). The differences in the clinicopathological parameters between patients with and without LNM were determined by the χ^2 test. A multivariate stepwise logistic regression analysis was performed subsequently in order to identify independent risk factors for LNM. Hazard ratio and 95%CI were calculated. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Of the 85 patients diagnosed with poorly differentiated EGC, 12 (14.1%) had LNM. As shown in Table 1, 8 (70.6%) were male and 13.3% of them had LNM.

Association between clinicopathological factors and lymph node metastasis

The association between various clinicopathological factors and LNM was first analyzed by the χ^2 test (Table 1). A tumor larger than 2.0 cm, submucosal invasion, and

Table 1 Univariate analysis of potential risk characteristics for lymph node metastasis *n* (%)

Factor	Lymph node metastasis	<i>P</i> value
Sex		
Male (<i>n</i> = 60)	8 (13.3)	0.781
Female (<i>n</i> = 25)	4 (16.0)	
Age (yr)		
< 60 (<i>n</i> = 76)	10 (13.2)	0.534
≥ 60 (<i>n</i> = 9)	2 (22.2)	
Family medical history		
Positive (<i>n</i> = 3)	0 (0)	0.509
Negative (<i>n</i> = 82)	12 (14.6)	
Number of tumors		
Single (<i>n</i> = 83)	12 (14.5)	0.591
Multitude (<i>n</i> = 2)	0 (0)	
Location		
Upper (<i>n</i> = 20)	4 (20.0)	0.566
Middle (<i>n</i> = 5)	0 (0)	
Lower (<i>n</i> = 60)	8 (13.3)	
Tumor size in diameter		
≤ 2 cm (<i>n</i> = 54)	3 (5.6)	0.011
> 2 cm (<i>n</i> = 31)	9 (29.0)	
Macroscopic type		
I (<i>n</i> = 2)	0	0.768
II (<i>n</i> = 64)	10 (15.6)	
III (<i>n</i> = 19)	2 (10.5)	
Depth of invasion		
Mucosa (<i>n</i> = 56)	3 (5.4)	0.007
Submucosa (<i>n</i> = 29)	9 (31.0)	
Lymphatic vessel involvement		
Positive (<i>n</i> = 14)	8 (57.1)	< 0.001
Negative (<i>n</i> = 71)	4 (5.6)	

the presence of LVI were significantly associated with a higher rate of LNM (all *P* < 0.05). However, gender, age, family medical history of gastric cancer, number, location, and macroscopic type were found not to be associated with LNM.

Multivariate analysis of potential independent risk clinicopathological factors for lymph node metastasis

The three characteristics that were significantly associated with LNM by univariate analysis were found to be significant and independent risk factors for LNM by multivariate analysis (both *P* < 0.05, Table 2).

Lymph node metastasis in poorly differentiated EGC

The LNM rates were 5.7%, 42.9% and 57.1%, respectively in cases with one, two and three of the risk factors respectively in poorly differentiated EGC. There was no LNM in 29 patients without the three risk clinicopathological factors.

DISCUSSION

Because an increased rate of accurate diagnosis of EGC, which in turn leads to an improved prognosis, an increased interest has been focused on the improvement of the quality of life and minimization of invasive procedures^[12-14]. LWR has been associated with less pain, quicker return of gastrointestinal function, better pulmo-

Table 2 Multivariate analysis of potential risk factors for lymph node metastasis

Characteristics	Hazard ratio	95%CI	<i>P</i> value
Tumor size	7.125	1.251-38.218	0.041
≤ 2 cm			
> 2 cm			
Depth of invasion	16.624	1.571-82.134	0.036
Mucosa			
Submucosa			
Lymphatic vessel involvement	39.112	1.745-123.671	0.011
Positive			
Negative			

nary function, decreased stress response, a shorter hospital stay and better postoperative quality of life than open gastrectomy^[15-19]. If the feasibility and safety of LWR in the treatment of EGC has been proven, it is also true that several reports have shown the efficacy of LWR in the cure of EGC with results comparable to those of an open gastrectomy^[20].

One of the critical factors in choosing LWR for EGC would be the precise prediction of whether the patient has LNM or not. To achieve this goal, several studies have attempted to identify risk factors predictive of LNM in EGC. Few reports, however, have focused on the applicability of laparoscopic treatment for poorly differentiated EGC.

The present multivariate analysis revealed that a tumor larger than 2.0 cm, submucosal invasion, and the presence of LVI were significant predictive factors for LNM in patients with poorly differentiated EGC. Our results together with the previous reports on undifferentiated EGC^[21-24] demonstrated a significant correlation between the high incidence of LNM and a tumor larger than 2.0 cm submucosal invasion, or presence of LVI^[25-27].

We then attempted to identify a subgroup among poorly differentiated EGC patients in whom the risk of LNM can be largely ruled out, i.e., candidates who can be curably treated by LWR. As a result, we found no LNM in patients with intramucosal cancer if the tumor is less than or equal to 2.0 cm in size without LVI. This may indicate that LWR could be sufficient to treat these cases, and that additional surgery is unnecessary.

We further examined the relationship between the positive number of the three significant predictive factors and the LNM rate in order to establish a simple criterion for an optimal strategy for treatment of poorly differentiated EGC. In the present study, the LNM rates were 5.7%, 42.9% and 57.1%, respectively in cases with one, two and three of the risk factors respectively in poorly differentiated EGC. Therefore, gastrectomy with lymphadenectomy is probably better for these patients with the risk factors.

However, there are some limitations in this study. First, this is a retrospective analysis. Second, the sample size is relatively small. Thus, the outcomes of the study may not be good enough to expose the truth.

According to our study, we would propose a treat-

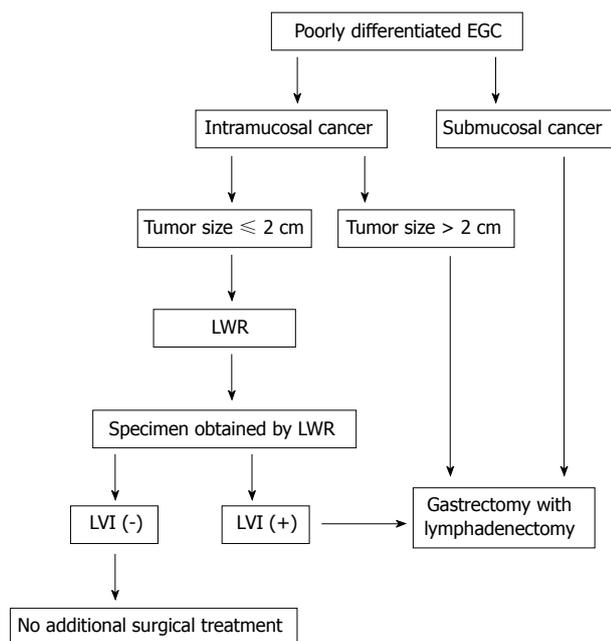


Figure 1 Flow chart of the therapeutic strategy for cases with poorly differentiated early gastric cancer. EGC: Early gastric cancer; LWR: Laparoscopic wedge resection; LVI: Lymphatic vessel involvement.

ment strategy for patients with poorly differentiated EGC (Figure 1). These predictive factors (tumor size and depth of invasion) were diagnosed by endoscopy and endoscopic ultrasound. The particular presence of LVI becomes first evident after the histological assessment of the entire specimen obtained by LWR. LWR alone may be a sufficient treatment for intramucosal poorly differentiated EGC if the tumor is less than or equal to 2.0 cm in size, and when LVI is absent at postoperative histological examination. When specimens show with LVI, an additional gastrectomy with lymphadenectomy should be recommended.

COMMENTS

Background

Gastrectomy with lymphadenectomy is the standard therapy for poorly differentiated early gastric cancer (EGC) with lymph node metastasis (LNM). However, because approximately 96.6% of in poorly differentiated intramucosal EGC have no LNM, gastrectomy with lymphadenectomy may be an over-treatment for such patients. The authors attempted to identify a subgroup of poorly differentiated EGC patients in whom the risk of LNM can be ruled out and treated them with laparoscopic wedge resection (LWR), which may serve as a breakthrough treatment of poorly differentiated EGC.

Research frontiers

Several studies have attempted to identify risk factors predictive of LNM in EGC. Few reports, however, have focused on the applicability of laparoscopic treatment for poorly differentiated EGC.

Innovations and breakthroughs

Tumor size, depth of invasion and lymphatic vessel involvement were found to be independently risk clinicopathological factors for LNM in poorly differentiated EGC. Furthermore, the authors established a simple criterion to expand the possibility of using LWR for the treatment of poorly differentiated EGC.

Applications

Based on the predictive factors for LNM, LWR is the treatment of choice for poorly differentiated EGC.

Terminology

LWR is a procedure based on local resection and a method of minimally invasive technique.

Peer review

This study analyzed the data from 85 patients with poorly differentiated EGC. They concluded that tumor size, depth of invasion and lymphatic vessel involvement are the significant risk factors for LNM. This is a well-designed retrospective study.

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Small intestine bleeding due to multifocal angiosarcoma

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Abstract

We report a case of an 84-year-old male patient with primary small intestinal angiosarcoma. The patient initially presented with anemia and melena. Consecutive endoscopy revealed no signs of upper or lower active gastrointestinal bleeding. The patient had been diagnosed 3 years previously with an aortic dilation, which was treated with a stent. Computed tomography suggested an aorto-intestinal fistula as the cause of the intestinal bleeding, leading to operative stent explantation and aortic replacement. However, an aorto-intestinal fistula was not found, and the intestinal bleeding did not arrest postoperatively. The constant need for blood transfusions made an exploratory laparotomy imperative, which showed multiple bleeding sites, predominately in the jejunal wall. A distal loop jejunostomy was conducted to contain the small intestinal bleeding and a segmental resection for histological evaluation was performed. The histological analysis revealed a less-differentiated tumor with characteristic CD31, cytokeratin, and vimentin expression, which led to the diagnosis of small intestinal angiosarcoma. Consequently, the

infiltrated part of the jejunum was successfully resected in a subsequent operation, and adjuvant chemotherapy with paclitaxel was planned. Angiosarcoma of the small intestine is an extremely rare malignant neoplasm that presents with bleeding and high mortality. Early diagnosis and treatment are essential to improve outcome. A small intestinal angiosarcoma is a challenging diagnosis to make because of its rarity, nonspecific symptoms of altered intestinal function, nonspecific abdominal pain, severe melena, and acute abdominal signs. Therefore, a quick clinical and histological diagnosis and decisive measures including surgery and adjuvant chemotherapy should be the aim.

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Key words: Gastrointestinal bleeding; Small intestine; Angiosarcoma; Small intestinal neoplasm

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INTRODUCTION

Primary malignant tumors of the small intestine are rare neoplasms, which comprise < 2% of all gastrointestinal tumors^[1], including adenocarcinoma, carcinoid, sarcoma, gastrointestinal stromal tumors, and lymphoma. The reason for the poor prognosis of small bowel malignant tumors is partly due to a late diagnosis. The difficulty diagnosing this type of tumor is associated with the nonspecific symptoms, including nausea, vomiting, abdominal pain, constipation, generalized weakness, fatigue,

malaise, weight loss, anemia, diarrhea, ileus, intestinal perforation, or hemorrhage^[2], as well as limited diagnostic methods for the small intestine.

Angiosarcoma is a rare mesenchymal tumor that most often arises from skin and subcutaneous tissues^[3-7] but can ultimately arise anywhere in the body. Angiosarcomas have been described in the liver^[8-11], spleen^[12,13], adrenal glands^[14-16], ovaries^[17-19], heart^[20-22], lung^[23,24], breast^[25-27] and, very rarely, in the gastrointestinal tract^[28-32]. Consequently, an intestinal angiosarcoma that is located in the small bowel rather than the upper or lower gastrointestinal tract is a very rare medical condition. Angiosarcomas are aggressive tumors with a high rate of lymph node and peripheral metastases. This tumor arises as a *de novo* primary tumor or secondary to irradiation or chemical exposure. Angiosarcoma of the small intestine presents unique diagnostic challenges and is often discovered late, leading to a very poor prognosis.

Additionally, the histological diagnosis is difficult and can be confused with other neoplasms such as poorly differentiated carcinoma^[4,33,34]. Diagnosis is facilitated by immunohistochemical expression analysis of the endothelial markers CD31 and CD34, as well as factor VIII-associated antigens.

Herein, we describe the case of an 84-year-old man with the first episode of gastrointestinal bleeding due to angiosarcoma of the small intestine.

CASE REPORT

The patient was transferred to the Department of Internal Medicine of a peripheral hospital with gastrointestinal bleeding, which required a blood transfusion. Three lesions with coagulum and vessels necessitating application of two clips were found by endoscopy of the distal duodenum and upper segment of the jejunum. A colonoscopy revealed old blood, so bleeding in the small intestine was suspected. The patient had been diagnosed with an aortic aneurysm 3 years previously, which was treated with juxtarenal stent-graft prosthesis. A prosthetic-enteric fistula was suspected on the emergency abdominal aortic computed tomography (CT) scan. With this suspected diagnosis, the patient was transferred to the University Hospital Düsseldorf, and emergency vascular surgery was performed. The stent-graft prosthesis was removed and desobliteration of the saccular aortic aneurysm and the renal arteries was implemented, followed by implantation of an aorto-biiliacal silver-graft prosthesis. However, a prosthetic-enteric fistula was not revealed intraoperatively. The gastrointestinal bleeding did not arrest postoperatively, and Forrest IIB bleeding in the proximal jejunum was endoscopically diagnosed and treated. The local bleeding was stopped with hemoclips.

An exploratory laparotomy was performed due to persistent gastrointestinal hemorrhage, which showed multiple intra-abdominal hemorrhagic lesions in the intestinal wall of the jejunum (Figure 1). Three segmental

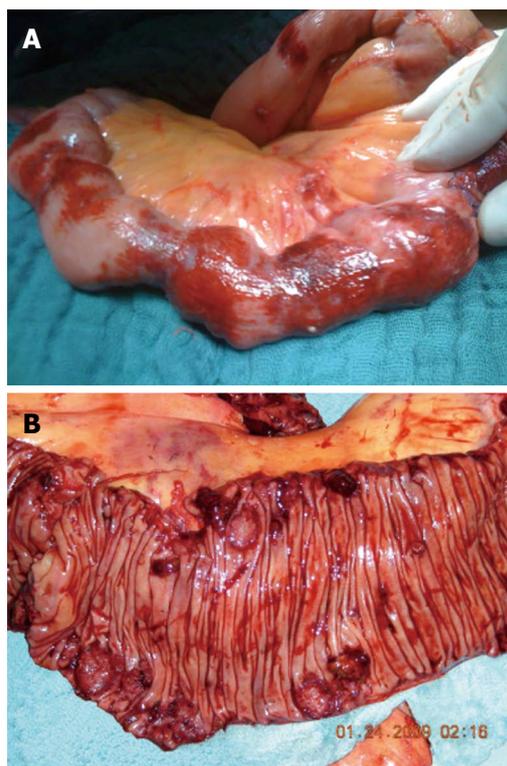


Figure 1 Multiple intra-abdominal hemorrhagic lesions in the intestinal wall of the jejunum. A: Intraoperative presentation of the jejunum with multiple subserous hemorrhages; B: Longitudinally lanced jejunum specimen with disseminated mucosal tumor manifestation.

resections and a distal loop jejunostomy were performed for a histological evaluation but achieved only temporal arrest of bleeding, which again became visible postoperatively after a loop jejunostomy. As no transanal bleeding was observed, and the histological analysis suggested a malignant angiosarcoma, a small bowel resection proximal to the loop jejunostomy with an end-to-end duodenoileostomy was subsequently performed.

Only approximately 1 m of small intestine could be preserved to achieve bleeding control. Adjuvant therapy was intended with paclitaxel, due to histological evidence of angiosarcoma. Unfortunately, a spontaneous intracranial hemorrhage with ventricular bleeding led to death of the patient, and the cause could not be determined. During hospitalization, the patient had received 75 erythrocyte concentrates, 49 units of fresh frozen plasma, 12 thrombocyte concentrates, and coagulation factors.

Histopathological findings

The small bowel showed an epithelium partly ulcerated with hemorrhage and infiltration of a mesenchymal fusiform tumor with parts of high-grade cells and nuclear polymorphism, including several mitoses and apoptosis formation (Figure 2). The tumor cells formed slit-shaped hollows, and they were predominantly grouped together with a solid appearance. The neoplastic cells were multifocal with macronucleoli. Eight mitoses were

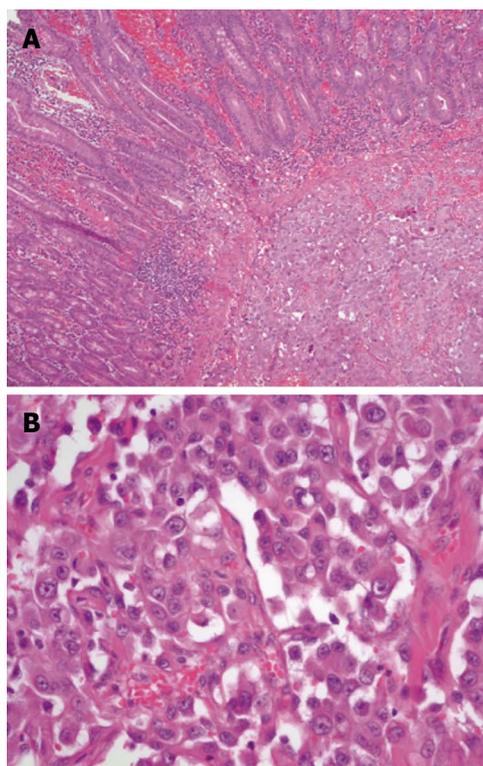


Figure 2 Histopathological findings. A: Ileum section stained with hematoxylin and eosin (HE) shows extensive tumor infiltration ($\times 100$); B: HE-stained detailed section of an angiosarcoma with typical vascular proliferation ($\times 400$).

identified per 10 high-power fields. The tumor cells stained positive for CD31, cytokeratin, and vimentin and slightly weaker for CD34. The tumor cells were also focally positive for factor VIII. The MIB-1 marker of proliferation was expressed in approximately 40% of nuclei. The Berlin blue reaction indicated siderin deposits and Elastica-van-Gieson staining revealed collagen fibers. In summary, the tumor showed a less differentiated, multifocally growing epithelioid angiosarcoma (degree of malignancy III Coindre) in the submucosa with infiltration of the subserosal fat tissue and extensive lymphatic spread.

DISCUSSION

A gastrointestinal hemorrhage is a potentially dangerous condition that warrants a quick diagnosis and decisive treatment. The vast majority of these bleeding events are due to either upper or lower gastrointestinal bleeding, and only 5% cannot be localized endoscopically^[35]. These bleeding events typically occur from the small intestine. The most common cause of small intestinal bleeding is a vascular abnormality such as angioectasia, followed by tumors and, more infrequently, small bowel ulcers and aortoenteric fistulas^[36]. Angiosarcoma of the small intestine is an extremely rare but potentially life-threatening cause of such bleeding.

Angiosarcomas typically occur in skin and superficial

soft tissue, rather than in the gastrointestinal tract, and compromise $< 2\%$ of all sarcomas^[37,38]. Consequently, only 33 cases of small intestinal angiosarcoma have been reported in the English literature over the past 42 years (Table 1).

The precise predisposing factors remain unknown. Exposure to vinyl chloride, thorotrast, arsene, and radiation have been associated with the pathogenesis^[9,31,39-41]. Of the 33 cases reported, 14 describe patients developing an angiosarcoma after being treated with radiation for a malignant tumor, including ovarian carcinoma^[39,42], ovarian dysgerminoma^[40], squamous cell carcinoma of the uterine cervix^[43-46], endometrial adenocarcinoma of the uterus^[41,47], and Hodgkin's disease^[48]. The first report of an angiosarcoma of the small intestine after postoperative irradiation was published in 1979^[39]. That patient developed an angiosarcoma in the terminal ileum 8 years after irradiation for an ovarian carcinoma. Since then, 13 more angiosarcoma cases following radiation have been published (Table 1). In one case, an angiosarcoma occurred after exposure to irradiation and polyvinyl chloride^[31], but predisposing factors could not be identified in the remaining 19 cases. The patient presented in this report also did not have any known malignancy or exposure to irradiation, vinyl chloride, or other chemicals known to induce angiosarcomas such as thorotrast or arsene.

Categorization by sex and age does not reveal any clear-cut distribution. The average age of patients with this type of angiosarcoma was 62 years (range, 25-87 years), and 18 patients were male and 15 were female (Table 1).

The clinical manifestations of patients with angiosarcomas of the small intestine include lethargy, weakness, altered intestinal function, nonspecific abdominal pain, severe melena, anemia, acute abdominal signs and/or ileus symptoms, and even nonspecific chest pain (Table 1). In 15 of the 33 cases, the patient had signs of gastrointestinal bleeding^[30,31,37,49,50], similar to the patient described in this report. This variability in clinical manifestations makes it even more difficult to reach a quick and correct diagnosis. Furthermore, currently available diagnostic modalities, including CT, capsule endoscopy, double-balloon enteroscopy, magnetic resonance imaging, and positron emission tomography-CT all fail to detect the bleeding site, let alone lead to a diagnosis.

Angiosarcomas are classified as well-differentiated, poorly differentiated, and epithelioid tumors. A histological diagnosis can be challenging because angiosarcoma of the small intestine shows high architectural and cytological variability. The epithelioid morphology is typical but can be easily confused with other entities such as a poorly differentiated carcinoma^[4,47]. Immunohistochemical expression analysis for the endothelial markers CD31, CD34, and factor VIII-associated antigen is crucial. The majority of cases listed in Table 1 were positive for these antigens. Other antigens show limited relevance and can

Table 1 Literature overview: Angiosarcoma of the gastrointestinal tract (modified from Grewal *et al.*^[32] and Policarpio-Nicolas *et al.*^[47])

Patient [age (yr)/sex]	Tumor manifestation	Histology	Radiation/ pre-disposition	Symptoms	Therapy	Outcome	Ref.
46/M	Duodenum, ileum, and stomach	Epithelioid	None	Abdominal pain, melena	Resection	Died after 6 mo	[50]
65/F	Ileum	Well-differentiated	Radiation	Abdominal pain, nausea, vomiting	Resection, chemotherapy	Died after 14 mo	[39]
64/M	NA	Epithelioid	None	Gastrointestinal bleeding	Resection	Died after 1 yr	[29]
57/F	Small intestine	Epithelioid	None	NA	Resection	Died after 4 mo	[29]
47/F	Ileum	NA	Radiation	Abdominal pain	Resection, chemotherapy, radiation	NA	[40]
64/M	Small intestine	Well-differentiated tumor	None	Gastrointestinal bleeding	Resection	Died with disseminated disease after approximately 1 yr	[30]
57/M	Ileocecal valve, small bowel, and mesentery	Well-differentiated tumor	None	NA	Resection	Died after several days	[30]
76/M	Ileum	Mixed, well-differentiated and epithelioid	None	Abdominal pain, poor appetite, fatigue	Resection	Died after 9 d	[59]
74/F	Jejunum	Well-differentiated	NA	Melena	Resection	Died due to multiple complications	[60]
51/M	NA	Well-differentiated	Radiation	Abdominal pain	Resection, chemotherapy	Died after 5 mo	[48]
76/F	Ileum	Well-differentiated	Radiation	Abdominal pain, weight loss, vomiting, diarrhea	Resection	Died after 5 mo	[45]
48/F	Ileum	NA	Radiation	Abdominal pain	Resection	Died from sepsis after 23 d	[43]
60/F	Small intestine	Well-differentiated	Radiation	Acute abdomen, and a distal jejunal perforation	Resection	Died after 3 mo	[44]
80/F	Small and large bowel	Well-differentiated	Radiation	Altered intestinal function	Resection	Died after 2 wk	[42]
69/F	Small and large bowel	Well-differentiated	Radiation	Weight loss, abdominal distention, hemochezia	Resection	Died after 23 d	[42]
NA/M	Duodenum, stomach	Epithelioid	None	Severe melena	Resection	Died of respiratory failure, metastases were found in various organs, including the lungs, bones, liver, gall-bladder, and lymph nodes	[61]
78/F	Small intestine	High-grade	Radiation	Relative bowel obstruction	Resection	Died after 2 yr	[41]
50/F	Ileum	Multifocal and infiltrating	Radiation	Repeated symptoms of intestinal obstruction	Resection, chemotherapy	Died after 21 mo	[41]
61/F	Ileum	Well-differentiated	Radiation	Fullness, abdominal pain	Resection	Died after 10 mo	[46]
67/M	Jejunum, ileum	Epithelioid	None	Weight loss, Intermittent severe abdominal pain, and melena	Resection	Died after 3 mo	[51]
85/M	Small intestine	High-grade	None	Weight loss, decreased appetite, generalized weakness, left upper quadrant abdominal pain	Resection, chemotherapy	Survived at least 1 yr	[52]
59/M	Ileum	Mixed epithelioid and well-differentiated	None	Gastrointestinal bleeding	Resection	Died after 11 d	[37]
70/M	Duodenum	Epithelioid	None	Melena, anemia	Chemotherapy	Died after 4 mo	[49]
84/F	Jejunum	Epithelioid	None	Melena, anemia, shortness of breath	NA	Died after 17 mo	[49]
47/M	Jejunum	Epithelioid	None	Melena, anemia, shortness of breath	NA	Died after 4 mo	[49]
25/M	Small intestine	Epithelioid	None	Gastrointestinal bleeding, hemoptysis, anemia	Chemotherapy, radiation	Alive 18 mo after diagnosis, palliative situation	[49]

70/M	Ileum	Mixed, well-differentiated	None	Abdominal pain, vomiting	Resection, chemotherapy, radiation	Survived at least 4 yr	[53]
68/M	Ileocecal	High-grade	Radiation, polyvinyl chloride	Gastrointestinal bleeding, melena	Resection	Died before starting chemotherapy	[31]
51/F	Ileum	Well-differentiated	Radiation	Decreased appetite and vague abdominal pain of several months duration	Resection	Died after 10 mo	[47]
87/M	Duodenum, jejunum	Epithelioid	None	Lethargy, weakness, and nonspecific chest pain	Endoscopy, argon plasma coagulation	Died after 6 wk	[62]
73/M	Duodenum, jejunum	Epithelioid	Radiation	Weakness, dizziness, constipation, and melena	Resection	Died after 4 mo	[32]
NA/M	Jejunum	NA	NA	Acute abdominal signs	Resection, chemotherapy	Survived at least 3 yr	[2]
25/F	Small and large bowel	NA	None	Intermittent abdominal pain, weight loss, and progressive abdominal distension, a 7-wk history of shortness of breath, hematemesis, and melena	Resection	Died after 2 wk	[63]

NA: Not available; M: Male; F: Female.

cause confusion with other carcinomas. There is some controversy about the relevance of cytokeratin, which has been reported positive by some authors^[32,37,49,51]. However, most authors have reported no such expression by intestinal angiosarcomas^[29-31,48,52].

The current therapy for angiosarcoma includes bleeding control and symptomatic therapy to stabilize the patient, followed by radical tumor resection.

Six patients in the literature received adjuvant chemotherapy^[39,41,48,49,52], and three patients were treated with combination chemotherapy and radiation^[40,49,53]. Adjuvant therapy with paclitaxel was intended in the present case; however, the patient died before starting chemotherapy. All adjuvant therapy protocols are generally empiric and based on studies of cutaneous angiosarcoma, as randomized clinical studies on gastrointestinal angiosarcomas are lacking due to their rarity. The first case published received combination chemotherapy consisting of doxorubicin, vincristine, dacarbazine, and cyclophosphamide, after operative resection of the terminal ileum. That patient survived 14 mo^[39]. Another combination therapy that has been used is doxorubicin and dacarbazine, which led to 5 mo survival after diagnosis^[48]. Monotherapy with doxorubicin showed survival of 21 mo, at which time the tumor was widely disseminated^[41]. Furthermore, thalidomide therapy was initiated as an experimental measure after operative resection in one case^[52]. That patient was still alive 1 year after the initial diagnosis. No recommendation can usually be made, but paclitaxel and/or thalidomide are currently commonly considered^[52,54,55]. The newest studies suggest administering doxorubicin and paclitaxel weekly for cutaneous angiosarcoma, which seem to provide longer progression-free survival^[56-58].

Despite all efforts, survival of patients with small bowel angiosarcoma is generally poor. Survival usually ranges from several days after surgical intervention to

2 years. The majority of patients die within 6 mo to 1 year after being diagnosed (Table 1). Only two reported patients survived > 2 years after resection and adjuvant (radio-) chemotherapy^[2,53].

One major cause of this poor outcome seems to be that the diagnosis is difficult, and many tumors are diagnosed only in the late stages of the disease. Therefore, a quick diagnosis using endoscopy and imaging procedures, as well as fast and decisive surgical intervention and adjuvant chemotherapy are necessary.

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Gastrointestinal bleeding caused by extrahepatic arterioportal fistula associated with portal vein thrombosis

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Nie L, Luo XF, Li X. Gastrointestinal bleeding caused by extrahepatic arterioportal fistula associated with portal vein thrombosis. *World J Gastroenterol* 2012; 18(44): 6501-6503 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i44/6501.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i44.6501>

Abstract

An extrahepatic arterioportal fistula (APF) involving the gastroduodenal artery and superior mesenteric vein is rare and mostly results from iatrogenic injuries. The clinical symptoms associated with APFs may include abdominal pain, gastrointestinal bleeding, ascites, nausea, vomiting, diarrhea, or even congestive heart failure. We present the case of a 70-year-old man who presented with chronic abdominal pain and gastrointestinal bleeding secondary to APF and portal vein thrombosis. The endovascular embolization of APF was accomplished successfully, and symptoms of portal hypertension resolved immediately after intervention. Unfortunately, the patient did not respond well to anticoagulation therapy with warfarin. Therefore, the patient underwent implantation of a transjugular intrahepatic portosystemic shunt, and the complications of portal hypertension resolved. In conclusion, the embolization of APF is technically feasible and effective and can be considered the first-choice therapy in selected patients.

INTRODUCTION

Upper gastrointestinal bleeding in patients with cirrhosis is usually due to portal hypertension, which is common in this population. Nevertheless, bleeding resulting from extrahepatic arterioportal fistulas (APFs) is extremely rare^[1-3]. Extrahepatic APF may cause severe portal hypertension, which leads to upper and lower gastrointestinal bleeding, refractory ascites, diarrhea, and hepatic encephalopathy. We present an unusual case of a 70-year-old man who presented with chronic abdominal pain and gastrointestinal bleeding secondary to APF and portal vein thrombosis.

CASE REPORT

A 70-year-old man with a history of cirrhosis resulting from hepatitis B virus infection was admitted to our hospital with complaints of chronic abdominal pain and melena. At the time of admission, his pulse rate was 63 beats/min, and his arterial blood pressure was 117/61

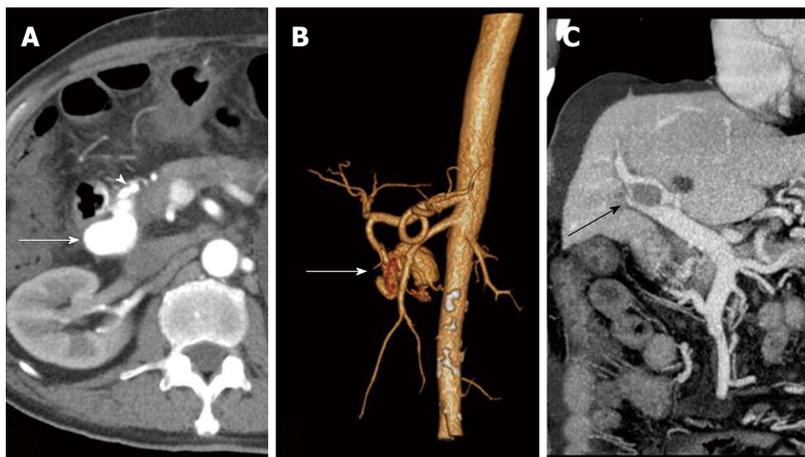


Figure 1 Contrast-enhanced computed tomography scan of the upper abdomen. A: Computerized tomography (CT) scan revealed a large aneurysm (white arrow) with dilated feeding gastroduodenal artery (GDA) (white arrowhead). Draining superior mesenteric vein (SMV) was observed at a lower level; B: Three-dimensional CT reconstruction demonstrated an arterioportal fistula (white arrow) arising from the GDA; C: CT scan showed a high-density mass in the right portal vein (black arrow), the patent main portal vein and SMV.



Figure 2 Selective angiography of the gastroduodenal artery. A: Angiography demonstrated an arterioportal fistula fistulating into the superior mesenteric vein; B: The varices were opacified.

Figure 3 After the placement of several metal coils, the arterioportal fistula was utterly abrogated.

mmHg. Laboratory tests disclosed the following values: red blood cells, $2.65 \times 10^{12}/L$; white blood cells, $5.65 \times 10^9/L$; PC, $207 \times 10^9/L$; prothrombin time, 15.9 s; total bilirubin, 1.1 mg/dL; albumin, 3.2 g/dL; aspartate aminotransferase, 47 IU/L; alanine aminotransferase, 43 IU/L; and alkaline phosphatase, 450 IU/L. The patient underwent cholecystectomy at the age of 67 because of acute cholecystitis.

Upper gastrointestinal endoscopy showed multiple severe esophageal and duodenal descendent varices and portal hypertensive gastropathy. Computerized tomography (CT) of the abdomen demonstrated the presence of a large aneurysm next to the head of the pancreas filled by the dilated gastroduodenal artery (GDA), draining into the superior mesenteric vein (SMV) (Figure 1). Other CT findings were cirrhotic liver, the presence of esophageal varices, portal thrombosis, moderate ascites and splenomegaly (Figure 1).

Under local anesthesia, a 5-F Cobra catheter (Teramo Co., Fijinomiya, Japan) was advanced into the GDA *via* the common femoral artery. Selective digital subtraction angiography showed an APF between the GDA and SMV, and the blood flow from the SMV did not go into the portal vein but into the collateral veins (Figure 2).

Embolization of the extrahepatic APF was performed to occlude the fistula and reduce portal pressure using stainless metal coils (Cook Incorporated, Bloomington, United States). A further angiography of the GDA confirmed immediate cessation of the fistula and the abrogation of the hyperkinetic portal flow (Figure 3). After the procedure, the gastrointestinal bleeding stopped, and the patient reported symptomatic improvement, with resolution of the chronic abdominal pain. Four days later, the patient was discharged in good condition. He was also started on anticoagulation therapy with warfarin, and the dose was adjusted to achieve an International Normalized Ratio of 2 to 3.

Three years after intervention, the patient presented to the emergency room with acute upper gastrointestinal bleeding. CT scan demonstrated multiple thromboses in the right and main portal vein and massive ascites, without any sign of APF (Figure 4). Because anticoagulation therapy was not successful, the patient underwent implantation of a transjugular intrahepatic portosystemic shunt (TIPS). Currently, two years after TIPS, despite several episodes of mild hepatic encephalopathy requiring conventional medical treatment, the patient remains clinically well.



Figure 4 Computerized tomography scan revealed massive intrahepatic portal vein thrombosis and main portal vein thrombosis.

DISCUSSION

Extrahepatic APF involving the GDA and SMV is an uncommon disorder of the mesenteric vasculature characterized by abnormal communication between the arterial and portal circulation. The most common causes of APFs are reported to be congenital vascular malformations, blunt or penetrating traumas and iatrogenic injuries^[1,4-6]. Our patient had no history of abdominal traumas or other vascular malformations, suggesting that the APF most likely arose as a result of iatrogenic vascular injury during the previous biliary surgery.

Both CT and magnetic resonance imaging with intravenous contrast material can provide useful information for the diagnosis of APFs, specifically, the exact anatomic location and extent of mesenteric vessel involvement. Doppler ultrasonography is another useful imaging modality for diagnosis, which can not only detect the position of an APF but also evaluate the flow direction. Arteriography should be performed as soon as possible for diagnostic purposes and evaluating APF hemodynamics, delineating the fistula site, and determining the size of the feeding artery. In the present case, CT of the abdomen clearly revealed a large aneurysm, which was supplied by the GDA and had fistulated into the SMV, with radiological evidence of portal hypertension.

APF is an uncommon but treatable cause of portal hypertension. The clinical symptoms associated with APFs may include abdominal pain, gastrointestinal bleeding, ascites, nausea, vomiting, diarrhea, or even congestive heart failure^[4,7]. In this case, extrahepatic APF led to secondary portal hypertension, resulting in upper gastrointestinal bleeding and moderate ascites. Portal hypertension disappeared immediately after cessation of the APF.

Generally, the early treatment of APF is mandated, even in asymptomatic patients with a high-flow fistula, to prevent complications. Both open and endovascular management of APFs has been well described^[5,8,9]. However, there is an increased use of endovascular techniques as first-choice therapy, including embolization or stent insertion^[8,10]. Endovascular techniques provide a less invasive alternative to surgical procedures, can be performed un-

der local anesthesia, and do not impede planned surgery. In our case, a 5-F Cobra catheter was used to extend the peripheral fistula position, and it allowed for the exact placement of the coils in the feeding GDA.

The endovascular embolization of APF was accomplished successfully, and symptoms of portal hypertension resolved immediately after intervention. Unfortunately, the patient did not respond well to anticoagulation therapy with warfarin. We elected to treat the patient with TIPS rather than endoscopic treatment for two reasons. The first reason is that because the patient did not respond well to warfarin, endoscopic therapy plus anticoagulation therapy seemed impossible to recanalize the portal vein. The second reason was that TIPS could not only reduce the portal pressure but also recanalize the portal vein and avoid rethrombosis by restoring portal flow through a low-resistance shunt.

In conclusion, extrahepatic APF is a rare cause of portal hypertension and may cause gastrointestinal bleeding and ascites. The embolization of APF is technically feasible and effective and can be considered the first-choice therapy in selected patients. Moreover, by embolizing the APF, chronic abdominal pain was also controlled.

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Novel *ATP8B1* mutation in an adult male with progressive familial intrahepatic cholestasis

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bile salt export pump expression due to the impaired FIC1 function. Our findings show that patients with intermittent cholestasis can develop progressive liver disease even after several decades and require regular follow up.

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Key words: *ATP8B1*; Bile salt export pump; Novel mutation; Progressive familial intrahepatic cholestasis type 1; Intermittent cholestasis

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Abstract

Progressive familial intrahepatic cholestasis type 1 is a rare disease that is characterized by low serum γ -glutamyltransferase levels due to mutation in *ATP8B1*. We present a 23-year-old male who experienced persistent marked pruritus for eighteen years and recurrent jaundice for thirteen years, in addition to cholestasis that eventually became fatal. Genetic sequencing studies of the entire coding (exon) sequences of *ATP8B1* and *ABCB11* uncovered a novel heterozygous missense 3035G>T mutation (S1012I) and a synonymous 696T>C mutation in *ATP8B1*. The patient's progression was associated with not only impaired familial intrahepatic cholestasis 1 (FIC1) function but also impaired

INTRODUCTION

Progressive familial intrahepatic cholestasis (PFIC) is a heterogeneous group of severe autosomal recessive liver disorders of childhood that result in cholestasis that can progress to end-stage in the first or second decade of life^[1,2]. PFIC-1 and PFIC-2 are characterized by low serum γ -glutamyltransferase (GGT) levels. Patients with PFIC-1 have mutations in *ATP8B1* (encoding FIC1), which maps to chromosome 18q21-22, and patients with PFIC-2 have mutations in *ABCB11* (encoding bile salt export pump, BSEP), which maps to chromosome 2q24. PFIC-3 is characterized by high serum GGT levels and is caused by genetic mutations in *ABCB4* (encoding multidrug resistant protein 3)^[3].

We report an unusual patient who presented with childhood-onset progressive intrahepatic cholestasis with low serum GGT levels and died in his third decade of life. Genetic sequencing analysis revealed a novel heterozygous 3035G>T mutation (S1012I) and a synonymous 696T>C mutation in *ATP8B1*.

CASE REPORT

A 23-year-old male presenting with an 18-year history of persistent pruritus and a 13-year history of recurrent jaundice was admitted to the department of infectious disease. He had gradually developed severe pruritus with pale yellow stools and dark urine 2 mo before admission. He had been admitted to hospital 13 years previously for pruritus, hyperbilirubinemia and mildly elevated alanine aminotransferase levels (Table 1). Skin pathology conducted at that time showed hyperkeratosis of epidermal cells, papilloma hyperplasia, acanthosis, and a slight increase of basal pigmentation (results not shown). The liver biopsy revealed intrahepatic cholestasis with ballooning degeneration of hepatocytes, no significant fibrosis, and multinucleated giant cells (Figure 1). Jaundice recurred four times during the subsequent 13 years. Serum biochemistry was returned to normal levels by treatment with a liver protectant. There was no family history of liver disease. On physical examination, the patient was 150 cm tall and 55 kg in weight. The skin and sclera were markedly icteric. The skin was rough and thickened (Figure 2). Palmar erythema and mild splenomegaly were noted. The rest of the examination was unremarkable.

Liver function tests showed conjugated hyperbilirubinemia, elevated serum aminotransferase levels, low serum GGT values, increased bile acids levels and hypoalbuminemia (Table 1). The patient was negative for markers of hepatitis virus. Antibodies to cytomegalovirus and Epstein-Barr virus were negative. Ceruloplasmin and serum copper were normal, and no Kayser-Fleischer ring was observed upon examination by an experienced ophthalmologist. A qualitative urinary porphyrin test was negative. Autoimmune antibodies including antimitochondrial antibody, antinuclear antibodies, antineutrophil cytoplasmic antibody were all negative, and serum α -1-antitrypsin concentration, thyroid function and other laboratory investigation results were normal.

A lesion in segment 4 of the liver, gallbladder stones, splenomegaly and an oval-shaped signal intensity in the spleen were observed upon magnetic resonance imaging (MRI) (Figure 3). No dilatation of the bile duct was observed by magnetic resonance cholangiopancreatography. A repeat biopsy was performed.

The liver biopsy revealed cholestasis, inflammation and fibrosis at the portal area. The mass consisted of normal liver tissue with normal liver architecture and mild fibrosis (data not shown). The fibrosis stained with hematoxylin and eosin and Masson's trichrome at portal area in the 23-year-old liver tissue was more severe than

that in the liver tissue sample taken from the same patient at 10 years of age. Atypical hyperplasia was not observed in the mass, and CD10, CD34 and α fetoprotein (AFP) immunohistochemistry staining were unremarkable (data not shown). BSEP expression was present only focally and was assessed as both faint and patchy in the liver biopsy tissue (Figure 4).

Ursodeoxycholic acid (UDCA) and vitamin supplements were prescribed. Serum total bilirubin was persistently elevated, and liver transplantation and partial external biliary diversion were advised. The patient declined surgery to resolve the cholestasis and was discharged. The patient was followed up but died of liver failure and hepatic encephalopathy two months later.

Mutation analysis

Genetic studies were performed with the informed consent of the patient, and the study was approved by the Ethics Committee of China Medical University. Genomic DNA was extracted from peripheral blood leucocytes. *ABCB11* and *ATP8B1* were analyzed by direct sequencing of polymerase chain reaction (PCR) products. PCR primers were designed to amplify the 27 exons of the entire coding sequence of the *ATP8B1* gene and the 28 exons of the *ABCB11* gene, followed by direct sequencing using a Genetic Analyzer 3730 instrument (Applied Biosystems). A synonymous 696T>C mutation and a novel heterozygous missense 3035G>T mutation (S1012I) were discovered in exons 7 and 24 of the *ATP8B1* gene. We obtained blood samples from the patient's parents and sisters, all of which were negative for the *ATP8B1* and *ABCB11* mutations. The mutations were absent in 100 control chromosomes (evaluated by direct sequencing). This missense mutation has not been reported in any other benign recurrent intrahepatic cholestasis (BRIC)-1 or PFIC-1 patient.

DISCUSSION

In this study, we present a patient with a novel mutation in *ATP8B1*, who experienced the onset of persistent severe pruritus at 5 years old and the onset of progressive intrahepatic cholestasis at 10 years old, eventually dying of liver failure at age 23. Common chronic cholestatic diseases such as primary biliary cirrhosis, sclerosing cholangitis and idiopathic ductopenia were excluded by laboratory, radiographic and histological examinations. Based on the absence of the missense mutation (S1012I) in 100 control chromosomes, the novel mutation S1012I is believed to be the disease-causing mutation. *ATP8B1* is also associated with BRIC1 and intrahepatic cholestasis of pregnancy, which are characterized by intermittent cholestasis without liver scarring^[4]. These clinical and histopathological features are generally considered diagnostic for PFIC-1 rather than BRIC type 1. The differences in clinical outcome and disease duration may be dictated by the variations of the structure and/or

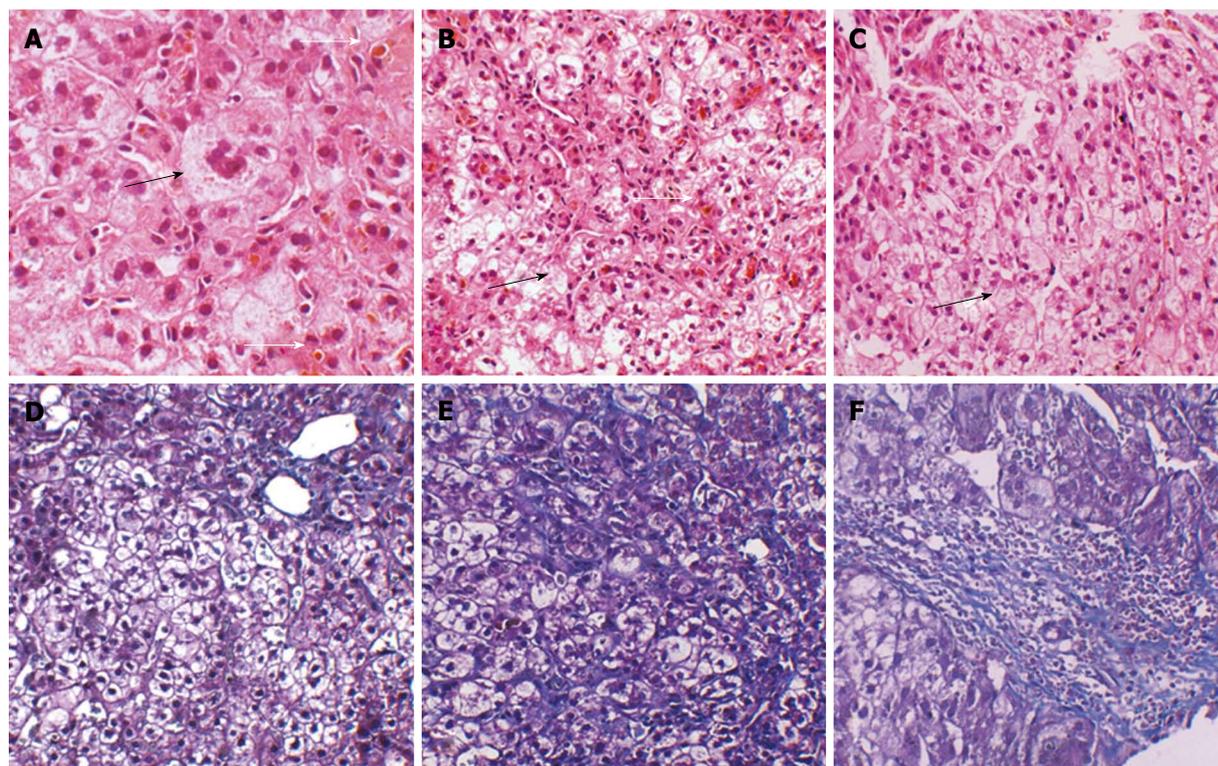


Figure 1 Histological features of samples taken at age 10 and current liver biopsy specimens (age 23 years). A: Multinucleated giant cells (black arrow) and ductal cholestasis (white arrow) in the previously sampled liver tissue (image, × 400); B: Ductal cholestasis (white arrow) and ballooning degeneration of the hepatocytes (black arrow) in the previously sampled liver tissue (image, × 200); C: Ductal cholestasis and hepatocytes ballooning degeneration (black arrow) in the recently sampled liver tissue (image, × 200); D: Mild portal and lobular fibrosis in the previously sampled liver tissue (image, × 200); E: Moderate lobular fibrosis in the recently sampled liver tissue (image, × 200); F: Moderate portal fibrosis in the recently sampled liver tissue (image, × 200).

Variables	Reference range	Age 10 yr	On admission	2 wk	1 mo	2 mo
Bilirubin, total (μmol/L)	3.4-20.5	213.5	250.1	303.7	180.5	420.1
BiLirubin, direct (μmol/L)	0.0-6.8	158.6	188.6	213.1	115.6	286.7
GGT (U/L)	10-47	8	7	31	26	NA
ALP (U/L)	40-129	237	345	322	300	NA
AST (U/L)	8-40	256	202	222	156	466
ALT (U/L)	5-40	375	196	183	130	148
ALbumin (g/L)	40-50	NA	35	33.3	NA	24.7
PT (s)	11.5-13.5	NA	14.9	NA	NA	28.5
PTA (%)	80-120	NA	80.6	NA	NA	24.7
BiLe acids (μmol/L)	0-10	NA	296	239	218	NA
PLasma ammonia (μmol/L)	0-23	NA	15	NA	NA	61.5
CHE (U/L)	NA	NA	1519	1720	NA	NA

ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; PT: Prothrombin time; PTA: Prothrombin activity; NA: Not available; GGT: γ -glutamyl transpeptidase; CHE: Cholinesterase.

function of the FIC1 protein due to the mutation in *ATP8B1*^[5]. Although the novel mutation 3035G>T is predicted to impair FIC1 function, the effects of the



Figure 2 Skin. The skin was rough and thickened.

missense 3035G>T mutation and the synonymous 696T>C mutation on the FIC1 protein are unknown.

Though the function of FIC1 protein is not yet known, it is thought to mediate the inward translocation of phosphatidylserine from the exoplasmic to the cytoplasmic leaflet of the plasma membrane^[5]. Immunohistochemistry for FIC1 protein was not performed due to the current lack of a commercially available specific antibody to detect the absence/alteration of the FIC1 protein. Impaired FIC1 function may lead to a loss of lipid asymmetry in the canalicular membranes of hepatocytes,

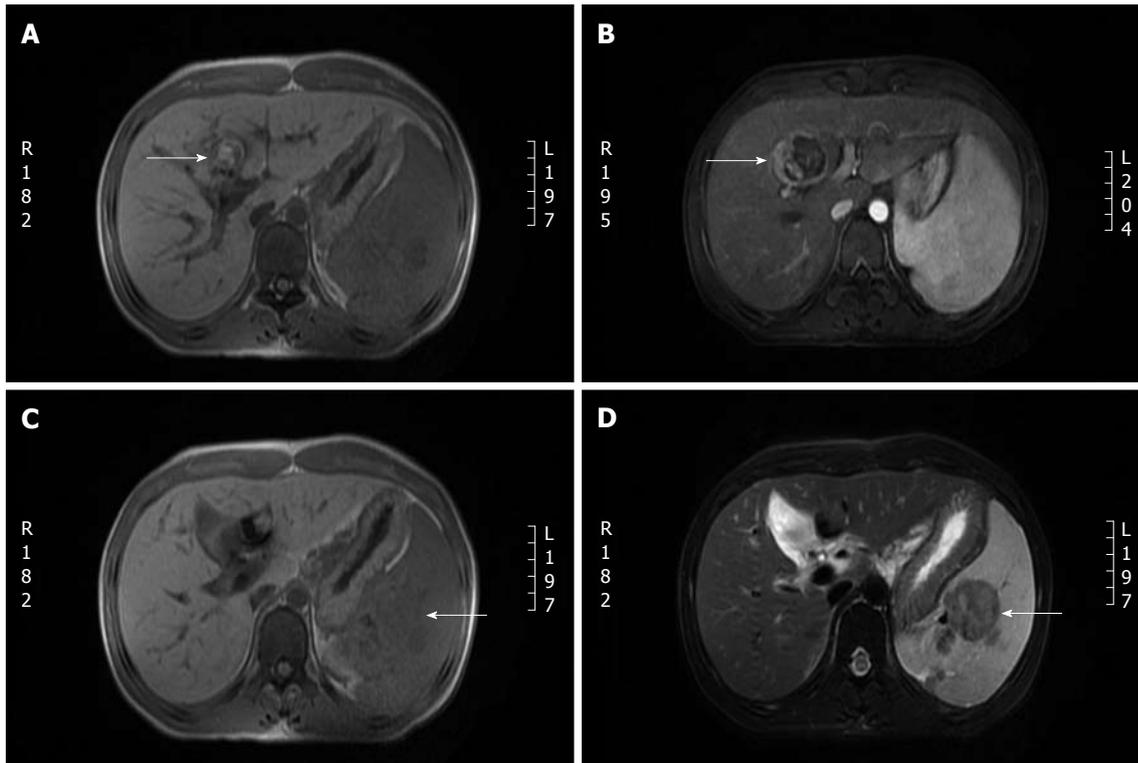


Figure 3 Liver magnetic resonance imaging. A: A 2.3 cm × 2.56 cm lesion was observed in segment 4 of the liver (T1-weighted). No obvious dilation of the intra or extrahepatic bile duct was observed; B: Following contrast magnetic resonance imaging (MRI), the capsule shows linear enhancement without enhancement of the lesion itself. Splenomegaly is observed, and several oval-shaped nodules in the spleen show slight enhancement; C: The oval-shaped nodules appear as low-intensity signals on a T1-weighted MRI image; D: The oval-shaped nodules in the spleen appear as a lower-intensity signal on T2-weighted MRI images than on T1-weighted images.

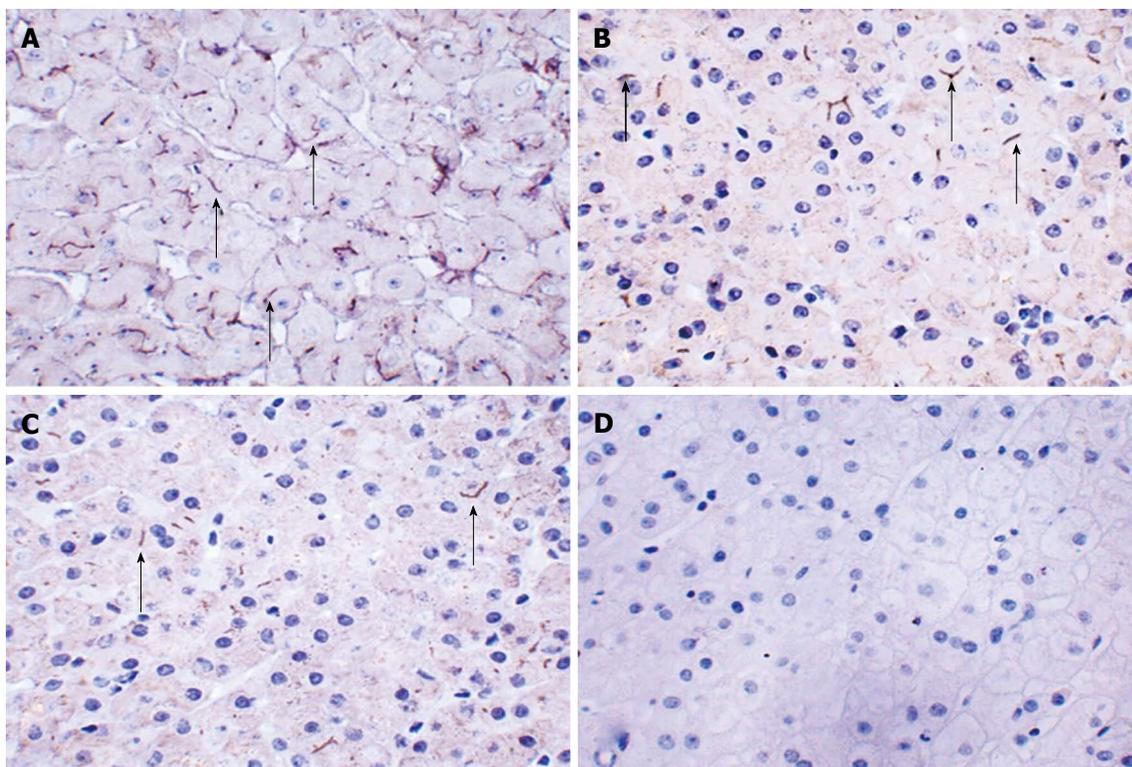


Figure 4 Expression pattern of the canalicular transporter bile salt export pump (rabbit anti-bile salt export pump polyclonal antibody, all images, × 400). A: Normal bile salt export pump (BSEP) staining in a control liver without cholestasis. Anti-BSEP antibody stains an orderly canalicular network; B: BSEP staining in liver tissue collected from the patient 13 years ago. BSEP is present only focally (arrows) and is both faint and patchy; C: BSEP staining in the recent sample of liver tissue BSEP expression is again faint and patchy; D: Canaliculi in negative control liver stained with phosphate-buffered saline.

resulting in BSEP dysfunction; decreased expression of BSEP (encoded by *ABCB11*) has been observed in livers from PFIC-1 patients, and this decrease is associated with the accumulation of bile salts in hepatocytes^[6,7]. It is also reported that a marked decrease in the activity of farnesoid X receptor, which can positively regulate the expression of BSEP and affects canalicular membrane composition to impair *ABCB11* activity, can be induced by a loss of FIC1 expression^[8,9]. Immunohistochemistry for BSEP in this patient revealed that BSEP expression was present only focally and was assessed as both faint and patchy in the liver biopsy sample. Thus, the chronic cholestasis in this patient was associated with not only impaired FIC1 protein function but also impaired BSEP expression. No mutation in *ABCB11* was detected, which suggested that impaired expression of BSEP in the patient was most likely induced by impaired FIC1 protein function.

PFIC-1 patients may exhibit extrahepatic features such as persistent short stature, diarrhea, pancreatitis and sensorineural hearing loss^[10], which were not present in this patient. Here, we show that a patient with intermittent cholestasis can develop progressive liver disease even after several decades. However, this does not imply that all patients with BRIC will progress to PFIC. Although van Ooteghem *et al.*^[11] reported that 4 of 63 patients with intermittent cholestasis developed progressive liver disease after several decades, only 2 of the 4 patients were diagnosed genetically, in assays that revealed the same splice site mutation resulting in skipping of exon 24. Here, we have uncovered a novel missense mutation in *ATP8B1* associated with progressive liver disease in a patient with intermittent cholestasis. A possible phenotypic continuum between BRIC-2 and PFIC-2 in a patient with mild disease, who experienced a complete remission of liver fibrosis following treatment with UDCA, was reported^[12]. The patient we presented did not respond to treatment with UDCA and died of liver failure, in contrast to the previously described patient with phenotypic continuum.

PFIC may induce hepatic malignancies, which can be coincident with hepatoblastoma^[13]. CD10, CD34 and AFP immunohistochemistry staining was performed to distinguish a well-differentiated hepatocellular carcinoma from normal and cirrhotic liver tissue or benign liver nodules were performed; the results were negative^[14,15]. The liver mass in this patient was found to be a benign lesion with normal liver architecture.

The current medical therapy for PFIC-1 is either non-existent or useful for only a limited duration, and some agents, including ursodeoxycholic acid, provide mainly symptomatic relief. Mutation-specific drugs may become a new tool for PFIC-1 therapy in the near future. Most patients with progressive cholestasis eventually require surgical intervention^[10]. Liver transplantation should be considered in patients who develop cirrhosis or progressive liver disease despite treatment.

In conclusion, we report a rare case that presented with onset persistent severe pruritus progressing to PFIC-1 due to a novel heterozygous *ATP8B1* mutation. Both impaired FIC1 function and impaired BSEP expression induced by the impaired FIC1 function were associated with this progression. This case shows that patients with intermittent cholestasis can develop progressive liver disease, even after several decades, and should be followed up regularly. Further studies are necessary to investigate the impact of the 3035G>T mutation on functional defects in FIC1 and BSEP.

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Laparoscopic resection of synchronous intraductal papillary mucinous neoplasms: A case report

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Abstract

We describe herein a 68-year-old woman who was diagnosed with a quite rare entity of intraductal papillary mucinous neoplasms (IPMNs) occurring simultaneously in the left lateral lobe of liver and the tail of pancreas. Abdominal computed tomography and magnetic resonance cholangiopancreatography showed a cystic dilatation of the pancreatic duct in the pancreatic tail, which suggested an IPMN, and multiple intrahepatic duct stones in the left lateral lobe. The patient underwent a laparoscopic left lateral hepatectomy and spleen-preserving distal pancreatectomy. Intra-operative finding of massive mucin in the dilated bile duct implied an intraductal mucinous tumor in the liver. The diagnosis of synchronous IPMNs in the liver and pancreas was confirmed by pathological examination. The patient was followed up for 6 mo without signs of recurrence. Although several cases of IPMN of liver without any pancreatic association have been reported, the simultaneous occurrence of IPMNs in the liver and pancreas

is very rare. To the best of our knowledge, it is the first reported case treated by laparoscopic resection.

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Key words: Laparoscopy; Pancreas; Liver; Intraductal papillary mucinous neoplasm; Synchronous neoplasm

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Xu XW, Li RH, Zhou W, Wang J, Zhang RC, Chen K, Mou YP. Laparoscopic resection of synchronous intraductal papillary mucinous neoplasms: A case report. *World J Gastroenterol* 2012; 18(44): 6510-6514 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i44/6510.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i44.6510>

INTRODUCTION

Intraductal papillary mucinous neoplasm (IPMN) is rare and mostly occurs in the liver or pancreas. Synchronous occurrence of IPMNs in the liver and pancreas is even more unusual. It is only described in the form of case reports in the literature^[1-5]. We present here a case of synchronous occurrence of IPMNs located respectively in the left lateral lobe of the liver and the tail of pancreas. These two lesions were resected by laparoscopic left lateral hepatectomy and spleen-preserving distal pancreatectomy. To the best of our knowledge, it is the first reported case treated by laparoscopic resection. We also summarized the features of five similar cases with detailed information reported in the English-language literature (Table 1).

CASE REPORT

A 68-year-old woman was admitted to our department in January 2012 because of epigastric pain for half a month. She had no fever, no nausea or vomiting, no hematemesis or melena, and no weight loss. She underwent open cholecystectomy for gallbladder stones 25 years ago. Physical examination on admission showed no abnormalities. Blood biochemistry and tumor markers (carcinoembryonic antigen, alpha fetoprotein, carbohydrate antigen 19-9, carbohydrate antigen 153, carbohydrate antigen 125) were all within normal ranges. Computed tomography (CT) of the abdomen with intravenous contrast revealed cystic dilatation of left intrahepatic bile ducts accompanied with stones (Figure 1A) and multiple cystic lesions in the pancreatic tail (Figure 1B). Magnetic resonance cholangiopancreatography revealed a diffuse dilated biliary tract with multiple filling defects in the left lateral lobe and segmental cystic dilatation of pancreatic duct in the pancreatic tail (Figure 2). According to the medical history and the imaging findings, left intrahepatic bile duct stones and pancreatic IPMN was first considered. Therefore, laparoscopic left lateral hepatectomy and spleen-preserving distal pancreatectomy were performed.

The patient was placed in supine position under general anesthesia. The operator and the second assistant who held the laparoscope stood on the right side of the patient and the first assistant stood on the left. Carbon dioxide pneumoperitoneum was established using a Veress needle to a pressure of 15 mmHg. One initial 10-mm trocar was placed for laparoscope below the umbilicus and another four trocars (one of 12 mm and three of 5 mm) were inserted into the left upper, left flank, right upper, and right flank quadrants, making the five trocars arrange in a V-shape (Figure 3). During laparoscopic exploration, severe adhesion in the right upper abdomen because of the former open cholecystectomy and the atrophy of left lateral lobe of the liver were found, but without abdominal metastasis.

After dissecting the adhesion, the gastrocolic omentum was divided for entrance to the lesser sac by ultrasonic coagulating shears (Harmonic Ace scalpel, Ethicon Endo-Surgery, Inc., Cincinnati, OH, United States). The stomach was reflected cephalad and the tumor was found in the tail of pancreas. According to the location of the tumor, the mobilization of the pancreas began at the upper border till the common hepatic artery and the proximal splenic artery were visualized. Then the pancreas was mobilized dorsally starting at the lower edge to visualize the splenic vein. Pancreas was transected 2 cm proximal to the right side of the tumor with an endoscopic linear staplers (Endocutter 60 staple, white cartridge; Ethicon, Endo-Surgery, Inc., Cincinnati, OH, United States) and the pancreatic tail was freely dissected from the splenic artery and vein by ligation of the small branches connected to the pancreas using small titanium vascular clips or ultrasonic coagulating shears.

The left lateral lobe of the liver was mobilized by



Figure 1 Computed tomography scan. A: A cystic dilatation of the left intrahepatic bile ducts accompanied with stones; B: Multiple cystic lesions in the pancreatic tail.



Figure 2 Magnetic resonance cholangiopancreatography reveals diffuse dilation of biliary tract with multiple filling defects in the left lateral lobe of the liver and segmental cystic dilatation of pancreatic duct in the pancreatic tail.

dissecting the round, falciform, left coronary and triangular ligaments. Liver parenchyma was also divided by ultrasonic coagulating shears 1 cm to the left side of the falciform ligament. The branches of left portal vein, hepatic artery and hepatic vein on the cut surface were ligated with hem-o-loks and divided. The dilated bile duct was cut and stones were found. However, it was surprising that a large amount of mucin outflowed from the bile duct, which implied the intraductal mucinous tumor. The common bile duct (CBD) was explored, because it was dilated to 2 cm. There was no stone but massive mucin.

Table 1 Summary of previous synchronous intraductal papillary mucinous neoplasms in the liver and pancreas

Source	Sex/age (yr)	IPMN-b					IPMN-p					Procedure
		Location	Size (cm ²)	Histology	CMD	MC	Location	Size (cm ²)	Histology	CMD	MC	
Joo <i>et al</i> ^[1]	M/60	Left lobe	1.5 × 1.5	Benign	Yes	Yes	Tail	2.5 × 2.5	Benign	Yes	Yes	LH + DP
Ishida <i>et al</i> ^[2]	M/67	Caudate lobe	4 × 3	Benign	Yes	Yes	Uncinate process	3.5 × 3	Benign	Yes	Yes	LH + Caudate lobectomy +segmental resection of uncinate process
Yamaguchi <i>et al</i> ^[3]	M/69	Left lateral lobe	6.5 × 3.5	Malignant	Yes	Yes	Head	3 × 2.5	Malignant	Yes	Yes	LLH + PPPD
Zalinski <i>et al</i> ^[4]	F/65	Left lobe	10 × 10	Malignant	Unknown	Yes	Head	Unknown	High-grade dysplasia	Unknown	Yes	Unknown
Park <i>et al</i> ^[5]	M/67	Left lateral lobe	Unknown	Benign	Yes	Yes	Tail	2.5 × 1	Benign	Yes	Yes	LLH + DP + splenectomy

CMD: Communicating with main duct; MC: Mucin contained; LH: Left hepatectomy; DP: Distal pancreatectomy; LLH: Left lateral hepatectomy; PPPD: Pylorus-preserving pancreaticoduodenectomy; IPMN: Intraductal papillary mucinous neoplasm; M: Male; F: Female.

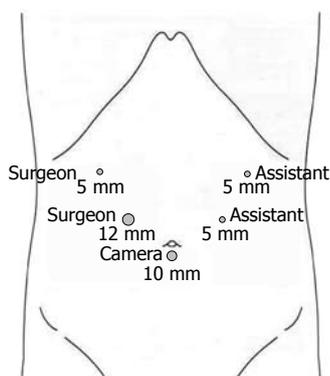


Figure 3 The location of trocars placement.

After inserting a No.24 T-tube, the CBD and bile duct in the cut surface were closed with a continuous suture of 3-0 Vicryl. The specimens were removed through the enlarged subumbilical port (Figure 4).

Intraoperative frozen section confirmed the mucinous tumors of the liver and pancreas and the resection margins were all negative. The operative time was 250 min and intraoperative bleeding was 150 mL. The post-operative course was uneventful, and the patient was discharged seven days later. She was followed up by abdominal CT six months later without signs of recurrence.

The gross finding was a 5 cm × 4 cm multiloculated cystic mass with stones in the left lateral lobe of liver, communicated with the dilated intrahepatic bile duct. Furthermore, multiple cystic lesions, ranging from 0.5 cm to 1.0 cm in diameter, were located in the pancreatic tail, in communication with the dilated pancreatic duct (Figure 4). All cystic lesions, in both the liver and pancreas, were filled with gelatinous transparent mucin. Microscopically, the cystic wall consisted of hyperplastic fibrous tissues lined by high columnar mucous epithelium demonstrating papillary growth, but no ovarian-like stroma was identified (Figure 5). There was no evidence of high-grade cellular dysplasia or stroma invasion suggesting malignancy in either specimen except for focal low-grade atypical



Figure 4 Resected specimens of intraductal papillary mucinous neoplasm-b (arrow head) and intraductal papillary mucinous neoplasm-p (arrow).

hyperplasia of epithelium in the liver lesion. The immunohistochemical staining of the liver lesion showed cytokeratin 7 (CK7): +/-, CK20: +, cadual type homeobox transcription factor-2 (CDX-2): +, mucin core protein 1 (MUC1): -, mucin core protein 2 (MUC2): + and mucin core protein 5 (MUC5): +, while the pancreas lesion showed CK7: +, CK20: -, CDX-2: -, MUC1: +, MUC2: - and MUC5: +, which were subsequently categorized into pancreaticobiliary and intestinal subtypes of IPMN.

DISCUSSION

IPMN is a rare but well-established disease, mostly occurs in the biliary tract or pancreas (IPMN-b or IPMN-p). It manifests as a cystic lesion in the imaging findings which make it difficult to differentiate from other cystic tumors preoperatively, such as cystadenoma or cystadenocarcinoma. The clinicopathological characteristics of IPMN can be summarized as follows: (1) Communicating with bile duct or pancreatic duct; (2) Dilated bile duct or pancreatic duct because of mucin hypersecretion and accumulation; (3) Lack of ovarian-like stroma characteristic of mucinous cystic tumor; and (4) Papillary proliferation

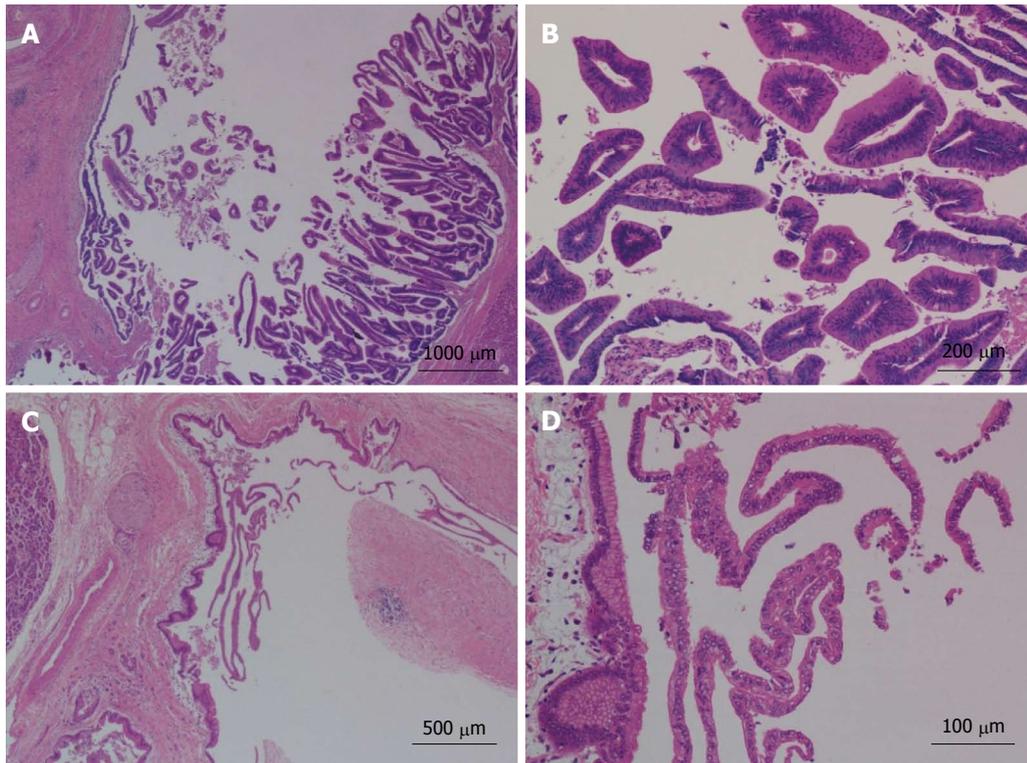


Figure 5 Histological features of intraductal papillary mucinous neoplasms by hematoxylin and eosin stain. A: Liver, $\times 20$; B: Liver, $\times 100$; C: Pancreas, $\times 40$; D: Pancreas, $\times 200$.

with delicate fibrovascular cores of ductal epithelium. The cystic lesions of the liver and pancreas in the present case possessed all the above characteristics, so a diagnosis of IPMN-b and IPMN-p was made. We believed that these two lesions were more likely to be the synchronous tumors arising in amenable ducts rather than metastasis, because each lesion was a definite benign tumor without lymph node involvement, and they were grossly and microscopically separate, without continuity between them. Besides, the IPMN-b and IPMN-p in the present case were categorized into different immunophenotypes, pancreaticobiliary and intestinal subtypes respectively, according to the results of immunohistochemical staining.

Although IPMN is frequently associated with malignancy of other organs, including the stomach, colon, esophagus and lung, the simultaneous occurrence of IPMN-b and IPMN-p is extremely rare^[6,7]. To the best of our knowledge, only five such cases have been reported in the English-language literature and the present case is the first one treated by total laparoscopic resection^[1-5]. Up till now, the origin of simultaneous occurrence of IPMN-b and IPMN-p is still unclear. However, similar clinicopathological findings supported the idea that both tumors might have the same pathogenesis. It was found that the multiple lesions of IPMN were consistent with the concept of field cancerization^[8], proposed by Slaughter^[9], which has been invoked to explain the occurrence of multiple, independent and primary neoplasms^[10]. Because the pancreas and the bile duct develop embryonically from the same primordium^[11], the present case may

also fit the concept of field cancerization.

IPMN-b and IPMN-p are slow-growing tumors with a lower malignant potential than ductal cell carcinoma and mucinous cystadenocarcinoma^[12]. Regional lymph node metastasis was rarely found in previously reported cases and excellent prognosis can be expected after complete resection of tumors with negative margins, with a 5-year survival rate higher than 80%^[13-15]. Therefore, an accurate preoperative or intraoperative diagnosis is very important in this disease to ensure the curative resection. In contrast to IPMN-p, IPMN-b is more difficult to be diagnosed because of lacking full recognition, especially when it is accompanied with stones. Missed diagnosis of IPMN-b also occurred in our case till massive mucin in bile duct was found during liver resection. Preoperative endoscopic retrograde cholangiopancreatography is more useful than routine CT and magnetic resonance imaging, for the diagnosis of lesions with the characteristic findings of a patulous ampulla, hypersecreted mucin and diffusely dilated biliary tract.

It has been reported that laparoscopic hepatectomy or pancreatectomy is safe, valid, and minimally invasive. However, there are few reports on laparoscopic resection of synchronous liver and pancreatic tumors except one case of laparoscopic resection of a pancreatic polypeptidoma with solitary liver metastasis^[16]. In our case, two tumors were located separately in the left lateral lobe of liver and pancreatic tail, making the laparoscopic left lateral hepatectomy and distal pancreatectomy an optimal choice of treatment for the patient, since the

complicated lymphadenectomy, hilar dissection and gastrointestinal reconstruction were not needed. The spleen was preserved, for there was no evidence of malignancy or splenic vessel invasion according to the imaging findings and intraoperative pathological diagnosis.

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Giant solitary fibrous tumor arising from greater omentum

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Abstract

Extrathoracic solitary fibrous tumors (SFTs) have been described at almost every anatomic location of human body, but reports of SFT in the abdominal cavity are rare. We herein present a rare case of SFT originating from greater omentum. Computed tomography revealed a 15.8 cm × 21.0 cm solid mass located at superior aspect of stomach. Open laparotomy confirmed its mesenchymal origin. Microscopically, its tissue was composed of non-organized and spindle-shaped cells exhibiting atypical nuclei, which were divided up by branching vessel and collagen bundles. Immunohistochemical staining showed that this tumor was negative for CD117, CD99, CD68, cytokeratin, calretinin, desmin, epithelial membrane antigen, F8 and S-100, but positive for CD34, bcl-2, α -smooth muscle actin and vimentin. The patient presented no evidence of recurrence during follow-up. SFT arising from abdominal cavity can be diagnosed by histological findings and immunohistochemical markers, especially for CD34 and bcl-2 positive cases.

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Key words: Greater omentum; Solitary fibrous tumor; Immunohistochemical markers

INTRODUCTION

Solitary fibrous tumor (SFT), a rare neoplasm occurring most often in the visceral pleura, was first described by Klemperer and Rabin in 1931^[1]. Extrathoracic SFT has been described at almost every anatomic location of the human body^[2-6], but reports of SFT in the abdominal cavity are rare^[7-11]. Five SFT cases involving omentum have been reported up till December 2011^[11-15]. Herein, we report a rare case of a giant SFT originating from greater omentum. The final diagnosis of the patient was established by pathological examination and immunohistochemical study after an open excision of the tumor.

CASE REPORT

A 29-year-old Chinese man was admitted to the Subei People's Hospital of Jiangsu Province, China on July 1, 2008. He complained of a mass in the upper abdomen and a gradual weight loss that started more than four months ago. He was a farmer. He had remained well until the day before admission, with no fever, no vomiting and no stomach-ache except for epigastric discomfort and compression. Physical examination showed a large abdominal mass lying between xiphoid process of the sternum and umbilicus without obvious tenderness. No abnormalities were found in laboratory data including tumor markers (Table 1). Abdominal contrast-enhanced

Tumor markers	Index	Normal range
CA199 (KU/L)	5.47	< 35.00
CA242 (KU/L)	1.31	< 20.00
CA125 (KU/L)	2.27	< 35.00
CA15-3 (KU/L)	2.28	< 35.00
NSE (ng/mL)	< 1.0	< 13.00
CEA (ng/mL)	1.21	< 5.00
Ferritin (ng/mL)	27.13	< 322.00
β-HCG (MIU/mL)	< 0.02	< 3.00
AFP (ng/mL)	0.88	< 20.00
Free-PSA (ng/mL)	< 0.22	< 1.00
PSA (ng/mL)	< 0.04	< 5.00
HGH (ng/mL)	2.15	< 7.50

CA: Cancer antigen; NSE: Neuron-specific enolase; CEA: Carcinoembryonic antigen; β-HCG: β-human chorionic gonadotropin; AFP: Alphafetoprotein; PSA: Prostate specific antigen; HGH: Human growth hormone.



Figure 1 Abdominal computed tomography demonstrating a giant solitary tumor of 15.8 cm × 21.0 cm in abdominal cavity.

computed tomography (CT) showed extrinsic multi-organ compression due to a giant solitary tumor of 15.8 cm × 21.0 cm occupying the majority of abdominal cavity (Figure 1).

Laparotomy was performed and a giant tumor originating from greater omentum was discovered. The tumor was partly surrounded by greater omentum, and tightly adhered to the spleen and stomach (Figure 2). Abundant and extremely expanded blood vessels of greater omentum were present along the surface of tumor, leading to a blood loss of nearly 2000 mL when the tumor was totally excised. The excised mass was solitary and tenacious compassed with a complete envelope. The mass measured 28 cm × 25 cm × 11 cm in size and 5002.4 g in weight. Microscopically, the excised tumor tissue was composed of non-organized and spindle-shaped cells exhibiting atypical nuclei, which were divided up by branching vessel and collagen bundles (Figure 3). Immunohistochemical staining showed that the tumor was negative for CD117, CD99, CD68, cytokeratin, calretinin, desmin, epithelial membrane antigen, F8 and S-100, but positive for CD34, bcl-2, α-smooth muscle actin (α-SMA) and vimentin (VIM) (Figure 4). According to the mitotic index, this case was considered to have a low risk of malignancy.

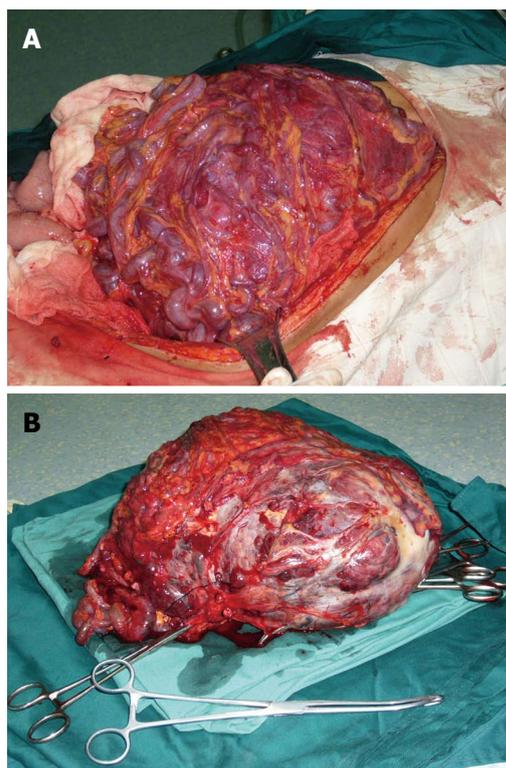


Figure 2 Giant tumor. A: A giant tumor originating from greater omentum; B: A giant tumor originating from resected specimen.

The patient experienced no postoperative complications, and was discharged 10 d after surgery. During a 48-moSS follow-up by ultrasonography or CT, there was no evidence of recurrence.

DISCUSSION

SFT is a rare mesenchymal neoplasm often originating from the pleura, but occasionally from other parts of the body, including the peritoneum, mediastinum, extremities, orbit, and parotid gland^[1-6]. Intra-abdominal SFT is very rare; and SFT with the involvement of greater omentum is even more uncommon. We searched the PubMed and reviewed the relevant papers published till December 2011, and found only 5 SFT cases involving the omentum^[11-15].

SFT is a neoplasm derived from mesenchymal cells located in the sub-mesothelial lining of the tissue space, predominantly composed of spindle-shaped cells and collagen bundles^[16]. Approximately 78%-88% of SFTs are benign and 12%-22% are malignant^[17,18]. The clinical and pathological properties of SFT were first reported by Klempere *et al*^[1]. The earliest criteria for a judgment of malignancy of SFT by England *et al*^[19] were (1) high cellularity with crowding and overlapping of nuclei; (2) high mitotic activity (more than 4 mitotic figures per 10 high-power fields); and (3) pleomorphism judged as mild, moderate, or marked based on nuclear size, irregularity, and nucleolar prominence. In addition, some authors suggested a potential association of tumor size,

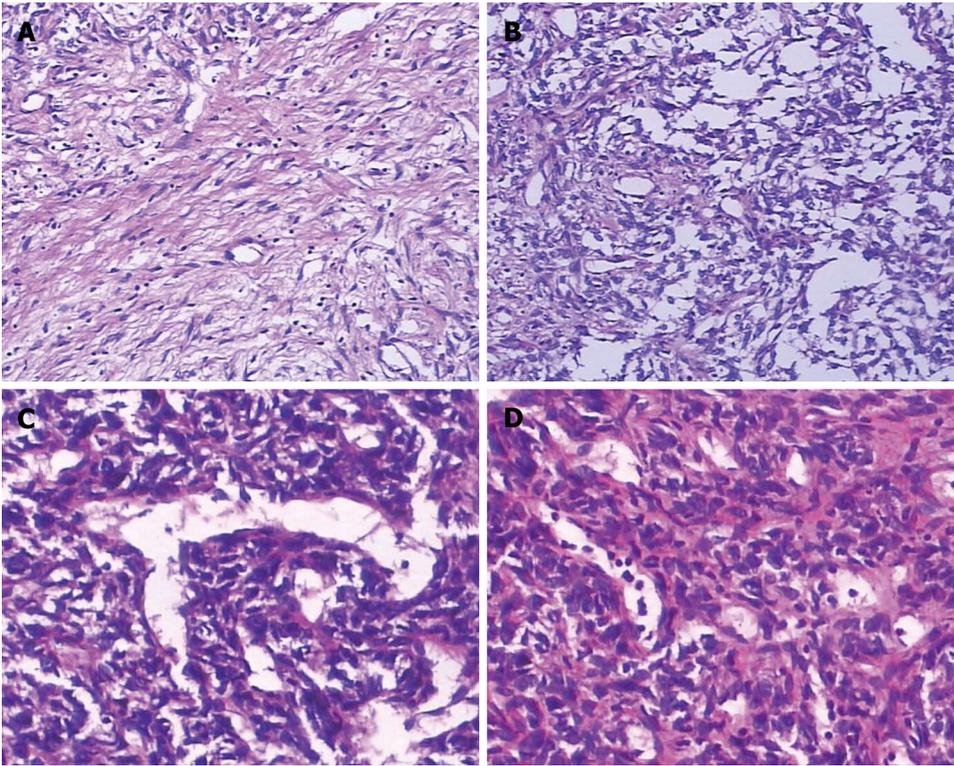


Figure 3 Hematoxylin and eosin stained sections. A: Collagen deposition, 10 cm × 10 cm; B: Abundant spindle cells, 10 cm × 10 cm; C: Branching vessel, 10 cm × 20 cm; D: Nuclear atypia, 10 cm × 20 cm.

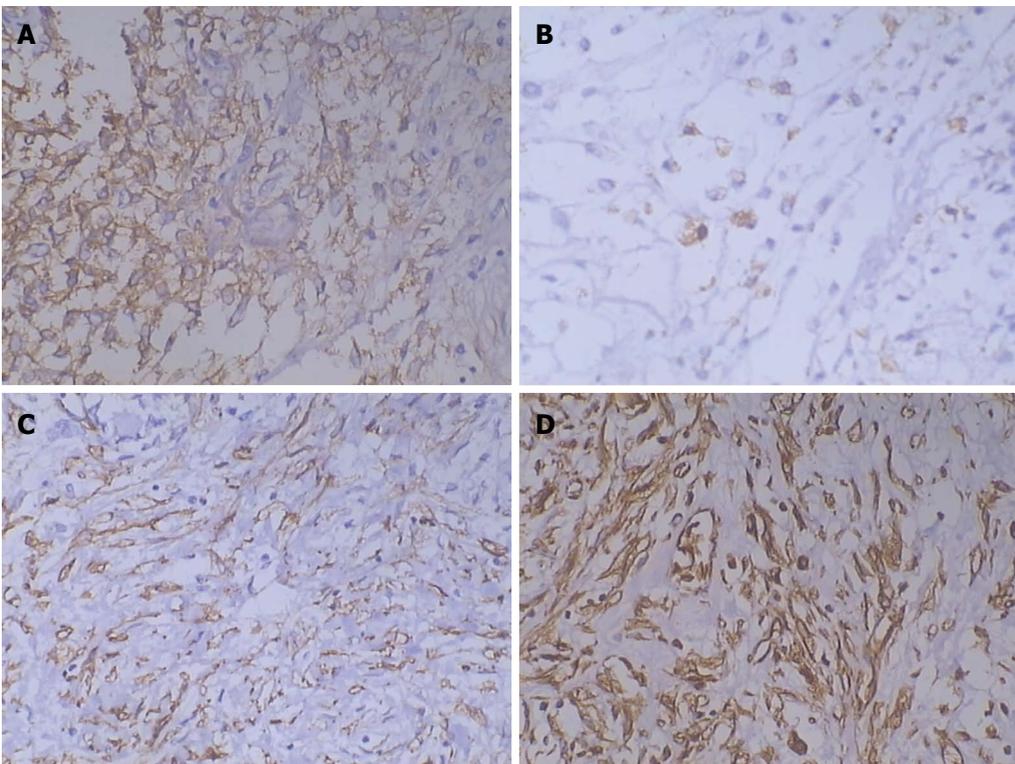


Figure 4 Immunohistochemical test. Immunohistochemical test showing the tumor was positive for CD34 (A), bcl-2 (B), α -smooth muscle actin (C), and vimentin (D) (10 cm × 20 cm).

Table 2 Clinical characteristics of the 5 reported cases of solitary fibrous tumor originating from omentum

Ref.	Site	Age (yr)	Size (cm)	Weight (kg)	Immunohistochemical analysis (pos/neg)	Mitotic count	Local recurrence /metastases	Status at last follow-up
Salem <i>et al</i> ^[11]	Omentum	60	24 × 19 × 10	3.87	CD34, CD99/(SMA), desmin, CD117	25/10HPF	No	NED 4 mo
Patriti <i>et al</i> ^[12]	Greater omentum	24	3.2 × 2.5	NA	CD34, bcl-2/Pan cytokeratin reaction	3/10HPF	No	NED 2 yr
Mosquera <i>et al</i> ^[13]	Omentum	40	9	NA	CD34, CD99, p16/ EMA, cytokeratin, S-100, desmin, p53	9/10HPF	Yes	DOD 34 mo
Ekici <i>et al</i> ^[14]	Lesser omentum	51	11.5 × 8.5 × 7.5	NA	CD 34/CD117, actin and S-100	0/10HPF	No	NED 10 mo
Gold <i>et al</i> ^[15]	Omentum	NA	NA	NA	NA	NA	NA	NA

NA: Not available; Neg: Negative; Pos: Positive; NED: No evidence of disease; DOD: Dead of disease; HPF: High-power fields; EMA: Epithelial membrane antigen; SMA: Smooth muscle actin.

Table 3 Immunohistochemical indexes for differential diagnosis of mesenchymal tumors

	CD34	bcl-2	CD99	S-100	Cytokeratin	EMA	Calretinin	Desmin	α-SMA
Solitary fibrous tumor	+	+	+	-	-	-	-	±	±
Neurofibroma	+	+	-	+	-	-	-	-	-
Spindle cell lipoma	+	+	-	-	-	-	-	-	-
Synovial sarcoma	-	+	±	-	+	+	-	-	-
Desmoid tumor	-	-	-	-	-	-	-	+	+
Hemangiopericytoma	+	-	±	-	-	-	-	-	-
Malignant peripheral nerve sheath tumor	±	±	-	+	±	-	-	±	±
Sarcomatoid mesothelioma	-	±	±	-	+	+	+	-	-
Schwannoma	±	+	-	+	-	-	-	-	-
Calcifying fibrous pseudotumor	±	-	-	-	-	-	-	-	-
Smooth muscle tumor	±	±	±	-	-	-	-	+	+

α-SMA: α-smooth muscle actin; EMA: Epithelial membrane antigen.

hemorrhage and necrosis with the clinical behavior of SFT^[19-21].

We summarized the clinical data of the 5 reported cases of SFT originating from omentum (Table 2). Of particular note, mitotic activity and tumor size may play a key role in predicting SFT. We suggest using risk assessment (very low risk, low risk, intermediate risk, and high risk) based on tumor size, mitotic activity, cellularity and pleomorphism to predict SFT behavior, rather than attempting to draw a sharp line between benign and malignant lesions. More frankly, malignant factors of SFT are associated with a higher risk for recurrence and metastasis. However, the small sample size limits us to draw a definite conclusion. And the detailed criteria still need to be discussed and validated by future studies.

In this case, no mitotic activity and necrosis were present, and there was low cellularity, resulting in a diagnosis of a possible benign entity according to the benign-malignant system. However, the only existing risk is the giant size, and multiple prognostic factors should be taken into account in predicting SFT behavior. In our opinion, it is reasonable to judge this case to be a low-risk lesion by clinical presentation, tumor size, mitotic activity, cellularity and pleomorphism. During a 48-mo follow-up, no evidence of local recurrence or metastasis was observed.

In cases of mesenchymal tumor presenting with

spindle-cell neoplasm, the differential diagnosis of SFT should be considered. It is absolutely necessary although sometimes it is difficult to clearly differentiate it from other malignant or benign entities such as hemangiopericytoma, neurofibroma, spindle cell lipoma, leiomyoma, fibrosarcoma, leiomyosarcoma, angiomyolipoma or fibroma (Table 3). Moreover, hemangiopericytoma must be included into differential diagnosis because of its vascular pattern. And electron microscopic examination can be used to exclude the presence of an external lamina, typically observed in hemangiopericytoma. Immunohistochemically, SFTs commonly express CD34 and bcl-2, and occasionally SMA. They are usually negative for S-100, desmin and cytokeratins^[22,23]. To our knowledge, few tumors of mesenchymal origin can express both CD34 and bcl-2, which are useful to differentiate SFT from other mesenchymal tumors, because approximately 82%-95% and 88%-100% of the SFTs are positive for CD34 and bcl-2, respectively^[24,25]. This report describes a giant SFT showing immunocytochemical reactivity for CD34, bcl-2, α-SMA and VIM, which is consistent with the results reported elsewhere. But what we are really concerned is how to make an exact diagnosis before operation.

The patients with SFT in abdominal cavity may complain of vomiting, abdominal pain or discomfort, but they are mostly asymptomatic. Tumor marker is not specific and sensitive for SFT. But in some patients with

the symptom of hypoglycemia, a high expression of serum insulin-like growth factor- II (IGF- II) was reported, which was found more in the tumor cystic fluid than in serum. Consequently, after total resection of the tumor, no abnormalities were found and the hypoglycemia was resolved^[26,27]. It was concluded that the tumor cells can secrete IGF- II, but the specificity and sensitivity need to be explored by further studies.

The best available diagnostic modalities are CT scanning and magnetic resonance imaging (MRI), which can demonstrate a proliferation of fibrous tissues in abdominal cavity and evaluate the relationship between the tumor and the neighboring structures so as to help the surgeons make a decision whether or not to excise the tumor. Nevertheless, CT scanning and MRI can not distinguish SFT from other mesenchymal tumors. Therefore, SFT may be misdiagnosed as stromal tumor as did in our case because of their homology.

Needle aspiration biopsy for SFT provides inconclusive results because the tumor is composed of acellular and hypercellular portions and it does not provide enough tissues for cytologic analysis. Although Apple *et al.*^[28] reported that accurate diagnosis could be established by fine needle aspiration, it still needs further investigation on a larger series of patients. The rare location of SFT often gives rise to difficulties in diagnosis or to misdiagnosis before operation. However, aspiration by Ryle's tube is a better option for large tumors and specimens could be obtained for immunohistochemical test, while for small tumors, samples can be collected through exploratory laparotomy for immunohistochemical test, thus a diagnosis can be done before operation.

Surgical treatment including local resection of this tumor is a definitive choice of treatment. Postoperative long-term follow-up is very important since SFT may recur locally. The malignant form pursues an aggressive course manifested by local invasion, recurrent growth, or metastasis^[29]. Therefore, postoperative chemotherapy is recommended for malignant SFT. Moreover, half of malignant SFT cases were positive for c-kit^[30] and tyrosine kinase inhibitors, such as imatinib and sunitinib, which are also effective against gastrointestinal stromal tumors^[31,32], have been used in the treatment of SFT^[33].

In summary, we report a rare case of a giant SFT originating from greater omentum. Abdominal imaging is helpful in the diagnosis of the tumor. If CT and MRI are not useful, immunohistochemical test can be performed in preoperative diagnosis using the tumor samples collected through Ryle's tube for small tumors and through exploratory laparotomy for large tumors. Long-term follow-up is necessary to assess the outcomes of the treatment, especially for the high-risk SFTs.

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 Asian Pacific *Helicobacter pylori*
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 Kuala Lumpur, Malaysia

January 19-21, 2012
 American Society of Clinical
 Oncology 2012 Gastrointestinal
 Cancers Symposium
 San Francisco, CA 3000,
 United States

January 19-21, 2012
 2012 Gastrointestinal Cancers
 Symposium
 San Francisco, CA 94103,
 United States

January 20-21, 2012
 American Gastroenterological
 Association Clinical Congress of
 Gastroenterology and Hepatology
 Miami Beach, FL 33141,
 United States

February 3, 2012
 The Future of Obesity Treatment
 London, United Kingdom

February 16-17, 2012
 4th United Kingdom Swallowing
 Research Group Conference
 London, United Kingdom

February 23, 2012
 Management of Barretts
 Oesophagus: Everything you need
 to know
 Cambridge, United Kingdom

February 24-27, 2012
 Canadian Digestive Diseases Week
 2012
 Montreal, Canada

March 1-3, 2012
 International Conference on
 Nutrition and Growth 2012
 Paris, France

March 7-10, 2012
 Society of American Gastrointestinal
 and Endoscopic Surgeons Annual
 Meeting
 San Diego, CA 92121, United States

March 12-14, 2012
 World Congress on
 Gastroenterology and Urology
 Omaha, NE 68197, United States

March 17-20, 2012
 Mayo Clinic Gastroenterology and
 Hepatology
 Orlando, FL 32808, United States

March 26-27, 2012
 26th Annual New Treatments in
 Chronic Liver Disease
 San Diego, CA 92121, United States

March 30-April 2, 2012
 Mayo Clinic Gastroenterology and
 Hepatology
 San Antonio, TX 78249,
 United States

March 31-April 1, 2012
 27th Annual New Treatments in
 Chronic Liver Disease
 San Diego, CA 92121, United States

April 8-10, 2012
 9th International Symposium on
 Functional GI Disorders
 Milwaukee, WI 53202, United States

April 13-15, 2012
 Asian Oncology Summit 2012
 Singapore, Singapore

April 15-17, 2012
 European Multidisciplinary
 Colorectal Cancer Congress 2012
 Prague, Czech

April 18-20, 2012
 The International Liver Congress
 2012
 Barcelona, Spain

April 19-21, 2012
 Internal Medicine 2012
 New Orleans, LA 70166,
 United States

April 20-22, 2012
 Diffuse Small Bowel and Liver
 Diseases
 Melbourne, Australia

April 22-24, 2012
 EUROSON 2012 EFSUMB Annual

Meeting
 Madrid, Spain

April 28, 2012
 Issues in Pediatric Oncology
 Kiev, Ukraine

May 3-5, 2012
 9th Congress of The Jordanian
 Society of Gastroenterology
 Amman, Jordan

May 7-10, 2012
 Digestive Diseases Week
 Chicago, IL 60601, United States

May 17-21, 2012
 2012 ASCRS Annual Meeting-
 American Society of Colon and
 Rectal Surgeons
 Hollywood, FL 1300, United States

May 18-19, 2012
 Pancreas Club Meeting
 San Diego, CA 92101, United States

May 18-23, 2012
 SGNA: Society of Gastroenterology
 Nurses and Associates Annual
 Course
 Phoenix, AZ 85001, United States

May 19-22, 2012
 2012-Digestive Disease Week
 San Diego, CA 92121, United States

June 2-6, 2012
 American Society of Colon and
 Rectal Surgeons Annual Meeting
 San Antonio, TX 78249,
 United States

June 18-21, 2012
 Pancreatic Cancer: Progress and
 Challenges
 Lake Tahoe, NV 89101, United States

July 25-26, 2012
 PancreasFest 2012
 Pittsburgh, PA 15260, United States

September 1-4, 2012
 OESO 11th World Conference
 Como, Italy

September 6-8, 2012
 2012 Joint International

Neurogastroenterology and Motility
 Meeting
 Bologna, Italy

September 7-9, 2012
 The Viral Hepatitis Congress
 Frankfurt, Germany

September 8-9, 2012
 New Advances in Inflammatory
 Bowel Disease
 La Jolla, CA 92093, United States

September 8-9, 2012
 Florida Gastroenterologic Society
 2012 Annual Meeting
 Boca Raton, FL 33498, United States

September 15-16, 2012
 Current Problems of
 Gastroenterology and Abdominal
 Surgery
 Kiev, Ukraine

September 20-22, 2012
 1st World Congress on Controversies
 in the Management of Viral Hepatitis
 Prague, Czech

October 19-24, 2012
 American College of
 Gastroenterology 77th Annual
 Scientific Meeting and Postgraduate
 Course
 Las Vegas, NV 89085, United States

November 3-4, 2012
 Modern Technologies in
 Diagnosis and Treatment of
 Gastroenterological Patients
 Dnepropetrovsk, Ukraine

November 4-8, 2012
 The Liver Meeting
 San Francisco, CA 94101,
 United States

November 9-13, 2012
 American Association for the Study
 of Liver Diseases
 Boston, MA 02298, United States

December 1-4, 2012
 Advances in Inflammatory Bowel
 Diseases
 Hollywood, FL 33028, United States

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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 PMCID: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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- 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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- 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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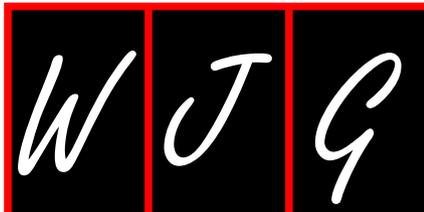
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Esophageal malignancy: A growing concern

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Abstract

Esophageal cancer is mainly found in Asia and east Africa and is one of the deadliest cancers in the world. However, it has not garnered much attention in the Western world due to its low incidence rate. An increasing amount of data indicate that esophageal cancer, particularly esophageal adenocarcinoma, has been rising by 6-fold annually and is now becoming the fastest growing cancer in the United States. This rise has been associated with the increase of the obese population, as abdominal fat puts extra pressure on the stomach and causes gastroesophageal reflux disease (GERD). Long standing GERD can induce esophagitis and metaplasia and, ultimately, leads to adenocarcinoma. Acid suppression has been the main strategy to treat GERD; however, it has not been proven to control esophageal malignancy effectively. In fact, its side effects have triggered multiple warnings from regulatory agencies. The high mortality and fast growth of esophageal cancer demand more vigorous efforts to look into its deeper mechanisms and come up with better therapeutic options.

Key words: Esophageal cancer; Gastroesophageal reflux disease; Obesity

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INTRODUCTION

While the incidence of most cancers is declining, esophageal cancer has been continuing its march as the fastest growing malignancy in the Western world^[1,2]. This rise is largely derived from gastroesophageal reflux disease (GERD), which is associated with the proliferation of obesity. The continuous growth of the obese population foreshadows a future increase of GERD and its associated esophageal cancer. This demands immediate and more rigorous research on the molecular mechanisms of this common disease and its pathways which lead to esophageal malignancy. Current GERD treatment mainly relies on acid suppression drugs which have not been proved to change the risk of cancer development. Although the debate is still going on whether gastric acid or bile acid is ultimately responsible for GERD malignancy, based on the data from human studies, animal modeling, and *in vitro* simulation, perhaps it is time to explore other options.

STATISTICS OF ESOPHAGEAL CANCER: RISING NUMBERS

Cancer is the second leading cause of death in the world^[3]

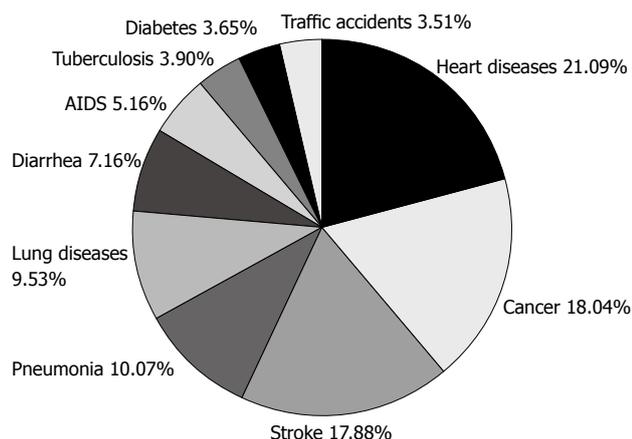


Figure 1 Top 10 leading causes of death worldwide. Cancer is the second highest one. Data extracted from World Health Organization documents. AIDS: Acquired immune deficiency syndrome.

after heart disease (21.09%), contributing 18.04% to the total number of deaths worldwide (Figure 1). Among all types of cancers, skin cancer is the most common one, which includes 2-3 million non-melanoma and 132 000 melanoma cases diagnosed each year, making up one third of the total cancer cases. According to Skin Cancer Foundation Statistics, one in every five Americans will develop skin cancer in their lifetime. This prevalence is largely due to depletion of ozone in the atmosphere, which weakens our planet’s protective shield from the brunt of the sun’s harmful rays. It is estimated that every 10% decrease in ozone levels will generate an additional 300 000 non-melanoma and 4500 melanoma cancer cases. However, the majority of skin cancer can be easily treated, while digestive cancers, such as esophageal cancer, are highly life-threatening.

Esophageal cancer is found more commonly in males than in females, with a ratio of approximately 7:1. Currently, it is the 7th leading cancer in men globally, contributing 6.51% to the total number of male cancer cases (Figure 2). There are two main subtypes of esophageal cancer: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). ESCC occurs most often in the middle portion of the esophagus and accounts for 90%-95% of all cases of esophageal cancer worldwide, while EAC is primarily found in the lower esophagus. The type and incidence of esophageal cancer varies dramatically depending on the geographical location (Figure 3). The top 10 countries with the highest age-standardized death rate due to esophageal cancer are Nauru (30.3), Sao Tome (26.4), Mongolia (18.6), South Africa (18.2), Malawi (18.2), China (15.5), Lesotho (15.5), Kenya (13.9), Mozambique (13.5) and Uganda (13.4) (the death rate being deaths per 100 000 people). The highest rates are found in Asia, stretching from northern Iran through the central Asian republics to north-central China, often referred to as the “esophageal cancer belt”. For instance, in China, the majority of esophageal cancer diagnoses are ESCC and it is ranked as the 8th leading cause of death nationwide (Table 1), mostly in northern

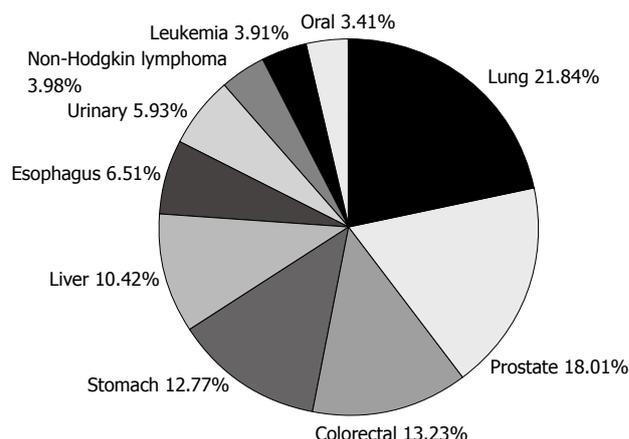


Figure 2 Top 10 of the most common cancers in men (up) and women (below) worldwide. Esophageal cancer is No. 7 in men. Data extracted from World Health Organization documents.

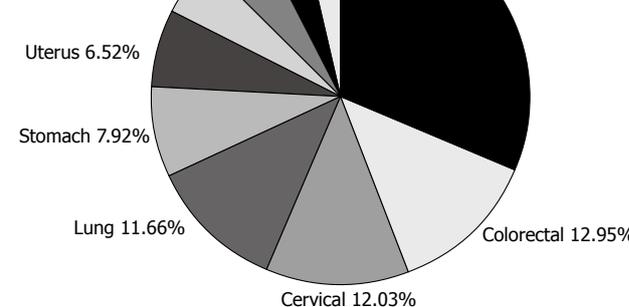


Figure 2 Top 10 of the most common cancers in men (up) and women (below) worldwide. Esophageal cancer is No. 7 in men. Data extracted from World Health Organization documents.

China where the incidence rate can be as high as 800 cases per 100 000 people. On the other hand, in the United States, more than 50% of esophageal cancer cases are EAC, and the rate is less than 5 in 100 000, making it the 29th leading cause of death. For this reason, esophageal cancer is not even on the current list of common cancers in the United States, according to the National Cancer Institute. In order to be on the list, esophageal cancer has to have at least 40 000 cases a year, while the current estimate for 2012 is only 17 460. Although the reason for this geographic variation still needs investigation, several factors have been suggested that might all contribute to the issue to a certain degree, such as *Helicobacter* infection, dietary pattern, and life habits^[4]. As Eastern and Western countries become increasingly open to each other and people adapt more to each other, we expect this discrepancy will become less and less. As evident in China, EAC incidence was doubled from the 1970s to the 1980s, according to an examination of the medical records of esophageal cancer patients diagnosed from 1970 to 2001 in a local hospital^[5].

These numbers only tell one side of the story. Based on the annual reports on the status of cancer^[1,2], although esophageal cancer is low in Americans, it has been rising by 6-fold annually and its increase rate now exceeds that

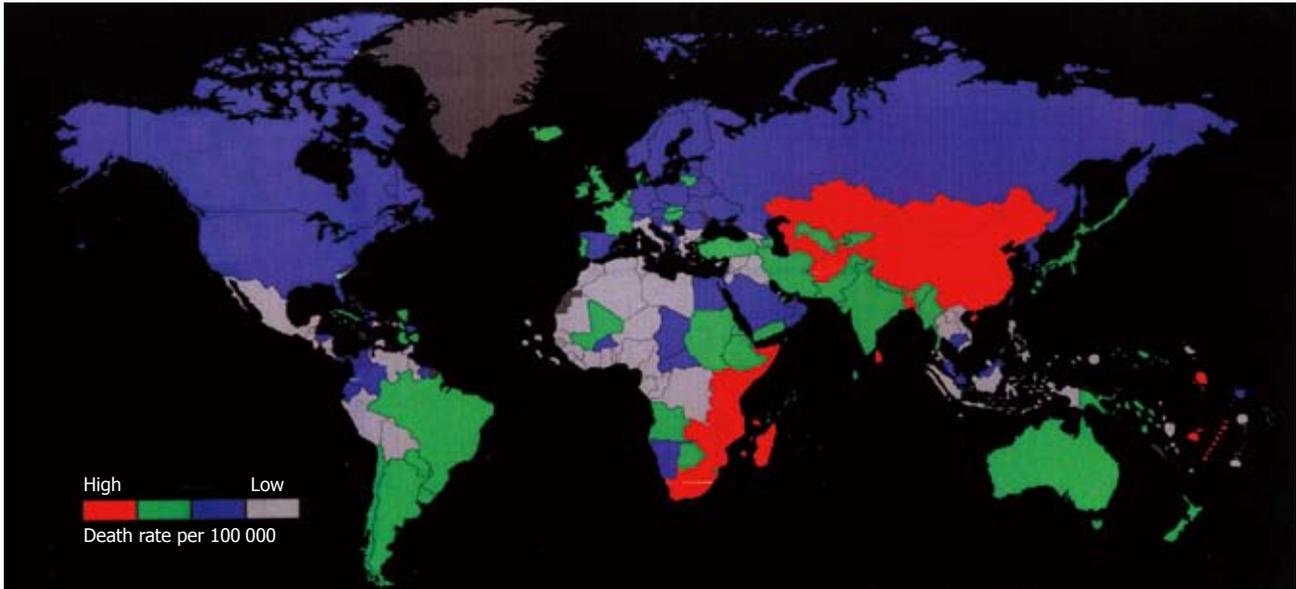


Figure 3 Geographic distribution of esophageal cancer. China is a hot spot. Data from World Health Organization documents.

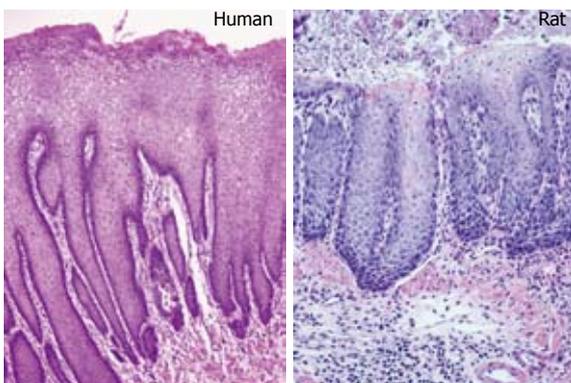


Figure 4 Gastroesophageal reflux disease-induced esophagitis in human and rat (hematoxylin and eosin staining). Gastroesophageal reflux disease rats were created by surgically anastomosing the duodenum to the gastroesophageal junction. These rats can develop esophageal adenocarcinoma within a year, in a pathological sequence similar to human esophageal malignancy.

for any other type of cancer. There are several possible reasons for this rise, such as excessive alcohol consumption, smoking, stress, and a diet low in vegetables, but the leading factor is GERD, a term that frequently appears in the media as well as in general conversations. A recent study showed that GERD increases the risk of esophageal cancer by 8.6 fold^[6].

GERD: NOT A SMALL PROBLEM

GERD is the most common gastrointestinal diagnosis given during office visits and its direct medical costs, which primarily include drug costs, exceed \$10 billion a year in the United States^[7], whereas indirect costs resulting from reduced work productivity are estimated to be as much as \$75 billion a year^[8]. GERD occurs when the esophageal sphincter at the bottom of the esophagus weakens and allows stomach acid (often mixed with duo-

denal contents) to back up into the esophagus. The refluxate erodes the epithelial lining of the lower esophagus and gives a burning sensation in the middle of the chest, which is commonly described as “heartburn”. Patients with long standing GERD can develop esophagitis, an inflammation characterized histologically by a markedly thickened epithelium, elongation of the lamina propria papillae into the epithelium, and basal cell hyperplasia (Figure 4). Over time, this inflammation/injury cycle can induce esophageal mucosa transformation from squamous to a more protective intestinal columnar phenotype, known as Barrett’s esophagus (BE). From a physiological point of view, the secretory columnar epithelium is better prepared to withstand the erosive action of the acidic refluxate than squamous epithelium; however, this metaplastic change confers an increased risk of transformation to EAC. Studies have shown that people with BE can have as high as a 400-fold increased risk of EAC^[9,10]. Today, over 60% of Americans experience occasional episodes of acid reflux, and about 25% deal with the problem on a weekly basis. The prevalence of the condition in Americans is increasing by approximately 5% annually^[11]. Hospitalizations for all GERD-caused esophageal disorders doubled from 1998 to 2005, according to the United States Agency for Healthcare Research and Quality.

OBESITY: THE DEVIL

The rise in GERD is associated with the rapidly growing obese population^[12], which is usually measured by body mass index (BMI). BMI is calculated based on the weight and height of a person [BMI = weight/(height)², kg/m²]. The World Health Organization regards a BMI of less than 18.5 as underweight and may indicate malnutrition, an eating disorder, or other health problems, while a BMI greater than 25 is considered overweight and above 30 is considered obese. A recent study showed that global obe-

Table 1 Top 10 leading causes of death in China vs the United States

Rank	China			United States		
	Diseases	Deaths	Death rate (%)	Diseases	Deaths	Death rate (%)
1	Stroke	2 125 802	23.92	Coronary heart disease	445 864	21.42
2	Lung disease	1 287 089	14.48	Alzheimer/dementia	172 765	8.30
3	Coronary heart disease	1 040 692	11.71	Lung cancers	165 402	7.95
4	Lung cancers	460 856	5.19	Stroke	146 664	7.05
5	Liver cancer	380 491	4.28	Lung disease	130 808	6.29
6	Stomach cancer	354 829	3.99	Diabetes mellitus	75 280	3.62
7	Road traffic accidents	292 481	3.29	Colorectal cancers	62 592	3.01
8	Esophageal cancer	212 537	2.39	Hypertension	62 156	2.99
9	Other injuries	209 836	2.36	Pneumonia	57 722	2.77
10	Hypertension	205 689	2.31	Kidney disease	50 889	2.45

Esophageal cancer is the No. 8 killer in China, while in the United States, it is No. 29. Here the death rate is the percentage of the total deaths nationwide. Data from World Health Organization, World Bank and National Institute of Health.

sity rates have doubled since 1980^[13]. The health care costs resulting from excess weight are estimated at greater than \$100 billion annually in the United States. According to the report released last year from the Centers for Disease Control and Prevention, more than 34% of adult Americans are obese, which is higher than Canadians (24%). It is predicted that by the year 2020, 77.6% of men will be overweight and 40.2% obese; the corresponding figures for women will be 71.1% and 43.3%, respectively^[14]. This problem has also affected children, whose obesity rate has tripled in the last 30 years^[15]. At the time of writing, 15.5% of children in the United States are obese. A recent study with a study population of 690 321 patients (age: 2-19 years) revealed that obese children have a 30%-40% higher risk of GERD, compared with children with a normal weight according to their BMI (BMI = 20 ± 3.8 kg/m²)^[16]. In adults, the situation is worse. In 2007, a study showed that the total number of GERD episodes was 48% higher in obese patients than those with a normal BMI^[17]. The link between increasing BMI and the presence of GERD was further strengthened by a meta-analysis of 20 independent studies, which established a dose-dependent association between these two conditions^[18]. A similar connection has also been drawn between increasing BMI and esophageal cancer^[19,23].

The precise pathophysiological pathway from obesity to GERD has not been fully elucidated. It has been shown that excess fat in the abdominal area can push on the stomach's contents to back up, relax the lower esophagus muscle^[24,25], disable the esophageal motor^[26], impair stomach accommodations^[27], and ultimately result in a higher frequency of esophageal acid exposure^[12,28,29]. Therefore, a potential causal pathway from body size to esophageal cancer may be from normal to GERD to esophagitis to BE, and ultimately to EAC. In such a direct pathway, obesity could act by increasing the prevalence of GERD, by increasing the prevalence of BE among the GERD population, or by enhancing the risk of malignant transformation from BE to EAC. Although obesity is a major contributor, other factors (e.g., smoking, drinking, diet, or genetics) may also influence the steps on this pathway. For example, GERD smokers

were found to have 12.3-fold higher risk of developing EAC than GERD non-smokers^[6]. While the issue is quite complex, since the main pathway starts with GERD, interventions aimed at GERD should be expected to proportionally lower the risk of the subsequent steps in the pathway: BE and EAC.

TREATMENT: NO WINNERS

Current treatment for GERD patients includes acid suppressive medications and lower esophageal repair surgery. Aside from traditional antacids (Alka-Seltzer and Tums) which have side effects such as diarrhea and constipation, there are now two categories of medications to treat GERD. H-2 blockers (e.g., Zantac 75, Pepcid AC, Tagamet HB and Axid AR) reduce the amount of histamine-2, which produces acid in the stomach, and are recommended for people with less frequent/severe heartburn. A second medication is the proton pump inhibitors or proton pump inhibitors (PPIs) (e.g., Prilosec, Prevacid, Protonix and Nexium), which directly shuts down the H⁺/K⁺ ATPase pump of the parietal cells in the stomach that produce acid. These drugs are stronger than H2 blockers and are recommended for people with more persistent/acute symptoms. Control of acid reflux with PPIs has been found extremely effective for healing reflux esophagitis, but not for prevention of BE development or its progression to EAC. As matter of fact, more and more evidence is emerging about the long-term side-effects associated with these drugs, such as decreased absorption of vitamins/minerals^[30], susceptibility to bacterial infections^[31], bone fracture^[32], and even elevated risk of developing EAC^[6,33]. The Food and Drug Administration of the United States has issued warnings repeatedly over the years on high-dose or long-term use of PPIs.

For people who have responded to medication but continue to experience GERD symptoms, surgery to reconstruct the lower esophageal sphincter is usually an option. However, only about 5% of GERD patients undergo surgery and a follow-up study showed that almost two-thirds of the surgical patients were back on medica-

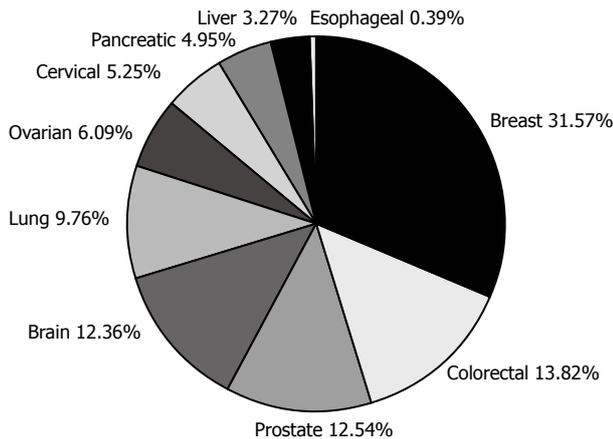


Figure 5 National Institute of Health expenditure on cancer-related studies in 2011. Esophageal cancer barely shows on the pie chart. Data from National Institute of Health documents.

tion^[34]. A more recent study reported that although surgical therapy achieved better remission of GERD symptoms than Prilosec, 36% of surgical-treated GERD patients ultimately received PPI medication, while 14% of PPI-treated GERD patients underwent subsequent surgery^[35]. One final way to treat GERD is through endoscopic procedures, including stitching or using radio-frequency waves to reconstruct the lower esophageal sphincter, but in 2002 this was not recommended by the American Gastroenterological Association for GERD treatment.

CAUSE OF EAC: ACID OR BILE? WHAT TO BLAME?

The inadequacy of treatment options raises the question on the real scientific basis of acid suppression in GERD treatment. A direct chemical analysis of esophageal fluid showed that GERD patients contain about 10 times more bile acid in their lower esophagus than normal people^[36]. This might give us a clue as to why regular use of acid suppressants has not lowered the risk of GERD malignancy. In support of this notion, animal studies showed that gastric reflux alone does not cause EAC at all; it is the duodenal contents per se that ultimately lead to esophageal malignancy^[37-39]. Furthermore, some animal studies even suggested that gastric acid may play a protective role in GERD against malignancy. For example, one study showed that 87% of rats with surgically-created duodenal reflux alone developed EAC, while the percentage of rats with gastric-duodenal reflux was only 30%^[40]. In agreement with the animal studies, a recent systematic review^[41,42] examined publications indexed in MEDLINE from 1950 to 2010, and found 82 original human studies on the association of bile acids with GERD, among which, all *in vivo* studies detected bile acids in the esophageal aspirates of GERD patients, and what's more, their concentrations were significantly higher than in normal people. It is clear that the refluxate of GERD patients frequently contains bile acids, some-

times even in millimolar concentrations. In addition to human studies and animal modeling, *in vitro* experiments have shown that bile acids, at equivalent concentrations to the ones found in the esophagus of GERD patients, can stimulate esophageal epithelial cells to produce inflammatory cytokines and chemokines, to generate reactive oxygen species, and to express intestinal genes. All these factors have the ability to facilitate esophageal epithelial metaplasia and even malignancy.

CONCLUSION

Although skin cancer is the most common cancer in the world, 95% of cases are either basal cell carcinoma or squamous cell carcinoma, which have less than 0.5% mortality. Even for the most deadly type of skin cancer - melanoma, which is very rare - the death rate is only about 15%. So is prostate cancer, the second most common cancer in the United States. On the other hand, although esophageal cancer patients have a mortality rate of approximately 85%, since it is less common than either skin cancer or prostate cancer, it receives little attention from government agencies or the research community. This can be seen by looking at the annual budget of the National Institute of Health (NIH) of the United States. In 2011, the NIH spent \$284 million on 772 prostate cancer research projects, while only funding 30 esophageal cancer studies with \$13 million (Figure 5). Through this article, we hope to attract attention to this disease since, due to its high mortality and fast growth, esophageal cancer could be catastrophic in the near future if we do not prepare ourselves with the proper knowledge.

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Does antecolic reconstruction decrease delayed gastric emptying after pancreatoduodenectomy?

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Abstract

Delayed gastric emptying (DGE) is a frequent complication after pylorus-preserving pancreatoduodenectomy (PpPD). Kawai and colleagues proposed pylorus-resecting pancreatoduodenectomy (PrPD) with antecolic gastrojejunal anastomosis to obviate DGE occurring after PpPD. Here we debate the reported differences in the prevalence of DGE in antecolic and retrocolic gastro/duodeno-jejunosomies after PrPD and PpPD, respectively. We concluded that the route of the gastro/duodeno-jejunal anastomosis with respect to the transverse colon; i.e., antecolic route or retrocolic route, is not responsible for the differences in prevalence of DGE after pancreatoduodenectomy (PD) and that the impact of the reconstructive method on DGE is related mostly to the angulation or torsion of the gastro/duodeno-jejunosomies. We report a prevalence of 8.9% grade A DGE and 1.1% grade C DGE in a series of 89 subtotal stomach-preserving PDs with Roux-en Y retrocolic reconstruction with anastomosis of the isolated Roux limb to the stomach and single Roux limb to both the pancreatic stump and hepatic duct. Retrocolic anastomosis of the isolated first jejunal loop to the gastric remnant allows outflow of the gastric contents by gravity through a "straight route".

INVITED COMMENTARY ON HOT ARTICLES

Delayed gastric emptying (DGE) is a major cause of early morbidity following pancreatoduodenectomy (PD). Although it has been recently reported that pylorus-preserving pancreatoduodenectomy (PpPD) and classical Whipple's PD are equal operations regarding the postoperative development of DGE^[1], the occurrence of this complication is usually considered to be associated with PpPD. DGE after PpPD was first described by Warshaw *et al*^[2] in 1985. DGE implies a state of postoperative gastroparesis and gastric stasis for which prolonged gastric drainage is necessary with delay to return to solid food intake. However, the pathogenesis of DGE is still unclear. Postoperative decrease in plasma motilin stimulation after duodenal resection^[3], devascularization and denervation of the pylorus with subsequent pylorospasm in PpPD^[4,5] and other operative factors such as the route of gastro- or

duodeno-enteric reconstruction (antecolic *vs* retrocolic)^[6] and the type of reconstructive technique (Billroth I *vs* Billroth II reconstruction)^[7] may contribute to the occurrence of DGE. Moreover, intra-abdominal postoperative complications such as pancreatic fistula, peripancreatic collections, intraabdominal abscess or postoperative pancreatitis may increase the prevalence of DGE^[8-13]. The reported prevalence of DGE after pancreatic surgery is remarkably variable due to different adopted definitions of DGE^[10,14,15]. In fact, a consensus definition of DGE based on the impact on the clinical course and on postoperative management was proposed by the International Study Group of Pancreatic Surgery only in 2007^[16]. Kawai *et al*^[17] reported a prospective randomized controlled trial (RCT) on the prevalence of DGE in pylorus-resecting pancreatoduodenectomy (PrPD) *vs* PpPD. The authors proposed PrPD, in which the stomach is nearly entirely preserved and divided just adjacent to the pyloric ring, to obviate DGE occurring after PpPD and avoid the impairment of nutritional status occurring after classical Wipple's PD. They highlighted that the results of their RCT significantly favored PrPD over PpPD, considering the prevalence of DGE (4.5% *vs* 17.2%): in these procedures an antecolic gastro- or duodeno-jejunal reconstruction was adopted^[18]. A recent RCT comparing the occurrence of DGE after subtotal stomach-preserving pancreatoduodenectomy in pancreaticogastrostomy with retrocolic gastro-jejunos- tomy reconstruction and in pancreaticogastrostomy with antecolic gastro-jejunos- tomy reconstruction concluded that antecolic reconstruction, and not retrocolic reconstruction, decreases DGE prevalence. However, in this study, Billroth I (retrocolic) reconstructions were compared with Billroth II (antecolic) reconstructions^[19]. After subtotal stomach-preserving pancreatoduodenectomy with pancreaticogastrostomy, Oida *et al*^[20,21] considered retrocolic gastrojejunal reconstruction preferable to antecolic reconstruction for preventing DGE because pancreaticogastric anastomosis is located behind the stomach and the retrocolic route in gastroenteric reconstruction enables the gastric contents to easily reach the jejunum. In the study by Eshuis *et al*^[22], DGE was more frequent in retrocolic reconstructions, but in multivariable analysis no association between the route of reconstruction and DGE was found.

After PD, Billroth I reconstruction is considered to have a higher incidence of DGE than Billroth II reconstruction^[7], but Billroth I is considered to be a more physiologic procedure than Billroth II because Billroth I preserves the proximal jejunum in the alimentary circuit and maintains the hormonal stimuli on the remnant pancreas^[23]. In evaluation of the prevalence of DGE in antecolic and retrocolic reconstruction in gastro- and duodeno-jejunos- tomy after classical Wipple's PD and PpPD, respectively, the two compared procedures should differ only in the manner in which the jejunum is brought up in respect to the transverse colon. Kawai participated in a previously reported prospective RCT in which the adopted reconstructive procedures after PpPD were different only regarding

the route; i.e., antecolic or retrocolic, for Billroth II type duodeno-jejunal anastomosis. The prevalence of DGE was significantly lower in the antecolic duodeno-jejunos- tomy group than in the retrocolic duodeno-jejunos- tomy group^[6]. However, another recent RCT showed no difference in the prevalence of DGE between antecolic and retrocolic gastro/duodeno-jejunos- tomy following classical Wipple's PD/PpPD after standardization of both the antecolic and retrocolic types of Billroth II gastro/duodeno- jejunos- tomy with respect to the distance from the hepato- co-jejunos- tomy and angulation of the jejunal loop. In this study, the occurrence of DGE was not affected by the type of performed PD; i.e., classical Wipple's PD *vs* PpPD, or the type of adopted reconstruction of the gastro/duode- no-jejunos- tomy; i.e., antecolic *vs* retrocolic^[24]. Ueno *et al*^[25] indicated that the transient torsion or angulation in the reconstruction of the alimentary tract is the main cause of DGE after PpPD. Several methods were proposed to promote the alimentary transit from the stomach through the jejunal loop, such as alignment of the stomach con- tour to avoid angulation of the jejunal loop distally to the duodeno-jejunal anastomosis in a Billroth I type of re- constructive procedure^[25], and straight antecolic duodeno- jejunos- tomy twisting the jejunum 30° counterclockwise to preserve the patency of the efferent jejunum and placing the stomach in the left subcolic fossa to straighten it in a Billroth II type of reconstruction^[26]. In the RCT by Chi- jiiwa *et al*^[27] no significant difference in the prevalence of DGE was found between retrocolic vertically performed duodenojejunos- tomy and antecolic duodenojejunos- tomy (Table 1).

Regarding the resection method, Kawai *et al*^[17,18] high- lighted that PrPD preserves the capacity of the stomach and obviates to pylorospasm, denervation and devascu- larization of the pylorus ring, which can occur in PpPD, and demonstrated that PrPD decreases the incidence of DGE in respect to PpPD. Recently, these surgical proce- dures of subtotal stomach-preserving (or pylorus-resect- ing) pancreatoduodenectomies have been adopted in sur- gical treatments of malignant tumors of the periampul- lary region and of the head of the pancreas. Our group has been adopting subtotal stomach-preserving pancre- atoduodenectomy since 1995 for several considerations. After pancreaticoduodenectomy, gastric preservation favors adequate weight gain due to higher caloric intake; moreover, and most of all, normal acid secretion acts as a physiologic stimulus promoting the intestinal secre- tion of secretin and CCK-PZ, as well as the subsequent stimulation of pancreatic exocrine secretion with better digestion of protein and fat (weight gain). Lastly, pres- ervation of the stomach with resection of the pylorus favors better gastric emptying^[28,29]. Regarding the impact of the reconstructive method on DGE, we think that the route of the gastro/duodeno-jejunal anastomosis with respect to the transverse colon (antecolic or retrocolic) or the type of reconstruction performed (Billroth I or Billroth II procedure) are not truly responsible for the dif- ferences in the prevalence of DGE after PD. We believe

Table 1 Summary of the cited studies on prevalence of delayed gastric emptying after pancreaticoduodenectomy

Ref.	Type of study	No. of patients	Studied groups	Significant difference in prevalence of DGE
Eshuis <i>et al</i> ^[22]	CCS	77	PD/PpPD + PJ-B II AG/DJ	Not found
		77	PD/PpPD + PJ-B II RG/DJ	
Oida <i>et al</i> ^[20]	CCS	14	MSSPPD + PG-B II AGJ	PG-B II RGJ < PG-B II AGJ
		28	MSSPPD + PG-B II RGJ	
Masui <i>et al</i> ^[26]	CCS	12	PpPD + PJ-B II ADJ	PJ-B II AMDJ < PJ-B II ADJ
		106	PpPD + PJ-B II AMDJ	
Kawai <i>et al</i> ^[17]	RCT	66	PrPD + PJ-B II AGJ	PrPD < PpPD
		64	PpPD + PJ-B II ADJ	
Kurahara <i>et al</i> ^[19]	RCT	22	SSPPD + PG-B I RGJ	PG-B II AGJ < PG-B I RGJ
		24	SSPPD + PG-B II AGJ	
Gangavatiker <i>et al</i> ^[24]	RCT	35	PD/PpPD + PJ-B II AG/DJ	Not found
		37	PD/PpPD + PJ-B II RG/DJ	
Chijiwa <i>et al</i> ^[27]	RCT	17	PpPD + PJ-B II ADJ	Not found
		18	PpPD + PJ-B II VRDJ	
Tani <i>et al</i> ^[6]	RCT	40	PpPD + PJ-B II ADJ	PJ-B II ADJ < PJ-B II RDJ
		40	PpPD + PJ-B II RDJ	

CCS: Case control study; RCT: Randomized controlled trial; PD/PpPD: Pancreaticoduodenectomy or pylorus-preserving pancreaticoduodenectomy; PJ-B II AG/DJ: Pancreaticojejunostomy with Billroth II antecolic gastro/duodenojejunostomy; PJ-B II RG/DJ: Pancreaticojejunostomy with Billroth II retrocolic gastro/duodenojejunostomy; MSSPPD: Modified subtotal stomach-preserving pancreaticoduodenectomy; PG-B II AGJ: Pancreaticogastrostomy with Billroth II antecolic gastrojejunostomy; PG-B II RGJ: Pancreaticogastrostomy with Billroth II retrocolic gastrojejunostomy; PJ-B II ADJ: Pancreaticojejunostomy with Billroth II antecolic duodenojejunostomy; PJ-B II AMDJ: Pancreaticojejunostomy with Billroth II antecolic modified reconstruction with straightening of the stomach and twisted duodenojejunostomy; PrPD: Pylorus-resecting pancreaticoduodenectomy; PJ-B II AGJ: Pancreaticojejunostomy with Billroth II antecolic gastrojejunostomy; SSPPD: Subtotal stomach-preserving pancreaticoduodenectomy; PG-B I RGJ: Pancreaticogastrostomy with Billroth I retrocolic gastrojejunostomy; PJ-B II VRDJ: Pancreaticojejunostomy with Billroth II retrocolic modified reconstruction with vertical duodenojejunostomy; PJ-B II RDJ: Pancreaticojejunostomy with Billroth II retrocolic duodenojejunostomy; DGE: Delayed gastric emptying.

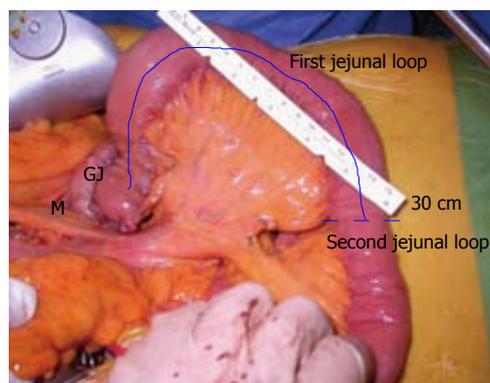


Figure 1 Retrocolic gastro-jejunal anastomosis in Roux-en-Y reconstruction after subtotal stomach-preserving pancreaticoduodenectomy. M: Mesocolic window; GJ: Gastro-jejunal anastomosis. Dashed line indicates the level of jejunal division.

that, after a PD, the impact of reconstructive methods on DGE is related mostly to the angulation or torsion of the reconstruction of the gastro/duodeno-jejunosomy because all the reported modified procedures associated with lower DGE, in Billroth I as well Billroth II types of reconstruction, are related to the reconstructive anatomy of the alimentary circuit and are aimed to facilitate the outflow of the ingests from the gastric/duodenal remnant. An antecolic gastro/duodeno-jejunosomy can favor a straight construction and gastric emptying by gravity in a Billroth II reconstruction after PD or PpPD^[24] as well as a retrocolic Billroth II gastrojejunostomy after a subtotal stomach-preserving pancreaticoduodenectomy with pancreaticogastrostomy reconstruction can favor

the transit of the gastric contents towards the jejunum in consequence of the retrogastric site of pancreaticogastric anastomosis^[20,21]. A Billroth II reconstruction can avoid the jejunal angulation produced by a Billroth I procedure in which the anastomosis of the proximal jejunum to the gastric/duodenal stump is performed at first, followed by pancreatico-jejunosomy and hepatico-jejunosomy^[25] (or by hepatico-jejunosomy in a case in which a pancreaticogastrostomy is carried out).

According to the ISGPS clinical criteria^[16], we have recently reported a prevalence of 8.9% (8 cases) of grade A DGE and 1.1% (1 case) of grade C DGE in a series of 89 subtotal stomach-preserving PD followed by Roux-en-Y retrocolic reconstruction with anastomosis of the isolated Roux limb (i.e., first jejunal loop) to the stomach and single Roux limb (i.e., second jejunal loop) to the pancreatic stump and hepatic duct^[30] (Figure 1).

We chose anastomosing the isolated proximal jejunum to the gastric remnant because, after removal of the duodenal source of CCK and secretin, preservation of the first jejunal loop in the reconstruction of the alimentary circuit maintains the physiologic jejunal secretion of secretin and CCK-PZ subsequent to alimentary transit and can compensate (at least in part) for the abolished duodenal hormonal release^[29]. Then, the anastomosis of the isolated first jejunal loop to the gastric remnant, although retrocolic, avoided any angulation and torsion allowed the outflow of the gastric contents by gravity through a “straight route” (Figure 2). It is widely known that postoperative complications are related to the occurrence of DGE. Therefore, controlling the prevalence

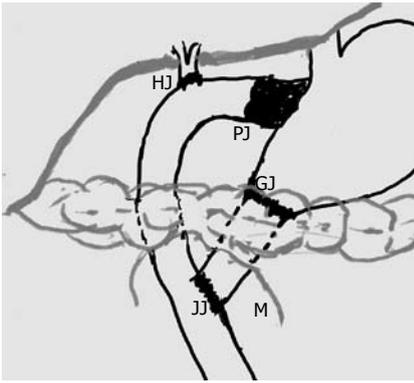


Figure 2 Roux-en-Y retrocolic reconstruction. HJ: Hepatico-jejunal anastomosis; PJ: Pancreatico-jejunal anastomosis; GJ: Gastro-jejunal anastomosis; JJ: Jejunio-jejunal anastomosis; M: Mesocolic window.

of other postoperative complications can contribute to reduce the occurrence of DGE. Postoperative pancreatic fistula occurred in seven patients (7.8%) of our series. Six cases of grade A fistula resolved spontaneously and in only one grade B fistula was percutaneous drainage necessary. Postoperative hemorrhage occurred in two of 89 (2.2%) patients, biliary fistula in eight (8.9%) patients and acute pancreatitis in one (1.1%). One patient with pre-existing stenosis of the hepatic artery developed thrombosis of the hepatic artery.

In conclusion, PrPD may contribute to a decrease in the prevalence of DGE due to pylorospasm, denervation and devascularization of the pylorus ring, which may occur after PpPD. A “straight” route, not necessarily an “antecolic” route, may obviate to the prevalence of DGE due to torsion or angulation in the reconstruction of the alimentary tract.

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Tumor budding as a potential histopathological biomarker in colorectal cancer: Hype or hope?

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Abstract

Colorectal cancer (CRC), the third most commonly diagnosed type of cancer in men and women worldwide is recognized as a complex multi-pathway disease, an observation sustained by the fact that histologically identical tumors may have different outcome, including various response to therapy. Therefore, particularly in early and intermediate stage (stages II and III, respectively) CRC, there is a compelling need for biomarkers helpful of selecting patients with aggressive disease that might benefit from adjuvant and targeted therapy. Histopathological examination shows that likely other solid tumors the development and progression of human CRC is not only determined by genetically abnormal cells, but also by intricate interactions between malignant cells and the surrounding microenvironment. This has led to reconsider the features of tumor microenvironment as potential predictive and prognostic biomarkers. Among the histopathological biomarkers, tumor budding (i.e., the presence of individual cells and small clusters of tumor cells at the tumor invasive front)

has received much recent attention, particularly in the setting of CRC. Although its acceptance as a reportable factor has been held back by a lack of uniformity with respect to qualitative and quantitative aspects, tumor budding is now considered as an independent adverse prognostic factor in CRC that may allow for stratification of patients into risk categories more meaningful than those defined by tumor-node-metastasis staging alone, and also potentially guide treatment decisions, especially in T2-T3 N0 (stage II) CRCs.

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Key words: Colorectal cancer; Tumor budding; Biomarker; Histopathology

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INVITED COMMENTARY ON HOT ARTICLES

We read with great interest the recent article by Lugli *et al*^[1] describing the morphology of "tumor budding" as a promising histopathological prognostic feature in colorectal cancer (CRC) and strongly recommend it to the readers.

Although in certain countries a decline in CRC incidence rate has been registered, attributed to increases in screening adherence rates and linked detection and removal of precancerous polyps^[2], CRC remains one of the most

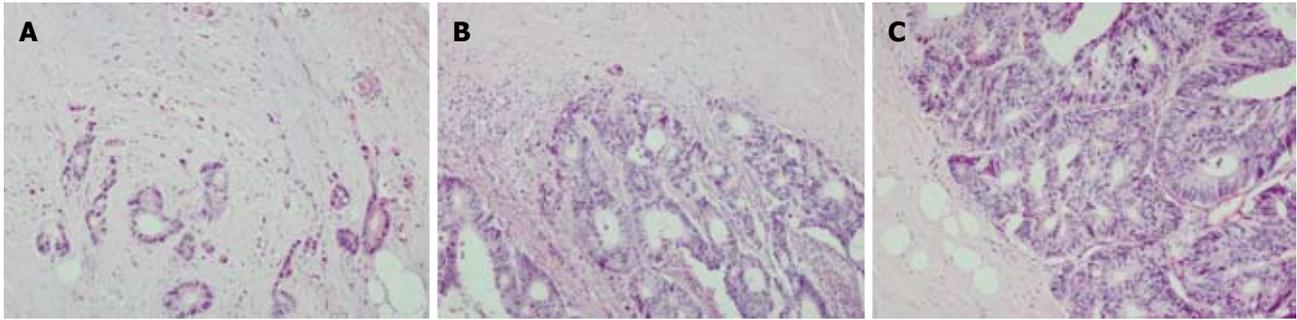


Figure 1 Colorectal cancer at the invasive front shows different growth patterns. Tumor budding denotes the presence of isolated single neoplastic cells or small clusters of cells scattered in the stromal compartment at the invasive tumor margin (A), although intra-tumoral budding is also reported. Tumor margin organized in larger tumor cell clusters (B) and a smooth infiltration tumor margin (C). Hematoxylin and eosin stain, objective magnification 20 \times .

common cancers^[3]. By its frequency, CRC ranks third in men and women worldwide^[3]. Explained as a multi-step dynamical disease in the last two decades, CRC develops slowly over several years and progresses through cytologically distinct benign and malignant states, from single crypt lesions through adenoma, to malignant carcinoma with the potential for local invasion and distant metastasis^[4,5]. According to the model of multi-step carcinogenesis, adenomatous cells accumulate a number of molecular abnormalities to eventually become fully malignant^[6,7]. In spite of unifying theories, genetic and epigenetic events during the carcinogenesis process differ considerably from tumor to tumor. Thus, CRC is not a unique disease; rather it encompasses different molecular and pathological entities with a wide range of clinical behaviors^[8]. At the molecular level, CRC encloses a complex array of gene alterations. Essentially, like individual fingerprints, each tumor arises and behaves in a distinctive fashion that is unlikely to be fully recapitulated by any other tumor. Nevertheless, molecular changes allow for a basic categorization of CRC, which is largely acknowledged, although likely over-simplistic. It has been demonstrated that genetic and epigenetic features, such as microsatellite instability (MSI), chromosomal instability, CpG island methylator phenotype or even global DNA hypomethylation, lead to alterations of gene function on a genome-wide scale. It is known that activation of oncogenes, including *KRAS*, *BRAF*, *TGFBR*, *PIK3CA* and *TP53*, affects complex intracellular signaling pathways^[9,10]. The suppressor pathway is disrupted in CRC with chromosomal instability occurring in the majority of CRCs (nearly, 85%), which have a molecular profile characterized by specific chromosomal amplifications and transformations, aneuploidy, and loss of heterozygosity^[8-10]. Differently, CRCs of the mutator pathway (roughly, 15%) have a defective DNA mismatch repair (MMR) system, which leads to accumulation of unrepaired mutations^[9], and harbor frameshift mutations in coding mononucleotide repeats of cancer-related genes (targets)^[11]. It is now accepted that MSI CRCs have a heterogeneous histological appearance, better prognosis due to a reduced metastatic potential, and a different response to 5-fluoro-uracyl^[12-14].

Histopathology of CRC

Histopathological examination shows that likely other solid tumors, CRCs are infiltrated by various innate and adaptive immune cells^[15-17], and that in the cancer context, epithelial cells coexist with different extracellular matrix components and non-neoplastic cell types, including fibroblasts, myofibroblasts, adipocytes, endothelial cells, pericytes, which collectively form the tumor microenvironment^[18].

It is well known that histopathology reports usually include various features and including tumor grade, histological sub-type, state of resection margins and information on vascular and perineural invasion, but the tumor-border configuration (i.e., growth pattern) and especially tumor budding remain rarely described^[1]. The term “tumor budding” denotes the presence of isolated single neoplastic cells or small clusters of cells (conventionally, up to 5 cells) scattered in the stromal compartment at the tumor invasive margin (Figure 1)^[19-21].

Tumor stage as stated by the American Joint Committee on Cancer/International Union Against Cancer (AJCC/UICC) tumor-node-metastasis (TNM) system is currently considered as the most robust prognostic criterion for CRC patients. The inability of the AJCC/UICC staging system to accurately predict the outcome of individual patients with stage II and stage III CRC might be overcome by adding morphological, molecular or treatment-related features, that could stratify patients more accurately into different risk categories^[22]. Depth local tumor infiltration (pT), loco-regional lymph-node involvement (N status), venous and lymphatic invasion, and tumor grade, are currently recognized as the main histopathological characteristics associated with worse patient outcome.

Tumor budding and CRC

Tumor budding first introduced by Jass *et al.*^[23], as a reliable histopathological hallmark to estimate the aggressiveness of rectal cancer, was initially shown to have a superior prognostic value when compared to other histopathological characteristics, including tumor differentiation and venous invasion. Tumor grading based on the nature of the advancing tumor margins, which in the scoring sys-

tem proposed by Giger *et al.*^[24] divided rectal tumors into expanding type and infiltrating type, obtained wide acceptance among surgical pathologists worldwide. Although subsequent studies revealed a scarce reproducibility of Jass scoring system, several authors highlighted the potential role of tumor budding as a valid prognosticator also in tumors other than CRC, including lung cancer^[25,26], invasive ampullary adenocarcinomas^[27], and oesophageal and gastro-oesophageal junction cancers^[28]. In CRC, tumor budding is considered as a stage II B prognostic factor, and strictly associated with lymph-node metastasis^[1]. It has been shown that the presence of “buds” at the tumor invasive front represents an independent predictor of lymph node metastasis in patients with sub-mucosal invasive or early pT1 CRCs^[29]. It has also been suggested that the frequency of tumor budding increases with more advanced TNM stage^[1].

Tumor budding is virtually absent in MMR-deficient cancers^[30,31]. MSI CRCs have significantly more pronounced tumor infiltrating lymphocytes (i.e., CD3⁺ or CD8⁺ cells), peritumoral lymphocytes inflammation, and bundling edge (i.e., the ability of cells to adhere and to migrate) compared with microsatellite-stable CRCs, all factors contributing to the absence of tumor budding in MSI CRCs.

Tumor budding and the epithelial-mesenchymal transition

A parallel between tumor budding and the epithelial to mesenchymal transition (EMT) has also been recently proposed. This (potentially reversible) process thought to occur physiologically during embryological development (EMT subtype I), has been also associated with wound healing, tissue regeneration, organ fibrosis (EMT subtype II), and tumor invasion (EMT subtype III). Cells in EMT lose their epithelial phenotype (i.e., lack of E-cadherin and cell polarity, expression of transcription factors including the zinc finger proteins SNAIL and SLUG, TWIST, ZEB 1/2 and SMAD) and dynamically acquire a mesenchymal phenotype (i.e., taking on a spindle-like, fusiform morphology, become motile, and start expressing mesenchymal markers including N-cadherin, fibronectin and vimentin)^[32]. While the mechanisms promoting distant metastasis are extremely wide and still under intense investigation, the presence of EMT features in cells of the tumor microenvironment has been associated with an increased metastatic potential^[32,33].

Assessing the tumor budding in colorectal cancer tissues

Rapidly growing insights into the cell biology of CRC and the recent developments of high-throughput technologies, gene sequencing and molecular diagnostics have led to practicable expectations for the identification of molecular biomarkers to be used in optimized and tailored treatment regimens. However, histopathological interpretation of CRC tissues remains the gold standard for cancer diagnosis. Tissue specimens, consisting of different cell types related to each other in complex spatial patterns, are important resources for both primary research

efforts and validation of biological findings that are made in laboratory^[34]. Working with human tissues poses several challenges to investigators, including: (1) tissue sampling (i.e., appropriate processing, histological variability, tissue heterogeneity with different areas of cancer, necrosis, inflammation and natural tissue); (2) selection of the proper preservation technique (i.e., maintenance of tissue morphology and molecular profile); (3) tissue complexity (i.e., requirement of an accurate histopathological interpretation); and (4) not least ethical and legal rules. However, an approach that integrates histopathology and molecular biology within a unique translational system is a mandatory strategy to pursue a better understanding of cancer. Such an effort can be achieved only through a more effective incorporation of pathology into clinical research, and conversely by integrating biological research into the pathological assessment, likely through efficient networks of translational researchers joining their data.

The morphology of the tumor invasive front has come into the focus of scientific studies because it appears to be intimately linked to cancer aggressiveness. Despite the established prognostic relevance of tumor budding in CRC, the reproducibility of actual methods proposed for its assessment, however, remains unstandardized, limiting its application in routine pathology practice^[35]. Diagnostic reproducibility is a prerequisite for the validation of a diagnostic test and is crucial for patient care. Tumor budding promises to be a histopathological prognostic factor in CRC, and although the level of agreement needs to be improved and further investigations are compulsory to confirm any association between the rates of tumor budding detection and clinical outcome, its evaluation can be improved first by an appropriate physician training. In addition, the use of immunohistochemistry (IHC) highlights budding cells by pan-cytokeratin antibodies leading to a significant increase of tumor budding-positive cases. Single tumor cells can be more accurately detected by immunological techniques than standard hematoxylin and eosin staining, even when they appear at the tumor boundary showing glandular disruption. Under these circumstances, dissociated tumor cells should not be interpreted as budding to avoid biasing tumor budding evaluation^[31].

As tumor budding has been shown as an independent prognostic factor in CRC, particularly in node-negative disease, its assessment has the potential to increase prognostic accuracy and influence treatment algorithms. When examined carefully, the majority of CRCs display some degree of budding; hence, attempts have been made at developing scoring systems to identify a prognostically significant degree of budding, commonly termed “high-grade” budding. Definitions of high-grade budding, however, vary substantially among different observers and even among different studies by the same observers^[31].

Final remarks

Consensus criteria for its evaluation must be better estab-

lished, to guide further research in this area and to provide the practicing pathologist with reporting guidelines. With respect to setting these criteria, studies focusing on budding should be designed to define objective cut-off for meaningful tumor budding. In mathematical terms, also tumor budding is a continuous variable; thus, a cut-off threshold should be less arbitrary as possible, and an attempt should be made to identify the budding threshold that results into relevant predictive information. Along this line, pathologist reporting on tumor budding should provide detailed information regarding the qualitative and quantitative criteria used to evaluate budding in order to allow for meaningful comparisons among different studies. Finally, the role of IHC in the evaluation of budding needs to be clarified. Although it might be impractical to perform IHC on all CRCs, there may be certain cases (i.e., in the context of a remarkable inflammatory reaction at the tumor invasive front), where it may reveal buds that are dubious when observed in standard hematoxylin and eosin stained histological sections.

It is indubitable that the substantial impediment to the adoption of tumor budding as a routinely reportable feature is the lack of a well defined, standardized and quantitative assessment. At any event, due to the forceful evidence that tumor budding is one of the most promising prognostic factors actually available, it is incumbent on the scientific community participating to the identification of CRC prognostic factors to move promptly to addressing it and removing the obstacles to its routine reporting and comparison with other predictive factors.

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Anti-tumor immunity, autophagy and chemotherapy

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Abstract

Autophagy or self-digestion of cells is activated upon various stressful stimuli and has been found to be a survival and drug resistance pathway in cancer. However, genetic studies support that autophagy can act as a tumor suppressor. Furthermore, defective autophagy is implicated in tumorigenesis, as well. The precise impact of autophagy on malignant transformation has not yet been clarified, but recent data suggest that this complex process is mainly directed by cell types, phases, genetic background and microenvironment. Relation of autophagy to anticancer immune responses may indicate a novel aspect in cancer chemotherapy.

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Key words: Cancer; Autophagy; Chemotherapy; Antitumor immunity; Cell death

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INVITED COMMENTARY ON HOT ARTICLES

Cancer is one of the major health problems worldwide, therefore constantly more effective therapeutic strategies are expected. Cancers arise from the uncontrolled proliferation and spreading of malignantly transformed cell clones with the obvious ability to evade protective immunity. In view of immune surveillance selective, specific and effective eradication of various cancer cells by a subsequent active host immune response serving as a widespread therapy option has still been remained unsolved.

Current therapies for cancer mainly are based on chemotherapeutic drugs that kill transformed, dividing cells or block cell division, but unfortunately these treatments may also attack normal proliferating cells, including immunocompetent ones. However, targeted immune responses (immunotherapy) to tumors may be specific, thus making the possibility to avoid normal cell injury. According to therapeutic vaccines killed tumor cells or tumor antigens can efficiently induce anticancer immunity.

So far less attention has been paid on the possible sub-cellular and molecular impact of chemotherapy-induced cell death regarding induction of host immune responses.

In a recent experimental study Michaud *et al*^[1] have underscored a new aspect of anticancer chemotherapy, that autophagy may contribute to action of certain drugs eliciting immunogenic tumor cell death. This type of cellular fate is characterized biochemically by pre- and postapoptotic events, like calreticulin exposure and high mobility group B-1 (HMGB-1) secretion, and by ATP release.

Autophagy

Besides the proteasomal degradation pathway autophagy represents an additional evolutionarily highly conserved multi-step process of cellular self-digestion due to sequestration of excessive, damaged, or aged proteins and

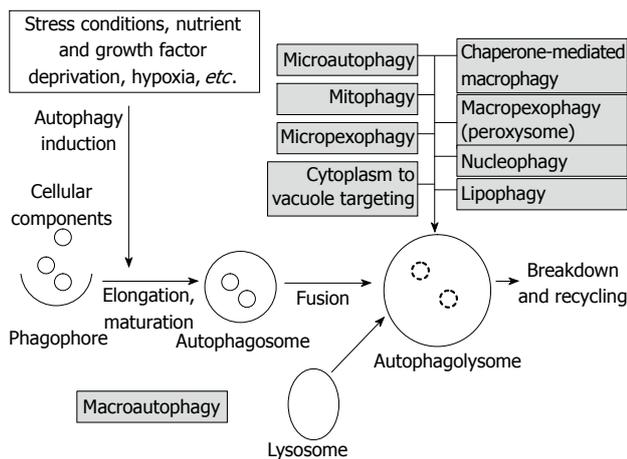


Figure 1 The process of macroautophagy and the types of autophagy (gray boxes).

intracellular organelles in double-membranous vesicles of autophagosomes, terminally self-digested in lysosomes^[2].

Different types of autophagy according to the route of delivery to lysosomes and the main physiological functions have been characterized, like macro- and microautophagy, and chaperon-mediated autophagy^[3]. Upon specific targeted degradation of cytosolic proteins, lipids, or organelles (e.g., ribosomes, nucleosomes, mitochondria), selective forms of autophagy can further be classified as lipophagy, or ribophagy, nucleophagy and mitophagy^[2].

Macroautophagy (hereafter simply termed autophagy) refers to cytoplasmatic bulk, non-selective degradation of subcellular constituents. Within this complex catabolic pathway regulated tightly by a limited number of autophagy genes (atgs) various morphologic stages are distinguishable starting with the formation of phagophore, followed by its elongation and maturation to autophagosome, and finally the fusion with lysosomes^[4]. The process of macroautophagy and the types of autophagy are summarized in Figure 1.

Autophagy is deeply implicated in regulation of numerous physiologic functions including cell development and differentiation, survival and senescence, and it also crucially affects inflammation and innate and adaptive immunity^[5]. On a basal level intact autophagy serves constantly and constitutively as a critical adaptive and surveillance mechanism in maintaining cellular homeostasis^[3]. However, autophagy is inducible, as well in response to different cellular metabolic stress conditions, including nutrient and growth factor deprivation in order to preserve cell viability. Defects in basal autophagy may yield accumulation of cytotoxic materials, damaged DNA, and thus, genomic instability, while alterations of induced autophagy especially lead to reduced cell survival^[4,5].

In general, defective autophagy by compromising cellular fitness has been ultimately related to several disease conditions, such as cancer, certain neurodegenerative, liver, and infectious disorders, aging, and inflammatory conditions, like Crohn's disease^[3,5-7].

Regarding tumorigenesis a dual-faced (Janus) role of

autophagy has been proposed, since on one side it may be critical for cancer cell survival and progression, in particular under stressful situations, however it may elicit tumor death signaling pathways. Direction of autophagy toward cytoprotection or tumor cell suppression, thus the pro-survival or pro-death function is context-dependent, and influenced by many intra- and extracellular factors, such as involved tissues, surrounding microenvironment, genetic background, and stages of tumor development, nevertheless its precise relation to cancer networks has not yet been fully elucidated^[5,6,8].

The involvement of autophagy in cell death, either in apoptosis (programmed, type I death) or in non-apoptotic or necrotic death, and their possible interactions are rather complicated. Autophagy in tumor cells usually displays a critical, programmed pro-survival function by inhibiting apoptosis or suppressing necrotic death, including programmed (or regulated) cell necrosis of caspase-independent necroptosis, and poly-ADP-ribose polymerase-mediated necrosis^[9].

In cases of autophagy deficiency, however, no tumor suppression, but on the contrary, accelerated tumorigenesis can be manifested. In autophagy-incompetent cells upon induced oxidative stress cell-autonomous mechanisms are exhibited in forms of accumulated DNA damage and chromatin instability^[10]. As a non-cell-autonomous mechanism, however, inflammatory events along with defective apoptosis could also contribute independently to cancer progression, partly by favouring cell necrosis^[11]. Similar situation has been found in human inflammatory bowel diseases (IBD) with high risk of malignancy, and in experimental cases of *atg5*^{-/-} or *atg7*^{-/-} mice displaying inflammatory Paneth cell abnormalities resembling human IBD^[7,12].

The *atg6/Beclin-1* gene, a Bcl-2/Bcl-xL interacting element has been found to be monoallelically lost in certain human cancers, and confirmed that it functions as a haploinsufficient tumor suppressor^[13]. However, this suppressive function of Beclin-1 may be tissue-specific, since even its higher expression has been detected in colorectal and gastric carcinomas^[14]. In addition to Beclin-1, alterations of other autophagy-associated genes, e.g., *atg4*, *atg5*, UV-irradiation resistance-associated gene (UVRAG), or Bax-binding protein-1 (Bif-1) have also been detected in various cancers, indicating that tumor suppression is attributed to different autophagy elements. Nonsense mutations of UVRAG, and downregulation of Bif-1 have been documented in colon and gastric carcinomas, and in colon adenocarcinomas, respectively^[15-17].

Hypothetically, increased autophagic flux *via* excessively induced autophagy may promote non-apoptotic (programmed, type II) autophagic cell death, acting like a tumor suppressor^[18]. Autophagy is also known to stimulate oncogene-induced senescence, thus providing another possible barrier against malignant transformation^[19]. Nevertheless, there is no direct evidence regarding the realistic anti-tumor capacity of autophagy.

In human cancers constitutive activation of Ras- and phosphoinositol 3-kinase/Akt-mammalian target of rapa-

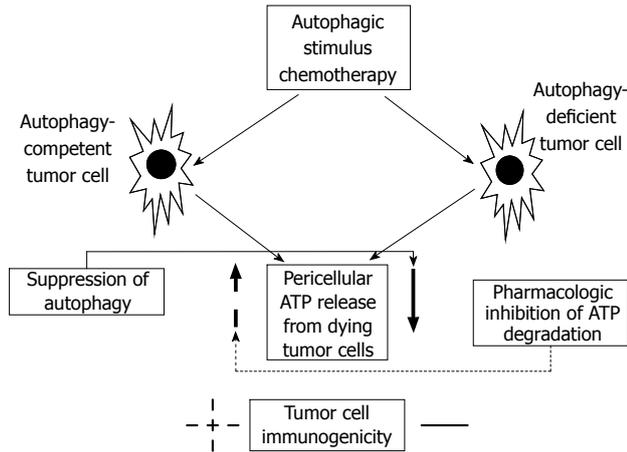


Figure 2 The relation of autophagy and anticancer immunity. Similarly to autophagy-deficient tumor cells inhibition of autophagy results in decreased pericellular ATP secretion, and thus suppressing anticancer immunity. Pharmacologic inhibition of ATP degradation, however, increases ATP level in the microenvironment of tumor cells, and favours tumor cell immunogenicity.

mycin (mTOR) pathway is a common phenomenon, and mTOR complex 1 seems to be the main negative regulator of autophagy^[20,21]. The tumor suppressor p53 gene exerts a typical dual role in autophagy regulation, depending primarily on its subcellular, nuclear or cytoplasmic distribution^[22]. Both stress-responsive cellular degradation pathways of intrinsic and extrinsic apoptosis and of autophagy can fundamentally affect, activate or inhibit each other *via* an extensive molecular crosstalk, and in fact, cell destiny is determined by their actual functional status and interplay^[6,23]. Their crosstalk is regulated primarily by the current status of the Bcl-2/Beclin-1 complex, dissociation of which can be achieved upon activation of mitogen activated phosphokinase-jun kinase or translocation of the damage-associated molecular pattern (DAMP) protein HMGB-1^[23]. Nuclear factor (NF)- κ B plays also a critical role in malignant transformation, and its constitutive, chronic activation has been observed in the majority of different tumor cells. There is also a complex interaction between autophagy and the NF- κ B signaling pathways *via* positive and negative feedback regulatory loops^[24]. The important autophagy selective substrate p62 acts as an adaptor protein to regulate NF- κ B, as well^[25].

Overall, there is no doubt that process of autophagy can be considered as an apparently quite difficult regulatory network, being in close connection with other signal transduction pathways and cellular programs. The complex and rather contradictory function of autophagy in tumorigenesis makes itself a promising but challenging therapeutic target both in cancer treatment and prevention. In autophagy-competent tumor cells autophagy increase can often be induced in response to different chemo- and radiotherapies, representing mainly an adaptive survival mechanism, but provoking simultaneously treatment resistance. Therefore it has been hypothesized that concurrent pharmacologic inhibition of autophagy, as an adjuvant may sensitize tumor cells to a spectrum of anticancer drugs^[22,26,27]. In cases of autophagy-deficient

tumors, however, due to their extreme susceptibility, metabolic stress- and DNA-damage-inducing therapeutic protocols are suggested. However, autophagy induction could also provide an alternative therapeutic option^[22,26,27]. Nevertheless, excessive autophagy can potentially act as an active cell death machinery, mainly along with inherent apoptosis defects, so induction of autophagy by antitumor drugs may also be considered as an efficient cytotoxic manipulation.

Michaud *et al*^[1] in their experiments, using transplantable murine tumors of CT26 colorectal carcinoma and of MCA205 fibrosarcoma treated either with mitoxantrone or oxaliptin have found that autophagy-competent tumor cells release more ATP comparing with autophagy-deficient ones. Furthermore, pharmacologic inhibition of autophagy reduced chemotherapy-induced ATP release, however induction of autophagy did not trigger it. ATP serves as a danger signal, it is a prominent DAMP molecule. In addition, unlike autophagy-deficient tumor cells chemotherapy in autophagy-competent cancer cells elicited a protective immune response, i.e., attraction of dendritic cells, CD4+ and CD8+ lymphocytes, and priming of T cells. Inhibition of autophagy decreased the immunogenic potential of tumor cells. The authors finally conclude, that upon chemotherapy pre-mortem autophagy is required for tumor immunogenicity by releasing ATP from dying apoptotic cells, and consequently, in case of autophagy deficiency the ability of tumor cells to induce an adaptive anticancer immune response is significantly restricted. In that transplantable model dying cancer cells function as a therapeutic vaccine. Nevertheless, in autophagy-deficient tumors of immunocompetent hosts by pharmacologic inhibition of ATP degradation a compensatory increase in pericellular ATP content was achieved, thus successfully restoring the immunogenic capacity, and suggesting a novel adjuvant therapeutic possibility (Figure 2).

Findings of Michaud *et al*^[1] not only highlight on the complexity and many faces of autophagy in tumorigenesis, but emphasize the rationality of analyzing subcellular, molecular consequences of chemotherapy in respect of influencing host immunity, and thus propose a promising therapeutic strategy to compensate autophagy deficiency-related altered tumor immunogenicity.

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Solitary rectal ulcer syndrome in children: A literature review

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tant problem as reflected by persistence of symptoms, especially rectal bleeding. In this review, we discuss current diagnosis and treatment for SRUS.

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Key words: Solitary rectal ulcer syndrome; Rectal bleeding; Children; Diagnosis; Treatment

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Abstract

Solitary rectal ulcer syndrome (SRUS) is a benign and chronic disorder well known in young adults and less in children. It is often related to prolonged excessive straining or abnormal defecation and clinically presents as rectal bleeding, copious mucus discharge, feeling of incomplete defecation, and rarely rectal prolapse. SRUS is diagnosed based on clinical symptoms and endoscopic and histological findings. The current treatments are suboptimal, and despite correct diagnosis, outcomes can be unsatisfactory. Some treatment protocols for SRUS include conservative management such as family reassurance, regulation of toilet habits, avoidance of straining, encouragement of a high-fiber diet, topical treatments with salicylate, sulfasalazine, steroids and sucralfate, and surgery. In children, SRUS is relatively uncommon but troublesome and easily misdiagnosed with other common diseases, however, it is being reported more than in the past. This condition in children is benign; however, morbidity is an impor-

INVITED COMMENTARY ON HOT ARTICLES

Solitary rectal ulcer syndrome (SRUS) is an uncommon chronic and benign rectal disorder often related to abnormal defecation or straining. It was first described by Cruveilhier^[1] in 1829, when he reported four unusual cases of rectal ulcers. The term "solitary ulcers of the rectum" was used by Lloyd-Davis in the late 1930s and in 1969 the disease became widely recognized after a review of 68 cases by Madigan *et al*^[2] and few years later, a more detail pathogenic concept of the disease was reported by Rutter *et al*^[3]. SRUS is an infrequent or unrecognized or misdiagnosed disorder, with an estimated prevalence of 1 in 100 000 persons per year^[4]. Solitary rectal ulcer is a misnomer as ulcers are found in 40% of patients, while 20% of patients have a solitary ulcer, and the rest of the lesions are different in shape and size, including hyperemic mucosa to broad-based polypoid lesions^[5]. The disease process also may involve the sig-

moid colon^[6].

Although it is uncommon, it is well recognized in adult populations^[3]. SRUS seems to be rare in childhood^[7-10] and may masquerade as other more common conditions, causing difficult-to-manage lower gastrointestinal symptoms. Opinion differs regarding the best treatment for this troublesome condition, varying from conservative management and enema preparations to more invasive surgical procedures such as rectopexy^[11].

In this review article, several aspects of this syndrome will be evaluated with an especial focus on the condition in children. Detailed risk factors, causes, and treatment methods will help guide future treatment and prevention strategies in children.

Pathophysiology and clinical presentation

The pathophysiology of SRUS is incompletely understood; however, rectal hypersensitivity leading to the persistent desire to defecate and sensation of incomplete evacuation may have a role in SRUS^[12]. Inappropriate contraction of the puborectalis muscle and rectal mucosal prolapse have been commonly implicated, although trauma and ischemia have been suspected in some children^[7,13]. In children, secondary to chronic mechanical and ischemic trauma, inflammation by hard stools, and intussusception of the rectal mucosa, some histological features of SRUS can be seen such as fibromuscular obliteration of the lamina propria and disorientation of muscle fibers^[14].

In our previous study of 256 children who were evaluated endoscopically for recurrent lower gastrointestinal bleeding, 4.7% had this syndrome^[15]. In adult patients, men and women are affected equally, with a small predominance for women^[16], but 75%-80% of children with SRUS are boys^[15,17]. Suresh *et al*^[18] have evaluated 325 children aged < 18 years during 8 years for various indications such as bleeding, polyps and anal fissure. Twenty-two (6.8%) children were diagnosed with SRUS and ranged in age from 18 months to 18 years (median: 10 years), and 18 (81.8%) of these were \geq 8 years of age. The male to female ratio in this group was 1.4:1. To date, the youngest patient with SRUS was a child of 1.5 years^[18]; Gabra *et al*^[19] also have reported two boys with SRUS who were 2 and 3 years old.

The average time from the onset of symptoms to diagnosis is 5 years, ranging from 3 mo to 30 years in adults, which is longer than in pediatric patients (3.2 years, range: 1.2-5.5 years)^[15,17,20-22]. This syndrome results from obstructed defecation secondary to internal rectal prolapse with a collection of symptoms including rectal bleeding, passage of mucus and straining on defecation, perineal and abdominal pain, tenesmus, feelings of incomplete defecation, constipation, and rectal prolapse^[23-25]. The amount of blood varies from a little fresh blood to severe hemorrhage that requires blood transfusion^[26-28]. Up to 26% of patients can be asymptomatic and may not show the bleeding that is discovered incidentally while investigating other diseases^[5]. The use of

digital manipulation to assist with a bowel movement is variably reported in patients with SRUS^[15,29]. Bright-red blood from the rectum or mucoid rectal discharge, tenesmus, proctalgia, and constipation are the major symptoms. Some children present with apparent diarrhea (because of prolonged visits to the bathroom) and the associated bleeding, abdominal pain, and tenesmus may suggest to clinicians the presence of inflammatory bowel disease^[25].

Diagnosis

SRUS is a relatively uncommon but bothersome and easily misdiagnosed condition of childhood. Clinical suspicion and paraclinical evaluations are needed and diagnosis is via symptomatology in combination with endoscopic and histological findings^[17]. A complete and thorough history is most important in the initial diagnosis of SRUS. It is essential to differentiate SRUS from other devastating, chronic, and potentially lethal disorders such as inflammatory bowel disease, amebiasis, lymphogranuloma venereum, chronic ischemic colitis, endometriosis, colitis cystica profunda, and malignancy. Obstructive symptoms (anismus) in children may be interpreted by parents as constipation. Concomitant haematochezia may be misinterpreted as originating from an anal fissure caused by constipation, or as other causes of rectal bleeding such as a juvenile polyp^[30-32].

Defecography is a useful method for determining the presence of intussusception or internal or external mucosal prolapse and can demonstrate a hidden prolapse, as well as a non-relaxing puborectalis muscle and incomplete or delayed rectal emptying^[33]. Barium enema shows granularity of the mucosa, polypoid lesion, rectal stricture and ulceration, and thickened rectal folds; all of which are nonspecific findings^[33,34]. Temiz *et al*^[35] have recommended that defecography and anorectal manometry should be performed in all children with SRUS to define the primary pathophysiological abnormality and to select the most appropriate treatment protocol.

The endoscopic spectrum of SRUS varies from simple hyperemic mucosa to small or giant ulcers to broad-based polypoid lesions of different sizes. Macroscopically, SRUS typically appears as shallow ulcerating lesions on a hyperemic surrounding mucosa, most often located on the anterior wall of the rectum at 5-10 cm from the anal verge. Ulcers may range from 0.5 to 4 cm in diameter but usually are 1-1.5 cm in diameter^[5,15,30].

Histological examination of biopsy material is necessary to confirm a diagnosis of SRUS. The histological criteria for diagnosis are as follows: fibrous obliteration of the lamina propria, streaming of fibroblasts and muscle fibers between crypts, thickening of muscularis mucosa, branching and distorted glandular crypts and diffuse collagen infiltration of the lamina propria^[15,36-38].

Recent studies have shown the usefulness of anorectal ultrasound in assessing internal anal sphincter thickness, which is shown to be increased in patients with this syndrome^[37,39], and it has been suggested that

sonographic evidence of a thick internal anal sphincter is highly predictive of high-grade rectal prolapse and intussusception in patients with SRUS^[39].

There is a need for a high index of suspicion for the possibility of SRUS in young children with clinical picture of nonspecific proctitis.

Treatment

The most frustrating aspect of SRUS is the difficulty in treatment; experiences have shown that most therapeutic regimens are inadequate. There are few data on treatment and its outcome in children with SRUS. In most reported pediatric case series, active intervention using enemas^[15], laxatives^[40], and surgical approaches have been used more frequently than behavioral modification, mainly biofeedback therapy in adults^[11,41-45].

Some suggestions for the treatment of SRUS include reassurance of the patient and parents that the lesion is benign, encouragement of a high-fiber diet^[46], avoidance of straining, regulation of toilet habits, and attempt to discuss any psychosocial factors^[20,36,37,47,48]. The use of a high-fiber diet, in combination with stool softeners and bulking laxatives, and avoidance of straining have had varying responses^[4,46].

In children, primary medical treatment is proposed for most cases^[49]. Topical application of sucralfate can be effective for treatment of SRUS in some patients^[15,50]. Many medications that are useful in the treatment of patients with inflammatory bowel disease have been tried in those with SRUS, such as sulfasalazine and corticosteroids, with varying responses^[16,51]. In one study, oral salicylate and other topical agents such as mesalamine and steroids were not effective^[52]. Endoscopic application of human fibrin sealant^[53], laser therapy^[54], and biofeedback^[45,47,48] are some of the effective treatment methods for SRUS.

A therapeutic role for botulinum toxin injection into the external anal sphincter for the treatment of SRUS and constipation associated with dyssynergia of defecation dynamics has been reported by Keshtgar *et al*^[55]. The effect of botulinum toxin lasts approximately 3 mo, which may be more beneficial than biofeedback therapy^[55].

Surgical methods for treatment of SRUS are rectopexy^[42,43,56], excision^[4,5,16] and Delorme's procedure^[41,44,57,58].

The choice of treatment protocol depends on acuteness of symptoms and whether there is an underlying rectal prolapse or not^[25,31]. Maintaining compliance in children may prevent progression to the type of long-term morbidity and treatment resistance sometimes seen in adults with this condition^[25]. Recommended treatment in children by Abbas *et al*^[59] is initially conservative, but, if that fails, transrectal resection followed by a high-fiber diet. Conservative management including behavioral modification and reduction of time spent straining at defecation has been reported as a good method^[25,46,60].

Compliance with simple behavioral modification appears to produce a good outcome in childhood SRUS, probably because of the short disease duration compared with adults.

Early recognition and management of these patients may avoid some of the chronic long-term morbidity often associated with this condition; however, late relapse because of noncompliance is a substantial risk and children should be followed up long term.

SRUS is thought to be part of the bigger disease process known as mucosal prolapse syndrome, which incorporates inflammatory cloacogenic polyps, inflammatory cap polyps, and gastric antral vascular ectasia. In fact, all these syndromes have the same histological features^[61]. As a result of the wide endoscopic spectrum of SRUS and the fact that the condition may go unrecognized or, more commonly, misdiagnosed, it is crucial to take biopsy specimens from the involved area to make a positive confirmation of the diagnosis and to exclude other diagnoses including malignancy^[31,62].

The exact etiology is unknown^[30,63,64] but it has also been noted that this syndrome is often associated with trauma resulting in focal ischemia and ulceration, pelvic floor disorders^[64], mucosal prolapse^[14,52,64,65] and/or a larger systemic process^[66]. Also, it has been associated with perineal descent, nonrelaxing puborectalis syndrome, and rectal prolapse^[21,52]. In children, paradoxical contraction of pelvic floor and external anal sphincter muscles contributes to constipation, rectal prolapse, ischemia and finally rectal ulcer.

Diversity of the clinical presentation of SRUS requires a high index of suspicion of both the clinician and the pathologist for the definite diagnosis^[30].

The clinicopathological similarities between SRUS and inflammatory bowel disease and the limited pediatric experience of these conditions may lead to difficulties in differentiating these conditions, and could result in under-reporting of SRUS in this age group.

It can present as more common childhood intestinal conditions such as inflammatory bowel disease or constipation, causing difficult-to-manage lower gastrointestinal symptoms. Also, it may present as polypoid mass lesions^[67]. A biopsy is required for confirmation of diagnosis, because ulceration may not be apparent at the time of endoscopy. SRUS should be considered in children presenting with rectal bleeding, mucorrhea and excessive straining during defecation.

Biofeedback^[44,45,47,48], sucralfate enema^[15,50] and surgery seem to be ideal strategies because they aim to correct the underlying processes^[57,58]. Behavioral modification or biofeedback therapy improves both rectal blood flow and symptoms and includes bowel habit training, avoiding excessive straining, and normalization of pelvic floor coordination^[46-48,68,69]. Surgery is indicated in children with persistent bleeding per rectum not amenable to medical therapy and includes rectopexy, excision of ulcer, and rarely colostomy^[42-44,56]. In children, a multitude of procedures have been advocated for rectopexy and a cure rate of at least 90% has been reported for posterosagittal rectopexy^[45]. Also, El-Hemaly *et al*^[70] have reported that the results of surgery and biofeedback are satisfactory in comparison to conservative treatment.

Most patients with SRUS in childhood have a satisfactory outcome using a simple behavioral modification approach. Ongoing follow-up to reinforce behavioral modification is important and may avoid long-term, treatment-resistant disease into adulthood. Despite the previous reports about SRUS in children that indicate low prevalence of the disease in childhood, recently we have been faced with higher prevalence rates in this age group. It seems that detailed and effective diagnosis methods such as endoscopy and histological examinations, as well as more attention by clinicians to this syndrome in children, have improved the diagnosis rate of the disease. Despite this being a benign condition in children, morbidity remains a problem as reflected by persistence of symptoms especially bleeding per rectum. Therefore, we are faced with an important condition that needs more attention and attempts for prevention and treatment.

More studies are needed to evaluate all of the aspects of the syndrome in children and to recommend the best treatment protocol. Every child with SRUS must be assessed individually using all modalities of investigation to define clearly the underlying pathophysiology, and to select the appropriate treatment strategies.

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Genetic and epigenetic variants influencing the development of nonalcoholic fatty liver disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is common worldwide. The importance of genetic and epigenetic changes in etiology and pathogenesis of NAFLD has been increasingly recognized. However, the exact mechanism is largely unknown. A large number of single nucleotide polymorphisms (SNPs) related to NAFLD has been documented by candidate gene studies (CGSs). Among these genes, peroxisome proliferator-activated receptor- γ , adiponectin, leptin and tumor necrosis factor- α were frequently reported. Since the introduction of genome-wide association studies (GWASs), there have been significant advances in our understanding of genomic variations of NAFLD. Patatin-like phospholipase domain containing family member A3 (PNPLA3, SNP rs738409, encoding I148M), also termed adiponutrin, has caught most attention. The evidence that PNPLA3 is associated with increased hepatic fat levels and hepatic inflammation has been validated by a series of studies. Epigenetic modification refers to phenotypic changes caused by an adaptive

mechanism unrelated to alteration of primary DNA sequences. Epigenetic regulation mainly includes microRNAs (miRs), DNA methylation, histone modifications and ubiquitination, among which miRs are studied most extensively. miRs are small natural single stranded RNA molecules regulating mRNA degradation or translation inhibition, subsequently altering protein expression of target genes. The miR-122, a highly abundant miR accounting for nearly 70% of all miRs in the liver, is significantly under-expressed in NAFLD subjects. Inhibition of miR-122 with an antisense oligonucleotide results in decreased mRNA expression of lipogenic genes and improvement of liver steatosis. The investigation into epigenetic involvement in NAFLD pathogenesis is just at the beginning and needs to be refined. This review summarizes the roles of genetics and epigenetics in the development of NAFLD. The progress made in this field may provide novel diagnostic biomarkers and therapeutic targets for NAFLD management.

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Key words: Nonalcoholic fatty liver disease; Epigenetic; MicroRNA; Methylation

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is one of the most common forms of chronic liver diseases and a cause of elevated serum aminotransferases worldwide. The prevalence of NAFLD in the general population of Western countries ranges from 20% to 30%^[1-3]. Due to the alterations of diet structure and life style, the prevalence of NAFLD in developing countries has been increasing rapidly^[4]. Recent studies, including one from our group indicate that the prevalence of NAFLD in Chinese population is about 15%^[5-7]. The term NAFLD encompasses a morphological spectrum of diseases, ranging from simple fatty liver (SFL) to nonalcoholic steatohepatitis (NASH) and hepatic cirrhosis, which may progress to hepatocellular carcinoma (HCC). SFL generally has a benign prognosis. Only a minority of them develop NASH, which is characterized by inflammation, fibrosis and liver cell injury^[8,9].

NAFLD has been shown to be associated with metabolic syndrome (MetS), which comprises obesity, type 2 diabetes, dyslipidemia and high blood pressure with insulin resistance being the central mechanism. NAFLD is presently considered the hepatic manifestation of MetS^[5,6,8,10].

It is generally believed that environmental and genetic factors interact to produce NAFLD phenotype and determine its progression. However, the detailed pathogenesis that determines which individual develops NAFLD remains unclear. Recently, the emerging field of epigenetics shed lights on the pathogenesis of chronic liver disease including NAFLD^[11,12]. Elucidation of genetic and epigenetic factors that predispose an individual to NAFLD may lead to development of noninvasive biomarkers for early diagnosis of NAFLD and may allow early preventive and therapeutic strategies for the people at the high risk. This review summarizes recent contributions to the field of the genetic and epigenetic variations that influence the development of NAFLD.

GENETIC VARIATIONS

Candidate gene studies

The genetic variations may result in conformational changes in the protein structures and functions of the genes. NAFLD is an exceedingly complex genetic disorder. Before 2008, the candidate genes based on the prior knowledge of MetS and NAFLD pathophysiology were selected for investigation^[11,13]. In comparison with NAFLD, the relationships between the genotypes and phenotypes of MetS have been examined more extensively. A large number of single nucleotide polymorphisms (SNPs) at the genes encoding proteins involved in insulin resistance has been revealed to be associated with the development of MetS^[14,15]. As there is substantial overlap in the pathogenesis of NAFLD and MetS, theoretically, many variations in candidate genes related to MetS may contribute to the pathogenesis of NAFLD: first, genes related to insulin resistance, such as adiponectin, resistin, insulin receptor, and peroxisome proliferator-activated receptors- γ

(PPAR- γ); second, genes influencing hepatic free fatty acid metabolism, such as hepatic lipase, leptin (or leptin receptor), adiponectin, microsomal triglyceride transfer protein, phosphatidylethanolamine N-methyltransferase (PEMT), PPAR- γ , cytochrome P 450, 2E1 and 4A; third, cytokine-related genes, such as tumor necrosis factor- α (TNF- α) and interleukin-10; fourth, genes affecting liver fibrogenic pathways, such as leptin, adiponectin, transforming growth factor beta1, connective tissue growth factor and angiotensinogen; and finally, genes encoding endotoxin receptors and oxidative stress responses, such as CD14, superoxide dismutase-2 and toll-like receptor-4. Among these genes, PPAR- γ , adiponectin, leptin and TNF- α were frequently reported in the field of MetS as well as NAFLD^[11,13,16]. It is noted that one gene may have a number of SNPs at several nucleotide loci. For example, the SNPs at the *PPAR- γ* gene involved in MetS may occur at the loci of C-681G, C-689T, Pro12Ala, G67222A, A69208G, G81556T, T95872C, T115432G, C127599T and C161T, but only a few of them have been investigated extensively^[17,18].

There is evidence supporting the theory that these genetic factors account for considerable variability in susceptibility to NAFLD. The SNPs may increase or decrease the function of the target genes and their encoding proteins. We have previously demonstrated that many candidate genes' SNPs mentioned above are associated with susceptibility to NAFLD. Some showed positive relationships (increased risk), i.e., TNF- α -238, adiponectin-45, leptin-2548, PPAR- γ -161 and PEMT-175. Other SNPs demonstrated a negative association (decreased risk), i.e., adiponectin-276 and hepatic lipase-514. Two were not relevant, i.e., TNF- α -380 and PPAR- γ coactivator-1a-482^[19]. Gene variations might affect the pathogenesis of NAFLD *via* blood cytokines (such as leptin and adiponectin) and insulin resistance pathways^[19,20]. Although many pathobiological candidacies of SNPs were reported, most studies in literature have not been well validated by larger replication cohorts. The findings in candidate gene studies might be influenced by specific ethnic groups or environmental conditions.

Genome-wide association studies

Since the introduction of genome-wide association studies (GWASs) to investigate genomic variations, there have been significant advances in our understanding of human genome and its clinical sequelae over a range of diseases. More than 3.1 million SNPs have been identified so far. The International HapMap Project (<http://hapmap.ncbi.nlm.nih.gov>) has characterized patterns of SNPs across individuals from diverse ethnic backgrounds^[16,21,22]. Although a number of GWASs has been published in the field of MetS (type 2 diabetes and insulin resistance)^[23], and other liver diseases (HCC, hepatitis B, hepatitis C, drug-induced liver injury and primary biliary cirrhosis)^[16,24,25], only a few studies were carried out on NAFLD.

In 2008, the first GWAS on NAFLD was reported by Romeo *et al*^[26]. In this population-based study, noninvasive

proton magnetic resonance spectroscopy (1H-MRS) was applied to assess hepatic steatosis. Totally, 2111 individuals comprising a mixed population of Hispanic, African American, and European American were enrolled. Non-synonymous sequence variations of 9229 SNPs were identified in NAFLD group compared with normal controls. An allele of patatin like phospholipase domain containing family member A3 (PNPLA3, SNP rs738409, encoding I148M), also termed adiponutrin, on chromosome 22 was shown to be strongly associated with increased hepatic fat levels and hepatic inflammation. This allele was most common in Hispanics, the group most susceptible to NAFLD, with hepatic fat content being more than twofold higher in G homozygous subjects than in non-carriers. G allele frequency was lower in people of European descents and lowest in African Americans, the group found to have the lowest level of hepatic triglyceride accumulation. These findings were validated by another GWAS. Totally 1117 individuals with histologically confirmed NAFLD were genotyped for six SNPs relevant to hepatic fat levels and liver enzymes. PNPLA3 was significantly associated with steatosis, portal inflammation, lobular inflammation, Mallory-Denk bodies, NAFLD activity score and fibrosis^[27]. Subsequently, the extension of the hepatic phenotype associated with the PNPLA3 genotype was independently replicated in both adult and pediatric subjects with simple steatosis, NASH and NASH-related fibrosis using different laboratory techniques^[28-33]. There was evidence that carriers of PNPLA3 exhibited more severe steatohepatitis and higher levels of fibrosis. PNPLA3 was consistent with the concept of NASH rather than the broader features of the MetS, such as body mass index, dyslipidemia, and type 2 diabetes mellitus. The influence of PNPLA3 on hepatic steatosis was not through insulin resistance pathway as assessed by hyperinsulinaemic, euglycaemic clamp and oral glucose tolerance testing^[27-29,32,34,35]. Recently a meta-analysis enrolling 16 studies (2937 subjects) was performed to evaluate the association of PNPLA3 with NAFLD. The results showed that PNPLA3 exerted a strong influence not only on liver fat accumulation (the GG homozygous subjects had a 73% higher lipid fat content compared with CC ones), but also on higher susceptibility to liver disorders (GG homozygous subjects had 3.24-fold higher risk of higher necro-inflammatory scores and 3.2-fold higher risk of developing fibrosis compared with CC homozygous ones). The PNPLA3 GG genotype vs the CC genotype was associated with a 28% increase in alanine transaminase (ALT) level. NASH was more frequently observed in GG than in CC homozygous subjects (odds ratio 3.488, 95%CI: 1.859-6.545). Nevertheless, carrying GG alleles did not seem to increase the risk of severe histological features^[36]. In a clinical study recruiting 302 subjects with 1H-MRS-confirmed NAFLD whose genotyping was determined with TaqMan polymerase chain reaction (PCR), a SNP (rs767870) at adiponectin receptors 2 (*ADIPOR2*), but not at *ADIPOR1* and *PPAR* gene, was found to link to a higher liver fat content. In this study, PNPLA3 was not tested^[37].

Although most studies supported the association be-

tween PNPLA3 and NAFLD, a few reports failed to validate this finding. In a GWAS enrolling 236 women with biopsy-confirmed NAFLD, no association for any feature of NAFLD with PNPLA3 was found. Another SNP (rs2645424) on chromosome 8 in the farnesyl diphosphate farnesyl transferase 1 gene, generating an enzyme with a role in cholesterol biosynthesis, was identified to relate to the severity of NAFLD histology including NAFLD activity score, liver fibrosis, lobular inflammation as well as increased ALT^[38].

The results from GWASs shed light on the understanding of the genetics in NAFLD, as the loci identified are frequently novel and have not previously been implicated. However, such findings require further detailed studies both to determine the activity and to validate the causality, as neither biological functions nor pathogenic mechanisms of these genetic variations are known.

EPIGENETIC MODIFICATIONS

During the past decade, the role of epigenetic mechanisms in the pathogenesis of disease has been increasingly recognized. Epigenetic modification, mainly including microRNAs (miRNAs, miRs), DNA methylation, histone modification and ubiquitination, refers to phenotypic changes caused by the mechanism that is unrelated to changes in the underlying DNA sequence. As an adaptive mechanism to alteration of genetic and environmental signal patterns and epigenetic regulation, which allows fine-tuning gene expression, is essential for the proper maintenance of cellular homeostasis. Disruption of the balance will lead to the development of a wide range of disorders. So far, epigenetic research has mainly focused on cancer, cardiovascular disease, mental illness and autoimmune disease. The roles of epigenetics in the pathogenesis of NAFLD are largely unknown^[39]. Among epigenetic modifications, miRs are studied most extensively in NAFLD. miRs are small naturally occurring single stranded RNA molecules regulating mRNA degradation or translation inhibition, subsequently altering protein expression of target genes. One miR can target multiple genes (multiplicity) and multiple miRs may target a single gene (cooperativity). Since the first discovery in 1993, many miRs in various organisms have been determined. To date, more than 1420 miRs have been identified in humans (miRBase v17). (<http://www.mirbase.org/>)^[40,41]. The expression of miRs is both organ-specific and dependent on the stage of development. miRs influence at least one-third of all human transcripts and are known regulators of important cellular processes, e.g., cell metabolism, cell proliferation, apoptosis, immune function, tissue development and differentiation^[42,43]. It has recently been shown that some 100 miRs are differentially expressed in human NASH. These miRs have diverse functions involved in the pathogenesis of NAFLD, including metabolisms of lipid and glucose, regulations of the unfolded protein response, endoplasmic reticulum stress, oxidative stress, cellular differentiation, inflammation, apoptosis and so on^[44,45]. In a

clinical study, the miR profiles of 15 patients with biopsy-proven NASH and 15 controls with normal liver histology were investigated. Out of a total of 474 tested miRs, 46 were differentially expressed in NASH with 23 being up-regulated (in particular, miR-34a and miR-146b), and 23 being down-regulated (in particular, miR-122). These differentially expressed miRs were further validated by quantitative real-time PCR^[45].

The miR-122, a highly abundant miR in the liver, has caught most attention in liver diseases. Accounting for nearly 70% of all miRs in the liver, miR-122 is significantly under-expressed (63%) in NASH subjects compared to controls^[45,46]. In addition to its role in lipid and cholesterol metabolism, miR-122 has been shown to promote adipocyte differentiation^[42]. Subsequently, the roles of miR-122 in the pathogenesis of NAFLD were confirmed by a number of studies. Inhibition of miR-122 in a diet-induced obesity mouse model with an antisense oligonucleotide treatment resulted in decreased mRNA expression of acetyl-coenzyme-A carboxylase-2, fatty acid synthetase, sterol regulatory element binding proteins 1-c, 2, stearoyl-CoA desaturase and 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG CoA) reductase, all of which were key lipogenic factors in human NASH. The histology also showed substantial improvement in liver steatosis^[47]. The results were validated by another study in mice, in which the plasma cholesterol level, hepatic fatty-acid and cholesterol synthesis rate as well as HMG-CoA reductase level were significantly decreased after silencing miR-122^[42]. All these findings strongly suggested the significance of miR-122 in the regulation of lipid metabolism and the contribution to the development of NAFLD. A further study suggested that miR-122 was closely linked to the output system of the circadian clock by regulating circadianly expressed genes^[48]. Besides miR-122, some miRs have been demonstrated to be involved in NAFLD development. miR-34a and miR-146b were shown to be significantly over-expressed (99% and 80%, respectively) in human NASH^[45]. The expression of miR-335 in the liver and white adipose tissue was up-regulated in mice. The increased miR-335 expression was associated with increased body, liver and white adipose tissue weight, as well as elevated hepatic triglyceride and cholesterol levels. Furthermore, hepatic miR-335 level was closely correlated with the expression of adipocyte differentiation markers, i.e., PPAR- α and FAS in adipocyte^[49]. The presence of miR-181d significantly decreased lipid droplets in the liver (60%), and subsequently reduced cellular triglyceride and cholesterol^[50]. miR-10b regulated steatosis level through PPAR- α pathway in a steatotic hepatocyte (L02 cell line) model. The post-transcriptional regulation of PPAR- α by miR-10b was maintained by a single binding site^[51].

Aberrant methylation patterns of genomic DNA have been studied in many diseases. Hypermethylation of CpG islands is generally associated with gene silencing, and hypomethylation of global genomic DNA affects genomic

stability. Hypermethylation of multiple genes in CpG islands has been demonstrated in human HCC, in which CpG island methylator phenotype was involved in the promoter hypermethylation of multiple genes^[52]. However, the relation of DNA methylation to NAFLD development has not been well documented. A recent study enrolling 63 NAFLD patients confirmed by liver biopsies and 11 controls showed a tight interaction between the presence of NAFLD and hepatic DNA methylation of CpG in PPAR- γ coactivator 1 α (PPARGC1A) and mitochondrial transcription factor A (TFAM) promoters. The proportion of DNA methylation in PPARGC1A and TFAM was significantly higher in the NAFLD livers than in the controls. However, the histological severity and activity scores of NAFLD were not correlated to methylation level and methylated DNA/unmethylated DNA ratio either in PPARGC1A or TFAM promoter^[53]. The development of hepatic steatosis in a mouse model was accompanied by prominent epigenetic abnormalities, which comprised pronounced loss of genomic and repetitive sequences cytosine methylation, increased level of repeat-associated transcripts, aberrant histone modifications and alterations in expression of the maintenance DNA methyltransferase 1 (DNMT1) and *de novo* DNMT3A proteins in the livers^[54].

Ubiquitination and sumoylation (sumo: abbreviation of small ubiquitin-like protein) are recently demonstrated to be novel forms of post-translational modifications (PTMs). PTMs of transcription factors through the course of protein processing play important roles in controlling many biological events^[55]. The research of ubiquitination related to NAFLD is just at the beginning. In a study investigating the hepatic gene networks in morbidly obese patients with NAFLD, hepatic fibrosis signaling was found to be the most significant pathway in the up-regulated NAFLD gene cluster, whereas the endoplasmic reticulum stress and protein ubiquitination pathways to be the most significant pathways in the down-regulated NAFLD gene cluster^[56]. Besides ubiquitination, transcription factors can undergo several types of PTMs, including acetylation, phosphorylation, and glycosylation. Little is known about their role in NAFLD so far^[55].

In conclusion, environmental and genetic factors interact to produce NAFLD phenotype and to determine its progression. This review summarizes the current knowledge of genetic and epigenetic determinations on NAFLD. Genetic variations (e.g., SNPs) account for only a small fraction of environmental and heritable disease risks, whereas epigenetic modifications (e.g., miRs, DNA methylation histone modifications and ubiquitination) affect a bigger proportion of disease phenotypes. The investigation into the potential roles of epigenetics in NAFLD is just at the beginning and needs to be refined. The accumulation of genetic and epigenetic knowledge related to NAFLD has provided novel insight into disease pathogenesis, and may help to develop new diagnostic biomarkers and therapeutic targets for NAFLD management.

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Way back for fructose and liver metabolism: Bench side to molecular insights

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Abstract

The World Health Organization recommends that the daily intake of added sugars should make up no more than 10% of total energy. The consumption of sugar-sweetened beverages is the main source of added sugars. Fructose, together with glucose, as a component of high fructose corn syrups or as a component of the sucrose molecule, is one of the main sweeteners present in this kind of beverages. Data from prospective and intervention studies clearly point to high fructose consumption, mainly in the form of sweetened beverages, as a risk factor for several metabolic diseases in humans. The incidence of hypertension, nonalcoholic fatty liver disease (NAFLD), dyslipidemia (mainly hypertriglyceridemia), insulin resistance, type 2 diabetes mellitus, obesity, and the cluster of many of these pathologies in the form of metabolic syndrome is higher in human population segments that show high intake of fructose. Adolescent and young adults from low-income families are especially at risk. We recently re-

viewed evidence from experimental animals and human data that confirms the deleterious effect of fructose on lipid and glucose metabolism. In this present review we update the information generated in the past 2 years about high consumption of fructose-enriched beverages and the occurrence of metabolic disturbances, especially NAFLD, type 2 diabetes mellitus, and metabolic syndrome. We have explored recent data from observational and experimental human studies, as well as experimental data from animal and cell models. Finally, using information generated in our laboratory and others, we provide a view of the molecular mechanisms that may be specifically involved in the development of liver lipid and glucose metabolic alterations after fructose consumption in liquid form.

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Key words: Obesity; Metabolic syndrome; Hypertension; Dyslipidemia; Nonalcoholic fatty liver disease; Clinical studies; Experimental studies; Sweetened beverages

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INTRODUCTION

At the end of 2011, the United Nations declared that, for the first time in the history of humanity, non-communicable diseases had outpaced infectious diseases as the main threat to human health globally. Among them, cardiovas-

cular diseases associated with metabolic disorders, such as obesity, metabolic syndrome, and type 2 diabetes mellitus, are of paramount importance. Changes in human dietary habits in recent decades have led to the consumption of hypercaloric diets that are rich in saturated fats and simple sugars (sucrose, glucose and fructose). This, combined with decreased physical activity, is one of the key factors contributing to the ever-increasing prevalence of metabolic disorders. This situation recently prompted Lustig *et al*^[1] to request the legal regulation of foodstuffs containing added sugars in a way similar to the control of tobacco and alcohol.

The World Health Organization recommends that the daily intake of added sugars should make up no more than 10% of total energy^[2]. The consumption of sugar-sweetened beverages is the main source of added sugars^[3]. Fructose, together with glucose, as a component of high fructose corn syrups (HFCSs) or as a component of the sucrose molecule, is mainly responsible for the metabolic disturbances associated with excessive consumption of added sugars. We recently reviewed evidence from experimental animals and human data that confirms the deleterious effect of fructose on lipid and glucose metabolism^[4]. Given the relevance of this issue to public health policies, in this review we update information on the effects of fructose on human health. We focus also on new experimental data from our laboratory and others on molecular mechanisms involved in the disturbance of liver metabolism by fructose.

FRUCTOSE: THE BENCH SIDE

Data from prospective and intervention studies clearly point to high fructose consumption, mainly in the form of sweetened beverages, as a risk factor for several metabolic diseases in humans. The incidence of hypertension, nonalcoholic fatty liver disease (NAFLD), dyslipidemia (mainly hypertriglyceridemia), insulin resistance, type 2 diabetes mellitus, obesity, and the cluster of many of these pathologies in the form of metabolic syndrome is higher in human population segments that show high intake of fructose. Adolescent and young adults from low-income families are especially at risk. We and others have recently reviewed the evidence of this relationship^[4-8]. In the present review, we provide an overview of recent data, from 2011 onwards that has not been discussed previously (Table 1). For readers interested in recent reviews on this subject, particularly regarding fructose consumption, uric acid metabolism and hypertension, we refer to two excellent reviews published in 2011^[9,10].

One of the ongoing controversies about fructose consumption in humans is related to the difficulty in identifying effects that are not strictly related to the simple consumption of an excess of daily calories. In a short (2 wk) dietary intervention study in NAFLD subjects, Browning *et al*^[11] showed that carbohydrate restriction (< 20 g/d) was significantly more effective in reducing hepatic triglyceride content than the restriction of calories

to 1200-1500 kcal/d (55% *vs* 28%, respectively), despite the fact that both interventions similarly reduced body weight (by about 4.3%). In a randomized intervention study comparing the consumption of sucrose-sweetened beverages (1 L/d for 6 mo) with other isocaloric beverages in obese subjects, Maersk *et al*^[12] demonstrated that sucrose significantly increased triglyceride deposition, not only in liver, but also in skeletal muscle and visceral adipose tissue.

In another intervention study in healthy people who consumed a balanced diet supplemented with 150 g/d fructose or glucose, Silbernagel *et al*^[13] showed that endogenous cholesterol synthesis was associated with visceral and liver fat content. However, in this study the strongest association was observed in glucose-consuming individuals. Nevertheless, in a well-conducted interventional study by Stanhope *et al*^[14], subjects who consumed fructose (at 25% of energy requirements), either as such or as HFCS, but not glucose, showed an increased fasting concentration of low density lipoprotein (LDL) cholesterol. Fructose consumption also increased the 24-h triglyceride area under the curve and the fasting apolipoprotein (apo)B concentration.

In a prospective cohort study that analyzed 40 389 healthy men over 20 years of follow up, de Koning *et al*^[15] clearly found an association between sugar-sweetened beverage consumption and an elevated risk of type 2 diabetes mellitus. Although it was suggested that fructose was mainly responsible for this association, Silbernagel *et al*^[16] did not find any differences between fructose and glucose in the reduction of insulin sensitivity when these sugars were administered to 20 healthy subjects in a small intervention study. However, plasma triacylglycerol concentrations only increased significantly in the fructose group.

Fructose-induced obesity is closely related to type 2 diabetes mellitus. In a well-conducted intervention study by Cox *et al*^[17] in overweight/obese male and female subjects, consumption of fructose (at 25% of energy requirements for 10 wk), but not glucose, clearly led to significant decreases in net postprandial fat oxidation and resting energy expenditure, thus contributing to the build-up of excess energy substrates. Furthermore, in one of the population segments at high risk of fructose-related obesity, Maier *et al*^[18] demonstrated that a significant reduction in fructose and/or general sugar intake over a short period of time (3 mo) in overweight and obese children may reduce the body mass index. Mainly through increases in visceral fat, fructose-induced obesity is positively associated in adolescents with cardio-metabolic risk markers, such as systolic blood pressure, fasting glucose, homeostasis model assessment-estimated insulin resistance index, and C-reactive protein^[19].

Cardiovascular accidents originate as thrombi deposits on atheromatous plaques, which obstruct blood circulation^[20]. Atherosclerosis is promoted by dyslipidemia, hypertension, and chronic low-grade inflammation. Besides increasing plasma triglycerides and LDL cholesterol^[14],

Table 1 Overview of fructose-related human studies

Authors	Subjects	Study characteristics	Sugar	Main results
Browning <i>et al</i> ^[11]	18 NAFLD (5 men, 13 women), BMI: 35 ± 7 kg/m ²	Intervention study 2 wk dietary carbohydrate and calorie restriction		Reductions in body weight (-4.6 ± 1.5 kg <i>vs</i> -4.0 ± 1.5 kg) and hepatic triglycerides (-55% ± 14% <i>vs</i> -28% ± 23%) were significantly greater with dietary carbohydrate restriction than with calorie restriction
Maersk <i>et al</i> ^[12]	60 overweight/obese nondiabetic subjects	Randomized intervention study Ingestion of 4 different drinks (1 L/d, SSB, isocaloric semiskim milk, aspartame-sweetened and water) for 6 mo	S	Daily intake of SSB with sucrose increased ectopic fat accumulation (liver, skeletal muscle) and lipids (blood cholesterol and triglycerides) compared with the other beverages
Silbernagel <i>et al</i> ^[13]	Healthy male (12) and female (8) adults	Dietary intervention study 150 g/d for 4 wk	F and G	Visceral and liver fat content associated to cholesterol synthesis Cholesterol synthesis appeared to be dependent on fructose/glucose intake
Stanhope <i>et al</i> ^[14]	48 adults, BMI 18-35 kg/m ²	Dietary intervention study Consumption of simple sugars at 25% of energy requirements for 2 wk	F and G	F consumption increased cardiovascular risk factors (AUC-Tg, fasting LDL and apo B) more than G
de Koning <i>et al</i> ^[15]	40 389 healthy men	Prospective cohort study 20 yr of follow-up of SSB and artificially sweetened beverages consumption	F, G and S SSB	After adjustment for several confounders, the hazard ratio for the association of SSB with incident type 2 diabetes was 1.24 for the comparison of the top with the bottom quartile of SSB intake
Silbernagel <i>et al</i> ^[16]	Healthy male (12) and female (8) adults	Dietary intervention study 150 g/d for 4 wk	F and G	Insulin sensitivity decreased in both intervention groups, while plasma triglycerides were increased in the F group
Cox <i>et al</i> ^[17]	Overweight/obese male (16) and female (15) adults	Intervention study 10 wk supplementation with SSB at 25% of energy requirements	F and G SSB	F-consuming subjects had a significant reduction in net postprandial fat oxidation and resting energy expenditure
Maier <i>et al</i> ^[18]	15 overweight/obese children (5-8 yr)	Dietary intervention study parental training to reduce dietary sugar content (-50% from baseline, 12 wk) and 12 wk of follow-up	F, G and S	Reductions in sugar intake were related to significant reductions in BMI and BMI standard deviation scores
Pollock <i>et al</i> ^[19]	559 adolescents (14-18 yr)	Association study of F intake and cardiometabolic risk factors	F	After adjustment, higher F consumption directly associated to BP, fasting glucose, HOMA-IR and C-reactive protein, and inversely to HDL-cholesterol and adiponectin. The introduction of visceral fat as a covariate attenuated these trends
Cox <i>et al</i> ^[21]	Overweight/obese male (16) and female (15) adults	Intervention study 10 wk supplementation with SSB at 25% of energy requirements	F and G SSB	Fasting concentrations of MCP-1, PAI-1 and E-selectin as well as postprandial concentrations of PAI-1 increased in subjects consuming F but not in those consuming G
Brown <i>et al</i> ^[22]	2696 people	Cross-sectional association study	F, G and S SSB	Direct and independent associations of SSB intake and BP Greater sugar-BP differences for persons with higher sodium excretion
Friberg <i>et al</i> ^[25]	61 226 women	Population-based cohort study 18.4 yr of follow-up of total sucrose, high-sugar-foods	F, G and S	Total sucrose intake and consumption of sweet buns and cookies was associated with increased risk of endometrial cancer
Ye <i>et al</i> ^[26]	737 non diabetic adults	Association study of sugar intake and cognitive function	F, G and S	Greater intakes of total sugars, added sugars and SSB beverages, but not of sugar sweetened solid foods, were significantly associated with lower MMSE scores, after adjusting for covariates

F: Fructose; G: Glucose; S: Sucrose; SSB: Sugar-sweetened beverages; BP: Blood pressure; NAFLD: Nonalcoholic fatty liver disease; BMI: Body mass index; MCP-1: Monocyte chemoattractant protein-1; PAI-1: Plasminogen activator inhibitor-1; HOMA-IR: Homeostasis model assessment-estimated insulin resistance; HDL: High-density lipoprotein; AUC-Tg: 24 h area under the curve for plasma triglycerides; LDL: Low-density lipoprotein; MMSE: Mini-mental state examination.

fructose seems to promote a proinflammatory milieu that favors atherosclerosis development. In an intervention study in overweight/obese subjects, Cox *et al*^[21] demonstrated that fructose supplementation in liquid form (at 25% of energy requirements for 10 wk), but not glucose, clearly increases proinflammatory and prothrombotic mediators, such as monocyte chemoattractant protein-1, plasminogen activator inhibitor-1, and E-selectin. Fur-

thermore, in a cross-sectional study including 2696 participants in the International Study of Macro/Micronutrients and Blood Pressure, Brown *et al*^[22] found a direct association between sugar-sweetened beverage intake and systolic and diastolic blood pressure increases. Thus, fructose seems to contribute directly to increased prevalence in the three main risk factors for atherosclerosis-related cardiovascular diseases.

Besides the association between fructose consumption and common metabolic diseases, there is growing evidence of a relationship with other diseases, such as cancer and Alzheimer's disease, that are also closely connected to the cellular metabolic status^[23,24]. Very recently, Friberg *et al.*^[25] analyzed data on total sucrose and high-sugar food consumption during 18.4 years of follow-up in 61 226 women. They found a direct association with increased risk of endometrial cancer. In addition, high sugar intake has recently been associated with lower cognitive function among middle-aged and older Puerto Ricans without diabetes, in an analysis of data from a substudy of the Boston Puerto Rican Health Study 2004-9^[26]. Although a high fructose diet does not affect spatial water maze learning and memory in female rats^[27], the presence of NAFLD, which is one of the main consequences of fructose consumption in men and experimental animals, seems to somehow impair hippocampal-dependent memory in male rats^[28].

Thus, overall, it seems that a high intake of sugar-sweetened beverages containing fructose places a metabolic burden on humans that facilitates the development of metabolic and cardiovascular diseases. What molecular mechanisms are involved in the production of these effects by fructose?

FRUCTOSE: MOLECULAR INSIGHTS FROM ANIMAL STUDIES

Fructose administration, mainly in drinking water, to laboratory rats and mice reproduces almost all of the features of metabolic syndrome and associated diseases in humans. These include left ventricular hypertrophy^[29,30], insulin resistance^[30-33], hypertension and related hyperuricemia^[34-36], NAFLD^[37,38], and metabolic syndrome itself^[39].

London *et al.*^[40] have investigated the role of increased 11-hydroxysteroid dehydrogenase type 1 in liver and visceral adipose tissue in rats after fructose, but not glucose, consumption. Their results indicate that deregulated local glucocorticoid production plays a role at the onset of fructose-induced obesity^[40]. Morris *et al.*^[41] put forward the hypothesis that the timing of fructose intake, mainly during the daylight period, could induce a mismatch in caloric consumption that favors the development of obesity and other metabolic alterations, at least in C57BL mice. Furthermore, several possible hypotheses related to the development of NAFLD by fructose consumption have been pursued, including increased oxidative and inflammatory stress through nitric oxide synthase induction^[42] and tumor necrosis factor α production^[43]. A very concise and interesting review on the issue of possible molecular mechanisms involved in fructose induced lipogenesis was published in 2011^[44].

In the past few years, our laboratory has researched three main issues regarding the molecular effects of fructose on liver fat and glucose metabolism: (1) possible drug therapies for the prevention and/or correction of

fructose-induced metabolic pathologies; (2) molecular mechanisms that are responsible for early induction of glucose intolerance in female rats, as a previous step to developing insulin resistance and type 2 diabetes mellitus; and (3) molecular mechanisms leading to reduced peroxisome proliferator-activated receptor (PPAR) expression and activity in livers of female rats.

NAFLD is by far the most common cause of liver dysfunction. It is a spectrum of diseases ranging from fatty liver (steatosis) to steatohepatitis^[45]. To date, the only effective treatment for NAFLD is modest calorie restriction and gradual weight loss^[46]. Statins, hypolipidemic drugs that act by inhibiting the hydroxymethyl-glutaryl-CoA reductase enzyme, can be safely used in NAFLD patients^[47], and there is evidence of improved liver histology in NAFLD patients treated with atorvastatin^[48,49]. In a recently published study, we proposed a possible molecular mechanism for the therapeutic effect of atorvastatin on NAFLD^[50]. Besides its well-known anti-inflammatory effect^[51,52], atorvastatin reduced the liver expression of fructokinase in male rats supplemented with a 10% w/v solution of fructose for 14 d. Fructose consumption induces the expression of liver fructokinase in experimental animals^[53,54] and in NAFLD patients^[55]. As fructokinase is essential in controlling fructose metabolism, its induction establishes a vicious circle that progressively increases the deleterious effect of fructose on liver metabolism. Atorvastatin effectively facilitates the breaking of this circle. It contributes to an increase in fatty acid metabolism^[56] and to a reduction in fatty acid synthesis that is driven by increased carbohydrate response element binding protein (ChREBP) transcriptional activity^[57,58], which are necessary to revert the deposition of triglycerides in liver tissue.

We used the same experimental model of rats supplemented with a 10% w/v solution of fructose for 14 d, to show that female rats were more sensitive to the deleterious effect of fructose on glucose homeostasis than male rats, as only females showed signs of glucose intolerance^[54]. In the same study, we found a marked reduction in insulin receptor substrate (IRS)-2 in the livers of fructose-supplemented female rats. IRS-2 is the main transducer of insulin signaling in hepatic tissue^[59]. We have further pursued research of molecular changes related to fructose consumption in liver. We have confirmed that female rats supplemented with liquid fructose for 14 d, but not 7 d, are glucose intolerant (as shown by glucose tolerance test; GTT). This situation correlates with a decrease in the amount of IRS-2 protein expressed in liver. The same animals showed a marked increase in mammalian target of rapamycin (mTOR) activity and mitogen-activated protein kinase (p38-MAPK) activity.

p38-MAPK is a stress-related kinase^[60] whose activity can be increased by the metabolic burden imposed by fructose metabolism in hepatocytes through two mechanisms: increased activity of protein phosphatase A2^[54,61], and the presence of bacterial toxins in blood, as a result of fructose-related alteration of the intestinal barrier

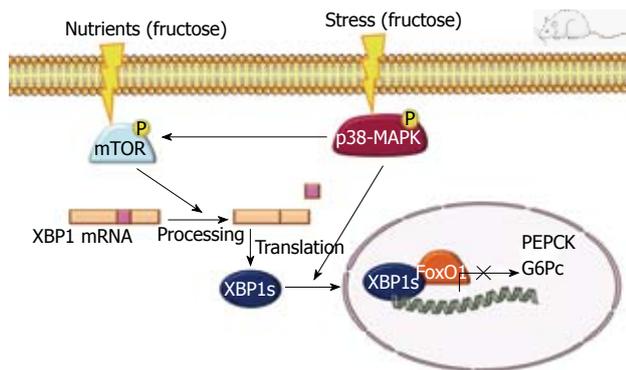


Figure 1 X-box-binding protein-1, an endoplasmic reticulum stress transcription factor, plays an essential role in maintaining plasma glucose concentration and glucose tolerance. Indeed, in the liver samples from the fructose-fed rats used in the study, there was a marked increase in the spliced form of X-box-binding protein (XBP)-1 mRNA and nuclear protein, in accordance with the increased activity of mammalian target of rapamycin (mTOR) activity and mitogen-activated protein kinase (p38-MAPK). Thus, although the decreased expression of insulin receptor substrate-2 in liver represents an impairment of insulin signaling, the increased expression and activity of XBP-1 could compensate for this deficit and maintain appropriate gluconeogenesis. PEPCK G6Pc: Phosphoenolpyruvate carboxykinase and glucose-6-phosphatase; FoxO1: Forkhead box protein O1.

permeability^[43,62]. Furthermore, increased p38-MAPK activity, by phosphorylating the tuberous sclerosis 2 gene product or tuberin, could release its inhibitory activity on mTOR complex 1 (mTORC1)^[63]. This would explain the observed increase in mTOR activity. The mTOR signaling pathway transduces information from different signals, such as growth factors, amino acids and energy overload of the cell^[64]. Finally, as Guo *et al.*^[65] have shown that mTOR activation causes IRS-2 degradation, the increase in mTOR activity could be the final molecular factor resulting in a decreased liver expression of liver IRS-2 protein, as we have found^[54].

Surprisingly, although female rats supplemented with liquid fructose for 14 d, had reduced liver expression of IRS-2, were hyperinsulinemic and showed an altered GTT, they were normoglycemic and their liver expression of gluconeogenic genes was unchanged (glucose-6-phosphatase) or even decreased (phosphoenolpyruvate carboxykinase). An explanation for this discrepancy can be found in a recent report indicating that X-box-binding protein (XBP)-1, an endoplasmic reticulum stress transcription factor, plays an essential role in maintaining plasma glucose concentration and glucose tolerance^[66]. It has been described that mTORC1 activity increases the splicing of XBP-1^[67], while p38-MAPK phosphorylates the spliced-derived protein, facilitating its nuclear localization and activity^[68]. Indeed, in the liver samples from the fructose-fed rats used in our study, there was a marked increase in the spliced form of XBP-1 mRNA and nuclear protein, in accordance with the increased activity of mTOR and p38-MAPK. Thus, although the decreased expression of IRS-2 in liver represents an impairment of insulin signaling, the increased expression and activity of

XBP-1 could compensate for this deficit and maintain appropriate gluconeogenesis (Figure 1). Data from skeletal muscle that indicate a deficit in adiponectin receptor and signaling in 14-d fructose-supplemented rats, could explain the fact that these animals do not have increased liver gluconeogenesis, but do have significant glucose tolerance impairment, as evaluated by an GTT.

We have previously shown that there is a state of leptin resistance in livers of male rats supplemented with liquid fructose. This results in increased binding of unphosphorylated active forkhead box protein (Fox)O1 to the transcription factor PPAR α , which causes the inhibition of PPAR α transcriptional activity and, as a consequence, reduces the liver capacity to oxidize fatty acids^[57,58]. FoxO-1 is a transcription factor that is regulated by insulin and deeply involved in the control of liver gluconeogenesis^[65]. Female rats equally supplemented with liquid fructose respond similarly with a reduction in liver PPAR α activity and fatty acid oxidation. However, there is no involvement of leptin resistance and FoxO-1 interaction^[54]. Thus, we have pursued the search for a possible molecular mechanism involved in the downregulation of the PPAR α system in the liver of fructose-supplemented female rats.

ChREBP is a transcription factor responsible for inducing liver lipogenesis after carbohydrate ingestion^[69]. We have previously reported that ChREBP is the main factor responsible for the increase in rat liver lipogenesis following fructose supplementation^[50,54,57,58,70]. Unpublished results from our group indicate that there is also a close relationship between ChREBP activation and PPAR α downregulation across different experimental settings (*in vivo* studies in female rats, cultured FaO and HepG2 hepatoma cells, primary cultures of human hepatocytes). It has been described that ChREBP controls the expression of regulator of G protein signaling (RGS) 16, a regulator of G protein signaling that inhibits hepatic fatty acid oxidation^[71]. Although fructose markedly increased the mRNA level of RGS16 in livers of female rats, there was no change in the amount of the expressed protein. This suggests that increased expression of RGS16 is not involved in downregulation of the PPAR α system. In rat hepatoma FaO cells cultured in the presence of a high concentration of fructose (25 mmol/L), we are performing knock-down experiments with siRNA against ChREBP to demonstrate clearly the direct involvement of ChREBP in the production of the fructose effect on the PPAR system. Confirmation of this hypothesis will indicate that fructose can simultaneously switch on liver fatty acid synthesis and switch off liver fatty acid catabolism by a single molecular mechanism: the intense activation of ChREBP. This would explain the effectiveness of fructose in inducing fatty liver and hypertriglyceridemia. We are also exploring possible mechanisms to explain why fructose stimulates the activity of ChREBP with such intensity. We have found that fructose supplementation markedly reduces the amount of the NAD-dependent deacetylase sirtuin 1 protein in livers of female rats,

but not males. This reduction increases the amount of acetylated ChREBP. As it has been shown that ChREBP hyperacetylation increases its transcriptional activity^[72], the reduction of sirtuin 1 expression could be one mechanism involved in the intense activation of ChREBP by fructose in the liver of female rats.

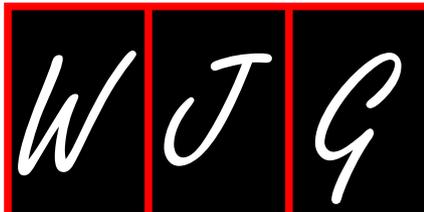
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Role of gastrin-peptides in Barrett's and colorectal carcinogenesis

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Abstract

Gastrin is the main hormone responsible for the stimulation of gastric acid secretion; in addition, gastrin and its derivatives exert proliferative and antiapoptotic effects on several cell types. Gastrin synthesis and secretion are increased in certain situations, for example, when proton pump inhibitors are used. The impact of sustained hypergastrinemia is currently being investigated. *In vitro* experiments and animal models have shown that prolonged hypergastrinemia may be related with higher cancer rates; although, this relationship is less clear in human beings. Higher gastrin levels have been shown to cause hyperplasia of several cell types; yet, the risk for developing cancer seems to be the same in normo- and hypergastrinemic patients. Some tumors also produce their own gastrin, which can act in an autocrine manner promoting tumor

growth. Certain cancers are extremely dependent on gastrin to proliferate. Initial research focused only on the effects of amidated gastrins, but there has been an interest in intermediates of gastrin in the last few decades. These intermediates aren't biologically inactive; in fact, they may exert greater effects on proliferation and apoptosis than the completely processed forms. In certain gastrin overproduction states, they are the most abundant gastrin peptides secreted. The purpose of this review is to examine the gastrin biosynthesis process and to summarize the results from different studies evaluating the production, levels, and effects of the main forms of gastrin in different overexpression states and their possible relationship with Barrett's and colorectal carcinogenesis.

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Key words: Gastrin; Progastrin; Glycine-extended gastrins; C-terminal flanking peptide; Hypergastrinemia; Proton pump inhibitors; Colorectal cancer; Esophageal adenocarcinoma; Barrett's esophagus

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INTRODUCTION

The polypeptide hormone gastrin was discovered in 1905 and described as a major stimulant of acid secre-

tion from the stomach antral mucosa. In the last few decades, several studies have reported on the role of gastrin in stimulating cell division and inhibiting apoptosis, suggesting that gastrin and its derivatives might promote carcinogenesis^[1-5]. Gastrin and cholecystokinin (CCK) are members of a family of neuroendocrine peptides and are both physiological ligands of the CCK-B receptor (CCKBR).

Gastrin is secreted by antral G cells and interacts with the CCKBR on enterochromaffin-like (ECL) and parietal cells to induce gastric acid secretion.

Gastrin release from G cells is stimulated by the presence of food - mainly peptides^[6] in the stomach, vagal release of gastrin releasing peptide, and an increase in stomach pH, as seen in achlorhydria^[7]. *Helicobacter pylori* (*H. pylori*) infection is also known to cause hypergastrinemia, increasing mainly plasma levels of its amidated form gastrin-17. After eradication of the bacteria, plasma gastrin levels decrease to normal^[8-10]. Gastrin release is inhibited by secretion of gastric acid, and this serves as a negative feedback control that prevents excess acid secretion. Low pH values in the stomach inhibit gastrin release by G cells, stimulating the secretion of somatostatin by antral D cells^[11].

Gastrin is expressed in a variety of tissues under both normal and pathological conditions. Its main site of production are G cells from the antral mucosa, but it is also synthesized at lower levels in duodenal mucosa, fetal and neonatal pancreases, in pituitary corticotrophs, melanotrophs, and neurons, in spermatogenic cells, and in a variety of cancers.

The main products of the gastrin gene in the antrum are its amidated forms gastrin 17 and gastrin 34 (G17-NH₂ and G34-NH₂).

GASTRIN BIOSYNTHESIS

As with other peptide hormones, gastrin is synthesized initially as a large precursor molecule, which undergoes extensive post-translational modification prior to secretion. The gastrin gene spans 4.1 kb and is located on chromosome 17 (17q21). It produces a single mRNA (0.7 kb), which encodes the 101 amino acid precursor, preprogastrin^[12]. Preprogastrin is translated at the endoplasmic reticulum, where the signal peptide is removed by signal peptidase, giving rise to progastrin (80 amino acids)^[13]. Progastrin (PG) then progresses through the Golgi complex.

If the cell has a regulated secretory pathway, as with differentiated endocrine cells such as G-cells in the antrum, progastrin is fully processed and transported by secretory granules. It is then released by exocytosis, which is induced by secretagogues after G-cell stimulation. This is the secretory pathway of most of the amidated products, because the enzymes and conditions necessary for the processing of the immature gastrin forms are found inside secretory granules from the Golgi stack.

Progastrin is cleaved at paired amino acids by endo-

proteases belonging to the prohormone convertases (PC) family. PC1/3 cleavages at the dibasic sites arginine36-arginine37 and arginine73-arginine74 lead to the formation of an intermediate, which undergoes processing by carboxypeptidase E and yields glycine-extended gastrins (G-Gly) and the C-terminal flanking peptide (CTFP). The peptidylglycine α -amidating monooxygenase converts G34-gly to its amidated form and PC2 cleaves at lysine53-lysine54, producing bioactive gastrins of varying sizes (e.g., gastrin-34 and gastrin-17)^[13,14] (Figure 1).

Preprogastrin derivatives can also exit the cell *via* another pathway, known as the constitutive pathway. Molecules exiting cells *via* this pathway are transported in secretory vesicles that take their contents from the Golgi apparatus and continuously fuse with the plasma membrane. Intermediate products of gastrin processing are secreted mainly by this pathway since peptides exiting this pathway do not undergo extensive posttranslational processing.

Processing and final secretion of progastrin products differ markedly depending on the expression location. In healthy adults, the main gastrin production site is antroduodenal G-cells, so the proportion of circulating gastrins depends largely on the products exiting these cells. In G-cells, the regulated secretory pathway predominates; thus, these cells mostly secrete a mixture of amidated products (95%), including G17-NH₂ (85%-90%), G34-NH₂ (5%-10%), and a mix of gastrin-14, gastrin-52, gastrin-71, and short amidated C-terminal fragments^[15]. The remaining 5% of the secreted products correspond to non-amidated processing intermediates (mainly progastrin and G-Gly).

Although the majority of gastrins secreted by G-cells correspond to the amidated G17 form, peripheral blood contains almost equal amounts of G17-NH₂ and G34-NH₂ because the metabolic clearance of large gastrins is slower than for smaller forms of the peptide^[16-18].

On the other hand, the proportions of the gastrin intermediates may vary in certain gastrin overexpression states, such as when proton pump inhibitors (PPIs) are used or in the presence of gastrin-producing tumors. Most of these tumors are not able to completely process gastrin, resulting in less conversion to the mature peptide^[19-22].

The causes of incomplete gastrin processing during hormone overexpression are still unclear; although, it has been proposed that it might be caused by saturation of the enzymes that catalyze progastrin modifications, leading to an inability to process increasing amounts of the gene product.

Another possible reason is the lack of a well-developed regulated pathway of secretion, as in some tumor cells. In that case, progastrin exits the cell *via* the constitutive pathway directly from the Golgi terminal.

GASTRIN RECEPTORS

The actions of amidated gastrins and CCK peptides are mediated by two different receptors: CCKA and CCKB

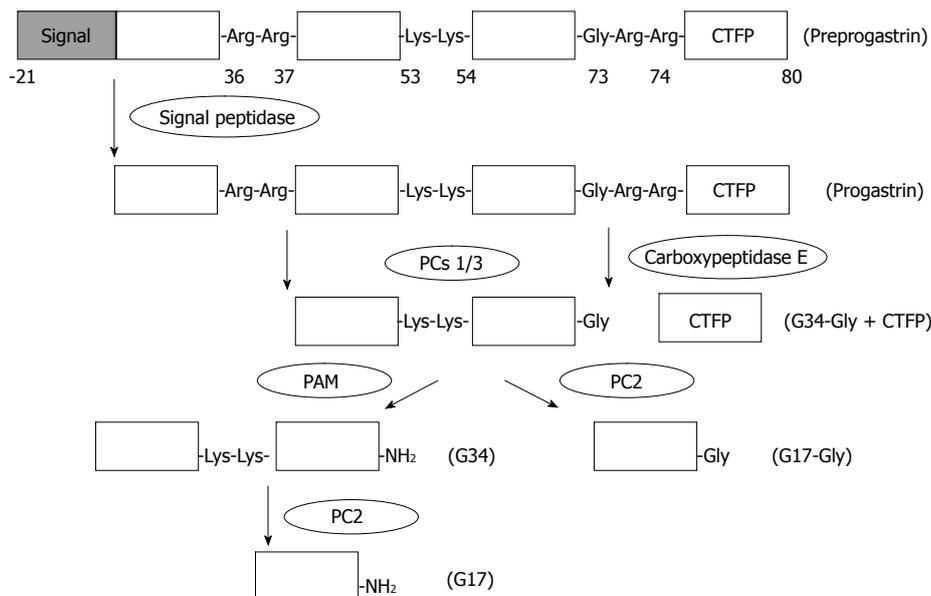


Figure 1 Main steps in preprogastrin processing in antral G cells. Arg: Arginine; Lys: Lysine; CTFP: C-terminal flanking peptide; PC: Prohormone convertases; PAM: Peptidyl-glycine α -amidating monooxygenase.

receptors, which differ pharmacologically by their affinity for gastrin (low for CCKA receptors and high for CCKB receptors)^[23,24].

Gastrin and CCK peptides share a common C-terminal sequence, which has been well preserved during evolution. This conserved C-terminal active site is related to most of the known effects of these peptides, especially the tetrapeptide Trp-Met-Asp-Phe-NH₂. The specificity of the receptor binding and biological potency depends on N-terminal extensions of this common tetrapeptide.

Sulfation of the tyrosyl residue (in position six in gastrin peptides, counted from the C-terminal position, and in position seven in CCK peptides) determines the specificity for CCKA or CCKB receptors. The residue is totally sulphated in CCK peptides, so they are able to bind either CCKA or CCKB receptors with high affinity. It is partially sulphated in gastrin peptides, so they can only bind CCKB receptors.

Gastrin and CCK display similar affinities for the CCKB receptor; however, the gastrin concentration in plasma is 10- to 20-fold higher than CCK; therefore, CCKB receptors in the periphery are, in physiological terms, mainly receptors for gastrin.

The CCKB receptor has seven transmembrane domains and belongs to the superfamily of G-protein coupled receptors. CCKBR is abundantly expressed on enterochromaffin-like cells in the stomach, in the central nervous system and in some tumors, principally in the gastrointestinal tract.

Gastrin, at physiological levels, is the main mediator of meal-stimulated acid secretion. Once secreted by the antral G cells, gastrin is transported to the oxyntic mucosa of the stomach, where it interacts with the CCKBR on ECL cells, stimulating the release of histamine. Both gastrin and histamine then interact with the parietal cells, through the gastrin CCKB and histamine H₂ receptors to induce gastric acid secretion^[25].

Only amidated gastrins exert their effects through CCK-

BR activation, while intermediate precursors such as progastrin or G-Gly interact with other receptors^[3,26-28].

Most PG effects are mediated *via* the monomeric 36 kDa form of the annexin II receptor (ANX II)^[29,30]. ANX II is a multi-functional protein that binds acid phospholipids and actin with similar affinity. It's expressed abundantly in rejuvenating cells, but not in quiescent cells; in addition, its expression is increased in many human cancer cells, including colon and pancreatic, and it's expressed in normal intestinal epithelial cells^[27,28]. ANX II is absent in the brain and liver, which supports that it is only expressed in proliferating cells.

The majority of effects of G-Gly and CTFP appear to be mediated by a cellular receptor distinct from CCKBR^[1,24,31-33]; yet, to date, the receptor or receptors remain unknown. Gly-G appears to be able to bind ANX II, but it is still unclear whether its action is mediated *via* this interaction^[1].

PPIs AND GASTRIN

PPIs are the most potent and widely used medications to reduce gastric acid secretion. These drugs are considered safe; although, some long-term side-effects have been identified, for example, all PPIs induce an increase in plasma gastrin levels. The reason for this increase remains unclear, but it may be due to the reduced activity in antral D-cells (shown by a three-fold decrease in antral somatostatin mRNA) in response to PPI-induced achlorhydria^[7]. There is also an increase in plasma gastrin levels with other antacids such as H₂ receptor antagonists, but only after long-term use^[34].

PPIs may induce a 2- to 4-fold increase in plasma gastrin^[35,36] (mainly G17-NH₂ and G34-NH₂) with short-term treatment, whereas, in long-term therapy, some patients will develop marked hypergastrinemia (often exceeding 400 pmol/L). The antral mucosa levels of amidated gastrins and G-Gly are not affected by PPI treatment, but

Table 1 Studies assessing expression levels and/or biological effects of gastrin through its interaction with cholecystokinin-B receptor

Ref.	Specimen	CCKBR expression	G17 expression	G17 effects
Haigh <i>et al</i> ^[41]	Esophageal biopsies from healthy, esophagitis, BE and EAC patients; <i>Ex vivo</i> culture of BE cells; OE33(E)GR cells	CCKBR is expressed in 3/9 of healthy, 5/7 esophagitis, 10/10 BE and 7/12 EAC samples	Not assessed	G17 stimulates cell proliferation through CCKBR
Konturek <i>et al</i> ^[46]	Tumor and plasma samples from CRC patients; Plasma and normal colonic mucosa biopsies from healthy subjects	All the tumor samples showed CCKBR expression	CRC patients showed normal G17 plasma levels, and higher progastrin levels than healthy subjects; Celecoxib diminished plasma gastrin and progastrin levels	Not assessed
Smith <i>et al</i> ^[49]	Healthy colonic mucosa and colonic polyps biopsies	Normal colonic mucosa didn't show CCKBR expression; Most of the polyps analyzed showed CCKBR expression	Most of the polyps showed higher expression of the gastrin precursors than amidated forms	Not assessed
Harris <i>et al</i> ^[70]	Healthy esophagus and BE biopsies; OE19 and OE33 cell culture; OE21 cell culture	All three esophageal cancer cell lines express CCKBR; BE biopsies show higher CCKBR expression levels than normal esophageal biopsies	BE samples express higher gastrin levels than healthy esophageal biopsies	G17 increases activation of the antiapoptotic factor PKB/Akt, through CCKBR

BE: Barrett's esophagus; EAC: Esophageal adenocarcinoma; CCKBR: Cholecystokinin-B receptor; G17: Amidated gastrin-17; CRC: Colorectal carcinoma; OE19 and OE33 cells: Esophageal adenocarcinoma cell lines; OE21 cells: Esophageal squamous carcinoma cell line; OE33(E)GR cells: Esophageal adenocarcinoma cells permanently transfected with the human CCKB receptor.

a 6-fold increase in the antral progastrin concentration was observed after PPI therapy^[57-39]. However, the gastrin levels in patients on PPIs are extremely variable, and not every subject will have a markedly increased plasma gastrin level after acid suppressive therapy.

PPIs rapidly stimulate antral gastrin secretion, but the overexpression of the gastrin gene, which is observed as increased gastrin mRNA concentrations in G-cells, is only seen after 24 h of achlorhydria^[7].

To date, circulating levels of gastrin precursors have not been evaluated in response to PPI intake.

GASTRIN AND CARCINOGENESIS

As mentioned above, gastrin is a major stimulant of acid secretion in the stomach mucosa, but it also has effects in different tissues promoting cell division and inhibiting apoptosis. There is now growing evidence suggesting that elevated gastrin levels could favor the development of certain neoplasias, especially in the gastrointestinal tract^[2,40-43]. To date, most of those studies have been focused on the possible relationship between elevated gastrin levels and colorectal and gastric cancers, but there is evidence that suggests a possible relationship with different tumors, even outside the gastrointestinal (GI) tract^[19,20,22,44,45].

CCKBR has been observed in several tumor types, but expression of the receptor in human gastrointestinal cancers is controversial. Although some groups found CCKBR expression in many GI neoplasias^[3,46], others found expression of the receptor in GI tumors only occasionally^[23,29] (Table 1).

It is well established that some tumors produce their own gastrin and that gastrin can promote tumor growth in an autocrine manner^[22,44,46-48], but there were conflicting findings from studies evaluating gastrin expression

in tumors. This may be because initial attempts focused only on the amidated forms of gastrin. We now know that certain tumors, such as colorectal carcinoma (CRC), produce high levels of gastrin intermediates while the amidated forms are not affected^[20,22]. Gastrin has been found in CRC extracts and also in adenomatous polyps^[49], but not in healthy colonic mucosa. A similar pattern was found with esophageal adenocarcinoma and Barrett's esophagus (BE) (a premalignant condition that is a major risk factor for esophageal adenocarcinoma), where gastrin and its receptor were expressed at higher levels than in normal epithelium^[4,50].

These observations suggest that activation of gastrin expression may be an early event in the adenoma-carcinoma or the metaplasia-carcinoma progression; thus, gastrin could favor neoplastic transformation.

Studies in animal models have demonstrated that a prolonged hypergastrinemic situation, such as in deep acid inhibition, is related to higher CRC rates and with gastric atrophy, metaplasia, gastric adenocarcinoma and carcinoid tumors^[34,41,43,51] (Table 2). *In vitro* studies demonstrated that gastrin and its derivatives increase the rate of cell proliferation and migration and reduce apoptosis, which are major steps in tumor development^[1,3,28,44,52]. Although both *in vitro* and *in vivo* animal model studies seem to demonstrate an association between a rise in gastrin levels and a higher risk of cancer development this is still unclear in human beings. While some epidemiologic studies showed an association between, elevated gastrin levels after use of PPIs and stomach ECL and argyrophil cell hyperplasia, but couldn't demonstrate that hypergastrinemia itself increases gastric adenocarcinoma rates^[35,53-55], others found higher cancer rates (gastric and gastrointestinal overall) in hypergastrinemic patients^[56,57] (Table 3).

Pernicious anemia could represent a human model

Table 2 Experimental studies in animal models exploring the impact of increased levels of gastrin peptides

Ref.	Animal model	Alteration on gastrin peptides levels	Hypergastrinemia effects
Cobb <i>et al</i> ^[2]	Fabp-wt mice; Fabp-mt mice	Fabp-wt mice express human PG in intestinal mucosa and Fabp-mt mice express a mutated form of human PG; Both mice show PG expression at similar levels as seen in hypergastrinemia	Mice overexpressing human PG (either the wild-type and the mutated form) are more likely to develop colonic tumors in response to AOM
Wang <i>et al</i> ^[5]	INS-GAS mice; hGAS mice	INS-GAS mice overexpress human amidated gastrin in the pancreatic islets; hGAS mice overexpress human PG in the liver	Both forms of gastrin showed similar proliferative effects on normal colonic mucosa
Havu <i>et al</i> ^[34]	Sprague-Dawley rats treated with ranitidine (2g/kg per day)	Rats showed a 3-fold increase in plasma gastrin levels	19/100 rats developed ECL carcinoids while no carcinoma was found in control animals
Watson <i>et al</i> ^[43]	APC ^{Min/+} mice (model of multiple intestinal neoplasia) treated with omeprazole (75 mg/kg in a single oral dose)	Omeprazole increased only amidated gastrin plasma levels	PPI-induced hypergastrinemia reduced mice survival; Hypergastrinemia increased colonic adenomas proliferation; Hypergastrinemia did not increase the incidence of intestinal tumors
Ferrand <i>et al</i> ^[90]	MTI/G-Gly mice; hGAS mice	MTI/G-Gly mice overexpress human G-Gly throughout the gastrointestinal tract; hGAS mice overexpress human PG in the liver	Both G-Gly and PG strongly up-regulate Src, JAK2 and STAT3 activation; PG produced significantly great ERK and Akt pathways activation and TGF- α overexpression
Koh <i>et al</i> ^[95]	MTI/G-Gly mice	MTI/G-Gly mice overexpress human G-Gly throughout the gastrointestinal tract	Goblet cells hyperplasia and colonic hyperproliferation; Hypergastrinemia did not increase the incidence of GI tumors, but 3/10 mice developed bronchoalveolar carcinoma
Ottewell <i>et al</i> ^[98]	G ⁻ /hg ^{+/+} mice; G ⁻ /hg ⁻ mice	G ⁻ /hg ^{+/+} mice express human PG and no murine gastrin; G ⁻ /hg ⁻ mice do not express any forms of gastrin	PG increased colonic proliferation; PG exerts mitotic effects on colonic epithelia but does not seem to affect the small intestine epithelia

PG: Progastrin; AOM: Azoxymethane; ECL: Enterochromaffin-like cells; PPI: Proton pump inhibitors; G-Gly: Glycine-extended gastrins; JAK2: Janus-activated kinase 2; STAT3: Signal transducer and activator of transcription 3; ERK: Extracellular-signal regulated kinase; Akt: Protein kinase B; TGF- α : Transforming growth factor-alpha; GI: Gastrointestinal.

to assess effects of long-term hypergastrinemia, since it causes a long-term hypergastrinemia as a consequence of sustained achlorhydria^[57]. Another human model of hypergastrinemia is Zollinger-Ellison syndrome. In this case, patients show higher rates of colonic proliferation^[58], but not a higher risk for developing CRC^[59].

Another study found a higher CRC incidence rate with higher serum gastrin levels^[60], while, one study found no association between PPI use and the risk of CRC^[61].

It has been suggested that the discrepancy between results observed in human studies could be explained by the variability of hypergastrinemia after use of PPIs among patients^[42], by differences in the duration of the follow-up period -since higher cancer rates have only been observed in long-time hypergastrinemic patients-, and by differences in the forms of gastrin being studied, given that most of the studies to date have been focused only on the amidated forms^[22,62].

GASTRIN, BE AND ESOPHAGEAL ADENOCARCINOMA

Gastroesophageal reflux disease (GERD) is a chronic state in which part of the acidic stomach contents backs up into the esophagus and may cause inflammation of its epithelium. In most patients, this damaged epithelium is

replaced by new squamous epithelium; however, in some subjects, this epithelium is substituted, through a metaplastic process, by an intestinal-type columnar epithelium. This condition is called BE, a premalignant state responsible for most esophageal adenocarcinoma cases (EAC). Patients with BE have a 30- to 40-fold higher risk for developing EAC than the general population^[63].

In the last few decades, the incidence rates for this tumor have increased significantly^[64], more than for any other type of cancer^[65] in developed countries.

BE may represent a good model to study the involvement of hypergastrinemia in carcinogenesis, because frequently high levels of gastrin can be observed in BE patients. PPIs are the main pharmacological treatment for BE and the sequence of neoplastic transformation is well known.

In the pathological state caused by the damaging effects of acid contents from the stomach in the esophagus, it seems that an increase in gastric reflux pH would have a potential benefit for the patient. However, the benefits of these drugs in the management of GERD and BE are not clear. Normalization of intraesophageal pH clearly relieves gastroesophageal reflux symptoms^[37], favoring differentiation and decreasing cell proliferation^[66]; yet, there has been an increasing incidence of EAC in BE patients in the last few decades, despite generalized use of PPIs^[67-69]. Studies addressing the potential

Table 3 Clinico-epidemiologic studies exploring the effects of proton pump inhibitors use in human beings

Ref.	Population studied	Treatment, dose and duration	Effects on gastrin levels	Physiopathological effects
Brunner <i>et al</i> ^[35]	143 patients with duodenal or stomach ulcer and GERD	Omeprazole 40 mg/d 1-5 yr	Plasma gastrin levels increased 4-fold after 4 mo of therapy	Hyperplasia of argyrophil cells from oxyntic mucosa; No increase in dysplasia or neoplasia rates was observed
Klinkenberg-Knol <i>et al</i> ^[37]	91 GERD patients	Omeprazole 20-40 mg/d 5 yr	Median serum gastrin levels increased from 60 to 162 ng/L and reached a plateau during maintenance treatment	Esophagitis symptoms ameliorated; Gastric hyperplasia rates increased from 2.5% at the beginning of the study to 20% at last biopsy
Nemeth <i>et al</i> ^[39]	10 patients with oesophagitis	Omeprazole 20 mg/d 6-8 wk	Plasma levels of amidated gastrins increased from 18 to 48 pmol/L; Antral levels of progastrin increased 6-fold while amidated gastrins and G-Gly remain unaltered	Not assessed
Wang <i>et al</i> ^[42]	82 BE patients; 13 GERD patients	All patients were on PPI therapy, once or twice daily during a median time of 74 mo	The median serum gastrin levels (40 pmol/L) was not related to the degree of dysplasia in BE	Higher serum gastrin levels were associated with high grade dysplasia and adenocarcinoma
Creutzfeldt <i>et al</i> ^[53]	74 patients with esophagitis or peptic ulcer	Omeprazole 40 mg/d 1-5 yr	Plasma gastrin levels increased 4-fold in 23% of patients	Patients with higher serum gastrin levels developed hyperplasia of the gastric argyrophil cells; This hyperplasia may not necessary be related to high gastrin levels
Kuipers <i>et al</i> ^[54]	177 GERD patients	105 patients treated with omeprazole 20-40 mg/d 5 yr; 72 patients treated with fundoplication	Not assessed	Patients treated with omeprazole and infected with H.pylori infection are at increased risk of atrophic gastritis
Lamberts <i>et al</i> ^[55]	74 peptic ulcer patients	Omeprazole 48 mo	Median gastrin levels moderately increased after 3 mo of therapy and reached a plateau during maintenance treatment	Significant argyrophil cell hyperplasia

GERD: Gastroesophageal reflux disease; G-Gly: Glycine-extended gastrins; BE: Barrett's esophagus; PPI: Proton pump inhibitors.

role of different molecular forms of gastrin in Barrett's carcinogenesis are discussed below.

AMIDATED GASTRINS

Amidated gastrins are, in healthy subjects, the final and most abundant product in the gastrin biosynthesis pathway. Through the interaction with their receptor, CCKBR, amidated gastrins might be involved in the neoplastic progression of BE.

Amidated gastrins and CCKB receptor expression in BE

Barrett's mucosa expresses its own gastrin. Patients with BE show higher levels of amidated gastrins than healthy subjects^[44], which might be a consequence of both PPI intake and autocrine gastrin production by Barrett's mucosa^[36,50]. This autocrine gastrin production diminishes with the progression to dysplasia and EAC^[44], and there is not a significant difference between serum gastrin levels in GERD and BE patients^[42]. The expression of its receptor increases in response to inflammation. In almost all BE biopsy samples studied, CCKBR mRNA and protein are detected; while, they are only occasionally present in healthy tissue and their presence in EAC is unclear^[4,36,50,70]. In addition, expression of the receptor increases cell proliferation^[26,31,44]; therefore, CCKBR may

have an important role in GERD ulcer healing^[36,44].

Biological effects of amidated gastrins

In vitro studies determined that amidated gastrins may promote cell proliferation and migration of BE and EAC cells, and those effects are mediated through the interaction with CCKBR^[4,26,36,44].

The effects of amidated gastrins are mediated, at least partially, by the induction of cyclooxygenases (COX)-2 expression and prostaglandins production^[3]. COX are membrane proteins that catalyze the limiting step in the prostaglandin synthesis pathway. Prostaglandins are molecules that may promote carcinogenesis through stimulation of cell division, induction of angiogenesis, and inhibition of apoptosis^[71,72]. As a consequence of the interaction between amidated gastrins and CCKBR, COX-2 is overexpressed in Barrett's mucosa, leading to an increase in prostaglandins synthesis and cell proliferation^[44,73].

COX-2 overexpression is related to the development of other GI cancers, and the use of COX-2 inhibitors, such as non-steroidal anti-inflammatory drugs is associated with a reduction in the frequency and mortality of those tumors^[74-78]. *In vitro* and *in vivo* studies have shown that COX-2 inhibitors decrease cell proliferation in BE^[79] and reduce the risk of developing EAC^[74], suggesting that COX-2 might be a key factor in Barrett's carcinogenesis.

COX-2 overexpression seems to be an early event in the neoplastic transformation of BE. Despite the great variability observed between subjects, COX-2 levels in biopsy samples are always higher in BE mucosa than in normal esophageal epithelium^[44,50,73,80].

Thus, amidated gastrins might have a role in the neoplastic progression of BE rather than in its initial development since BE cells express higher levels of gastrin, CCKB receptor and also COX-2 than EAC^[44] and normal esophageal cells.

GASTRIN SYNTHESIS INTERMEDIATES: PROGASTRIN, G-GLY AND CTFP

The biological activity of gastrin synthesis intermediates was unknown until 1994^[81]. Experiments carried out to determine their ability to stimulate gastric acid secretion showed negligible or less potency than fully processed amidated forms^[82,83]; therefore, those investigations concluded the intermediates were inactive peptides and focused mainly on the known bioactive forms. However, in the last few decades, numerous studies have demonstrated that these molecules are far from inactive precursors. Gastrin intermediates are secreted in higher proportions than their amidated forms in certain gastrin overexpression states^[39,84]; thus, knowledge of their recently known biological effects has led to several studies on these intermediates in the last few decades. To date, most of these studies have been focused on CRC; although the relative abundance of these precursors in other tissues supports that it is necessary to extend research to other organs as well.

PROGASTRIN

PG is the first gastrin synthesis intermediate after signal peptide cleavage. A study demonstrated that PG levels in antral biopsies from patients undergoing PPI treatment were up to 6-fold higher than in untreated patients^[39]; although, there are currently no studies showing PG plasma levels in response to PPI administration

Progastrin expression

Studies carried out on healthy colonic and CRC tissue have shown a higher proportion of products from the early stages of gastrin synthesis (PG above all) than those from later stages (G-Gly and amidated forms) in cancer samples^[20,85]. Plasma PG levels, but not amidated gastrin, are elevated in CRC patients compared with healthy subjects and those with colonic polyps, suggesting a possible tumor origin for this PG and an incomplete processing of the peptide in tumor cells^[22]. Other tumors, such as pancreatic, ovarian, and lung cancer, also overexpress PG^[45,47,48].

Biological effects of PG

Progastrin may exert greater proliferative effects than amidated gastrins on normal and tumor cells (CRC, pancreat-

ic) in culture^[28,86] and also has an antiapoptotic effect^[87]. *In vivo* studies using mice overexpressing both G17-NH₂ and PG showed increased colonic proliferation compared to wild-type control mice. At plasma concentrations similar to those observed in certain disease states, PG can act as a co-carcinogen and significantly increases the risk for colon carcinogenesis in response to azoxymethane^[2,5].

PG has negligible affinity for the receptor for amidated gastrins (CCKBR) and its effects are mediated by a different receptor: ANX II^[27-30]. This receptor is not expressed on quiescent cells and it is necessary to mediate at least 50% of exogenous PG effects on intestinal cells and more than 80% of the effects of autocrine gastrins on CRC cells^[29]. ANX II is overexpressed in human CRC and may be related to a poor prognosis^[88]. It is also overexpressed in a wide variety of tumors^[88,89]. The mitogenic and antiapoptotic effects of PG seem to be mediated through activation of several signaling pathways including nuclear factor- κ B (NF- κ B), Src, Janus-activated kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3), extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt kinases^[86,90].

G-GLY

G-Gly are some of the last gastrin processing intermediates. They result from the cleavage of the C-terminal arginyl residues of progastrin by carboxypeptidase E, before the amidation step.

G-Gly expression

As with other gastrin intermediates, there are few studies focused on assessing possible changes in plasma or tissue levels of G-Gly in different situations. It seems that PPIs don't significantly affect its levels because antral mucosa levels of G-Gly remain unaltered after treatment with PPIs^[39]. In addition, CRC doesn't alter plasma G-Gly concentrations^[22]; while, tumor biopsies and cell lines derived from CRC show higher levels than healthy tissue^[20,22]. G-Gly levels are also increased in the gastric mucosa from patients with gastrinoma^[91]. Outside the GI tract, only a small proportion of lung cancer cases analyzed showed G-Gly overexpression, which was inversely related to survival rates^[45].

Biological effects of G-Gly

Even small variations in the levels of G-Gly may affect proliferation and apoptosis. G-Gly effects can be observed at concentrations at least one order of magnitude less than for amidated forms^[33,92]. G-Gly can act as a growth factor for many cultured cells, including gastric, pancreatic, colonic cancer cells, and non-transformed cells^[33,52,81,93,94]. It also decreases apoptosis in CRC and EAC^[1,92] cells and increases migration in CRC cells^[40]. *In vivo* experiments using transgenic mice overexpressing this intermediate demonstrated that higher G-Gly levels are related to colonic hyperproliferation, but were not

able to cause tumors alone^[90]. Surprisingly these experiments showed higher bronchoalveolar cancer rates in the animals overexpressing the molecule^[45,95].

The proliferative effects of G-Gly are dependant, at least partially, on COX-2 expression in EAC cells because the use of COX-2 inhibitors abolishes the proliferative effects of the molecule^[52].

To date, the G-Gly receptor remains unknown but the majority of its effects seem to be mediated *via* a different receptor than CCKBR^[1,33,96]. Although G-Gly has been shown to bind to ANX II, no effects were observed^[29]. The G-Gly interaction with a receptor distinct from CCKBR leads to JAK2/STAT3, Akt, NF- κ B, PI3k, and ERK activation^[52], increasing COX-2 expression. In contrast, the antiapoptotic effects of G-Gly occur independently from COX-2 expression^[1].

CTFP

CTFP is the gastrin synthesis intermediate generated after cleavage of progastrin into its dibasic residues, generating G-Gly and the 6-amino acid CTFP. After the discoveries that the gastrin intermediates PG and G-Gly are present in normal antrum and certain tumors^[20-22,48] and have effects enhancing cell proliferation and inhibiting apoptosis^[5,28,29,33,52], several studies focused on those molecules. However, little attention has been paid to CTFP.

CTFP expression

In plasma and human antral extracts from healthy subjects, CTFP is the most abundant peptide (four-fold higher than the next most abundant peptide in antral samples and 30-fold higher in plasma). In CRC patients, CTFP levels are elevated in tumor mucosa and remain unaltered in plasma^[32].

CTFP biological effects

CTFP effects have been tested on colonic and gastric cancer cell lines *in vitro*. CTFP showed higher potency in stimulating cell growth than G-Gly in colon tumor cells and a similar potency in gastric cancer cells. CTFP also stimulated cell migration in a non-transformed mouse gastric cell line and activated MAPK phosphorylation in colon cancer cells *via* a different receptor than CCKBR^[32]. CTFP also exerts anti-apoptotic effects^[97]. To date, only a few studies have focused on CTFP, but it has been determined that it is a biologically active molecule that is secreted in higher amounts than any other product of the gastrin gene. Thus, further experiments with this peptide should be carried out.

CONCLUSION

In summary, data derived from *in vitro* studies and animal models strongly suggest that high levels of gastrin may exert carcinogenic effects, on BE and colorectal epithelia, but also elsewhere. In addition, certain tumors produce their own gastrin, which might contribute to support

tumor growth. However, it is currently not clear if high gastrin levels have the same effects in human beings. Most studies have been focused on amidated gastrins. Although, intermediates of gastrin synthesis can exert even greater carcinogenic effects than the amidated forms and in certain situations they become the most abundant forms of gastrin. Therefore, more studies evaluating these molecules are needed to elucidate the potential role of gastrins in human carcinogenesis.

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Relatedness of *Helicobacter pylori* populations to gastric carcinogenesis

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Abstract

Helicobacter pylori (*H. pylori*) is a Gram-negative bacterium that infects half of the human population. The infection is associated with chronic inflammation of the gastric mucosa and peptic ulcers. It is also a major risk factor for gastric cancer. Phylogenetic analysis of global strains reveals there are seven populations of *H. pylori*, including hpAfrica1, hpAfrica2, hpEastAsia, hpEurope, hpNEAfrica, hpAsia2 and hpSahul. These populations are consistent with their geographical origins, and possibly result from geographical separation of the bacterium leading to reduced bacterial recombination in some populations. For each population, *H. pylori* has evolved to possess genomic contents distinguishable from others. The hpEurope population is distinct in that it has the largest genome of 1.65 mbp on average, and the highest number of coding sequences. This confers its competitive advantage over other populations but at the cost of a lower infection rate. The large genomic size could be a cause of the frequent occurrence of the deletion of the *cag* pathogenicity island in *H. pylori* strains from hpEurope. The incidence of gastric cancer varies among different geographical regions. This can

be attributed in part to different rates of infection of *H. pylori*. Recent studies found that different populations of *H. pylori* vary in their carcinogenic potential and contribute to the variation in incidence of gastric cancer among geographical regions. This could be related to the ancestral origin of *H. pylori*. Further studies are indicated to investigate the bacterial factors contributing to differential virulence and their influence on the clinical features in infected individuals.

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Key words: *Helicobacter pylori*; Population genetics; Gastric cancer; Virulence; Genome

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Dong QJ, Zhan SH, Wang LL, Xin YN, Jiang M, Xuan SY. Relatedness of *Helicobacter pylori* populations to gastric carcinogenesis. *World J Gastroenterol* 2012; 18(45): 6571-6576 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i45/6571.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i45.6571>

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative bacterium which colonizes the human stomach. As a pathogen, *H. pylori* induces inflammation of the gastric mucosa^[1]. It plays a causal role in the ulceration and recurrence of peptic ulcer^[2]. Eradication of the bacterium heals ulcers and prevents recurrence of the disease. The infection is also associated with an increased risk of gastric cancer^[3,4].

The incidence of gastric cancer shows geographical variation. This is attributed in part to the difference in the prevalence of the *H. pylori* infection among geo-

graphical regions. In Africa and South Asia, however, the incidence of gastric cancer in these areas is much lower than in other countries in spite of the high prevalence of the *H. pylori* infection^[5]. Such a disparity has also been found in other local regions^[6]. Analysis of global strains reveals seven populations of *H. pylori* that are consistent with their geographical origin^[7-10]. These current populations derive from six ancestral populations^[7]. It appears that the ancestry, genomic contents and carcinogenic potentials are diversified among *H. pylori* populations. Studies at a population level have improved our understanding of gastric carcinogenesis associated with the *H. pylori* infection.

GENETIC DIVERSITY AND POPULATIONS OF *H. PYLORI*

There are three types of bacterial population structure: clonal, panmictic and endemic^[11]. If intra-species or inter-species recombination is rare, the genetic diversity of a bacterial species predominantly comes from evolution of the ancestry. This species has a clonal population structure. In a species with high frequency of recombination, introduction of foreign gene fragments into the genome occurs frequently in the evolution history. As foreign genes have a different evolution history, the evolution speed of individual genes is different. In this case, the species possess a panmictic structure. For a bacterial species with a panmictic structure, a temporal clonal structure may occur if it rapidly spread among naïve hosts. In this situation, a bacterial species has an endemic structure.

H. pylori shows great inter-strain variation in genetic content^[12]. None of the individual strains is identical as demonstrated by multiple fingerprinting methods^[13,14]. Sequence divergence is the main cause of this variation. Comparison of two sequenced genomes revealed occurrence of substantial silent mutation in the genetic loci^[15]. A number of mechanisms are involved in the generation of the sequence variation: *H. pylori* shows a higher mutation rate than *Escherichia coli*^[16]. Approximately a quarter of strains possess a mutator-like phenotype. This is attributed to the lack of a functional DNA repair system^[16,17] and error-prone DNA polymerase in *H. pylori*^[18]. Recombination in *H. pylori* is more frequent than in any other organism studied to date^[19]. Foreign DNA from the same species or phage has been found in the bacterium^[15]. Strand slippage mispairing is another mechanism responsible for genetic diversity. A number of homopolymeric tracts and dinucleotide repeat regions are present in the *H. pylori* genome^[20,21], which may cause replication error and subsequently sequence variation. *H. pylori* has a specialized type IV system for uptake of foreign DNA from the same species or other species^[22]. Foreign DNA fragments are subsequently integrated into the genome by recombination. A high frequency of recombination and a high mutation rate in *H. pylori* result in a panmictic structure of the bacterium^[23].

Recombination is a rare genetic event in house-keeping genes. Phylogenetic analysis of highly conserved house-keeping genes (*atpA*, *efp*, *mutY*, *ppa*, *trpC*, *ureI* and *yphC*) has then been used to study populations of *H. pylori*^[24]. Examination of global strains of *H. pylori* reveals that it has seven populations: hpAfrica1, hpAfrica2, hpEastAsia, hpEurope, hpNEAfrica, hpAsia2 and hpSahul^[7-10,25,26]. Some populations could be further divided into subpopulations. For hpEastAsia, there are three subpopulations including hspEAsia, hspAmerind and hspMaori^[27], while hpAfrica1 is split into hspWAfrica and hspSAfrica^[28]. These populations and subpopulations reflect not only their geographical origin but also ethnic groups of their hosts. Spatial separation reduces bacterial recombination between different geographical regions. A weakly clonal population may thus be generated in strains from a particular geographical region^[29]. *H. pylori* spreads mainly through a mode of family transmission^[30,31], leading to a reduced chance of recombination between strains from different ethnic groups. Therefore, strains from different ethnic groups could be distinguished in the phylogenetic analysis. This is one of the features that allows phylogenetic analysis of *H. pylori* to be used to trace the history of human migration.

GENOMIC DIVERGENCE BETWEEN *H. PYLORI* POPULATIONS

H. pylori has been accompanying human hosts for more than 58 000 years^[10]. The genome of *H. pylori* is thus shaped by its human hosts due to the long co-existence^[32]. *H. pylori* strains differ in their affinity to bind blood group antigens expressed in the gastric mucosa^[33]. Strains from Europe bind all three blood group antigens, while Amerindian strains have higher affinity for O blood antigen as this antigen is predominant in Amerindians^[32]. Therefore, the genomic content of *H. pylori* may vary in different populations.

To date, a number of strains of *H. pylori* have been sequenced^[15,34-37]. Of these, the origin and other information of 30 strains are publicly available. These include 14 strains from hpEastAsia (7 from hspEAsia and 7 from hspAmerind subpopulations, respectively), 10 from hpEurope, 5 from hpAfrica1 and 1 from hpAfrica2^[38-41]. All, except for strain B38 from hpEurope, possess *cagA* and the *cag* pathogenicity island (*cag* PAI). The genomic size of *cagA*-positive *H. pylori* ranges from approximately 1.55 mbp to 1.71 mbp with an average of 1.61 mbp. For *cagA*-negative strains, their genome is generally smaller because of the lack of the *cag* pathogenicity island of about 40 kbp. We analyzed the average genomic size of *cagA*-positive *H. pylori* strains from different populations^[42-47]. The average genomic size of strains from hpEurope is approximately 1.65 mbp, which is significantly larger than that from hpEastAsia (1.60 mbp, $P < 0.05$) or hpAfrica1 (1.60 mbp). Consistent with this, strains of hpEurope have the highest number of coding sequences. There was a statistically significant difference between hpEurope and

hpEastAsia in the average genomic size and number of CDS. The size of bacterial genomes is primarily determined by two counteracting processes: the gain of new genes by gene duplication or by horizontal gene transfer; and the decay of non-essential genes^[15]. Both of these processes have been observed in *H. pylori*. Recombination, conjugation, insertion elements, mutation and slipped-strain replication lead to gene gain or loss^[34]. They may be involved in the variability of genomic size among *H. pylori* of different populations.

For bacteria, a larger genome requires more metabolic activities and consumes more energy^[48,49]. Therefore bacteria containing a larger genome may have a lowered capacity of growth, reducing its competitive ability with other bacteria in the same ecological niche. This leads to a decreased spread of bacteria. It is well known that *H. pylori* is less prevalent in Western countries than in other parts of the world^[50]. Hygiene, economical incomes and social status have been suggested to contribute to this differential prevalence^[51]. It is arguable that a larger genome of *H. pylori* strains in Western countries may also contribute to the low prevalence of the infection in this region.

Comparison of the genomic content of *H. pylori* from different populations revealed differences in the compositions of outer membrane proteins and central metabolism^[39]. Compared with hpEurope, strains from hpEastAsia tend to have fewer genes of these two categories. There are a total of 12 genes in *H. pylori* involved in molybdenum metabolism, including those encoding proteins for molybdenum transport and cofactor synthesis and a molybdenum-containing enzyme. A massive decay of molybdenum-related genes occurs in strains from hpEastAsia. At least five genes are fragmented due to mutations. The molybdenum-containing enzyme functions in electron transfer and responses to oxidative and acid stress^[52]. It is probable that in hpEastAsia populations, *H. pylori* use alternative pathways for the purpose^[39]. Outer membrane proteins consist of several paralog families interacting with the human host^[53,54]. In the hpEastAsia population, there is a tendency for a reduced number of these proteins resulting from mutations and recombination. Therefore it appears that *H. pylori* from hpEastAsia have evolved to possess a reduced genome.

The *cag* PAI is a 40-kb DNA fragment which contains 27 to 31 genes flanked by 31-bp direct repeats^[55]. It encodes CagA, the major virulence determinant of *H. pylori* and components of a type IV secretion system^[56,57]. The latter translocates CagA into host cells^[58]. Once inside the host cells, CagA binds to a number of host cell proteins disrupting intracellular signaling systems *via* tyrosine phosphorylation-dependent or -independent pathways^[59]. This causes elongation and loss of polarity of host cells, promoting proliferation and inflammation. The presence of the *cag* PAI in *H. pylori* is associated with increased risk of severe gastritis, atrophic gastritis, and distal gastric cancer compared with strains that lack the *cag* island^[60-62].

A marked difference lies between hpEurope and hpEast-

Asia in the prevalence of strains possessing the *cag* PAI. Approximately 60% to 70% of Western *H. pylori* strains express CagA^[61,63], indicating the presence of the *cag* PAI. In East Asia, however, almost 100% of strains possess the *cag* PAI irrespective of pathology^[64,65]. It is believed that the *cag* PAI is deleted in Western strains resulted from recombination between the repeats flanking the island^[66]. This results in a reduced genomic size by approximately 40 kbp. In addition, it has been demonstrated that the prevalence of strains with an intact *cag* PAI is the lowest in Western countries^[67]. As described above, strains from hpEurope are coincidentally 40 kbp larger than the average genomic size of *H. pylori*. Thus, the occurrence of *cagA*-negative strains in hpEurope is probably due to the evolution of the bacterium towards a smaller genome.

VARIATION IN THE CARCINOGENIC POTENTIAL OF *H. PYLORI* POPULATIONS

The incidence of gastric cancer varies in different geographical regions. It is higher in East Asian countries than in any other countries when age-standardized rates are considered^[68]. In some countries of West Africa and South America, there is also an increased incidence of gastric cancer^[69,70]. The geographic difference in the incidence of gastric cancer can be attributed partially to the difference in the prevalence of *H. pylori* infection^[71]. A high prevalence of virulent strains of *H. pylori* is another contributing factor to the high incidence of gastric cancer in East Asia. Virulent strains possess the *cag* PAI and express *VacA*. There is, however, a disparity between the prevalence of *H. pylori* or virulent strains and the incidence of gastric cancer. In Linq County, China, the incidence of gastric cancer is extremely high, while in its neighboring county Cangshan the incidence is very low^[72,73]. The rate of *H. pylori* infection and the proportion of virulent strains in these two counties, however, show no significant difference^[74]. Similar results have been found when comparing two regions in Mexico with contrasting incidence of gastric cancer^[75]. These results indicate differential incidence of gastric cancer among different geographical regions is attributable to other bacterial factors.

To explore other bacterial factors related to carcinogenesis, the phylogeographical origin of *H. pylori* has been investigated^[76]. In the Andean mountain region of Colombia, habitants have a high incidence of gastric cancer (150 per 100 000 people per year)^[77,78], while habitants in the coastal line 200 kilometers away, have a very low incidence of gastric cancer (6/100 000)^[77,78]. The prevalence of the infection and virulent strains of *H. pylori* in these two regions are similar^[75]. All *H. pylori* strains isolated from the Andean region, however, are from hpEurope, in contrast to strains isolated from the coastal line which are mainly from hpAfrica1^[76]. Furthermore, strains from the Andean region caused more severe mucosal inflammation and more DNA damage in epithelial cells. This suggests that strains of hpEurope probably have an

increased carcinogenic potential compared with those of hpAfrica1^[76]. Ancestral origin of the bacterium could be an important factor contributing to gastric carcinogenesis. This conclusion is further supported from a study conducted in Malaysia^[7]. There are three ethnic groups in the country: Malay, Indian and Chinese. The infection rate of *H. pylori* in Malays is lower than that in Indian and Chinese subjects^[79]. The incidence of gastric cancer, however, is similar in Malays and Indians, but is much lower than in the Chinese^[80]. Analysis of the ancestral origin of *H. pylori* found that strains isolated from both Malay and Indian subjects belonged to hpAsia2, whereas those isolated from Chinese subjects belonged to hpEastAsia. This suggests a different potential for carcinogenesis between hpAsia2 and hpEastAsia. *H. pylori* populations generally reflect the geographical regions from which they are isolated. Differences in the incidence of gastric cancer among geographical regions could be in part attributed to different populations of *H. pylori*. Further study is required to investigate other bacterial factors involved in the carcinogenesis.

CONCLUSION

In summary, geographical separation reduces the frequency of recombination between *H. pylori* strains from a local area and those from outside. This leads to the formation of a clonal population structure of *H. pylori* in the local area. Thus, populations of *H. pylori* could be identified through examination of global strains. For each population, *H. pylori* have experienced relatively separate evolution processes, resulting in genomic diversity and differential potential for carcinogenesis. Further study to characterize these differences may help elucidate mechanisms involved in the development of gastric cancer induced by *H. pylori*.

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Alterations in the human epidermal growth factor receptor 2-phosphatidylinositol 3-kinase-v-Akt pathway in gastric cancer

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Abstract

AIM: To investigate human epidermal growth factor receptor 2 (HER2)-phosphatidylinositol 3-kinase (PI3K)-v-Akt murine thymoma viral oncogene homolog signaling pathway.

METHODS: We analyzed 231 formalin-fixed, paraffin-embedded gastric cancer tissue specimens from Japanese patients who had undergone surgical treatment. The patients' age, sex, tumor location, depth of invasion, pathological type, lymph node metastasis, and pathological stage were determined by a review of the medical records. Expression of HER2 was analyzed by immunohistochemistry (IHC) using the HercepTest™ kit. Standard criteria for HER2 positivity (0, 1+, 2+, and 3+) were used. Tumors that scored 3+ were considered HER2-positive. Expression of phospho Akt (pAkt) was also analyzed by IHC. Tumors were considered pAkt-positive when the percentage of positive tumor cells was 10% or more. PI3K, catalytic, alpha polypeptide (PIK3CA) mutations in exons 1, 9 and 20 were analyzed by pyrosequencing. Epstein-Barr virus (EBV) infection was analyzed by *in situ* hybridization targeting EBV-encoded small RNA (EBER) with an EBER-RNA probe. Microsatellite instability (MSI) was analyzed by polymerase chain reaction using the mononucleotide markers BAT25 and BAT26.

RESULTS: HER2 expression levels of 0, 1+, 2+ and 3+ were found in 167 (72%), 32 (14%), 12 (5%) and 20 (8.7%) samples, respectively. HER2 overexpression (IHC 3+) significantly correlated with intestinal histological type (15/20 vs 98/205, $P = 0.05$). PIK3CA mutations were present in 20 cases (8.7%) and significantly correlated with MSI (10/20 vs 9/211, $P < 0.01$).

The mutation frequency was high (21%) in T4 cancers and very low (6%) in T2 cancers. Mutations in exons 1, 9 and 20 were detected in 5 (2%), 9 (4%) and 7 (3%) cases, respectively. Two new types of PIK3CA mutation, R88Q and R108H, were found in exon1. All PIK3CA mutations were heterozygous missense single-base substitutions, the most common being H1047R (6/20, 30%) in exon20. Eighteen cancers (8%) were EBV-positive and this positivity significantly correlated with a diffuse histological type (13/18 *vs* 93/198, $P = 0.04$). There were 7 cases of lymphoepithelioma-like carcinomas (LELC) and 6 of those cases were EBV-positive (percent/EBV: 6/18, 33%; percent/all LELC: 6/7, 86%). pAkt expression was positive in 119 (53%) cases but showed no correlation with clinicopathological characteristics. pAkt expression was significantly correlated with HER2 overexpression (16/20 *vs* 103/211, $P < 0.01$) but not with PIK3CA mutations (12/20 *vs* 107/211, $P = 0.37$) or EBV infection (8/18 *vs* 103/211, $P = 0.69$). The frequency of pAkt expression was higher in cancers with exon20 mutations (100%) than in those with exon1 (40%) or exon9 (56%) mutations. One case showed both HER2 overexpression and EBV infection and 3 cases showed both PIK3CA mutations and EBV infection. However, no cases showed both PIK3CA mutations and HER2 overexpression. One EBV-positive cancer with PIK3CA mutation (H1047R) was MSI-positive. Three of these 4 cases were positive for pAkt expression. In survival analysis, pAkt expression significantly correlated with a poor prognosis (hazard ratio 1.75; 95%CI: 1.12-2.80, $P = 0.02$).

CONCLUSION: HER2 expression, PIK3CA mutations and EBV infection in gastric cancer were characterized. pAkt expression significantly correlates with HER2 expression and with a poor prognosis.

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Key words: Human epidermal growth factor receptor 2; Phosphatidylinositol 3-kinase; Catalytic; Alpha polypeptide; Epstein-Barr virus; Akt; Gastric cancer

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INTRODUCTION

Gastric cancer is one of the most common cancer types

and the second leading cause of cancer-related deaths worldwide^[1]. Genetic and epigenetic alterations play important roles in the development and progression of these tumors^[1,2]. Considerable attention has been given to the potential role of the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway in gastric cancer^[3,4]. Various alterations, such as activation of growth factor receptors, PI3K, catalytic, alpha polypeptide (PIK3CA) mutations and inactivation of phosphatase and tensin homolog (PTEN) lead to activation of the PI3K-Akt signaling pathway. With regards to growth factor receptors, there is growing evidence that human epidermal growth factor receptor 2 (HER2) is a key driver of tumorigenesis and an important biomarker in gastric cancer. The amplification or overexpression of HER2 is observed in 7%-34% of these cases^[5-9].

PIK3CA is mutated in a wide variety of human tumor types^[10,11], including gastric cancers^[12-15]. Activating mutations in this gene up-regulate the PI3K-Akt signaling pathway, making it a potentially useful therapeutic target. For example, oncogenic mutations of PIK3CA reportedly render breast cancers more resistant to treatment with the anti-HER2 receptor antibody trastuzumab^[16]. Thus, this signaling pathway is thought to be one of the mechanisms underlying resistance to trastuzumab. Trastuzumab has recently been approved for treatment of advanced gastric cancers^[5,6].

Pyrosequencing-based methods facilitate the identification of low-frequency tumor mutations and allow a more accurate assessment of tumor mutation burden^[17]. PIK3CA mutations have been detected in 4%-25% of gastric cancers^[12-15]. However, in most previous studies, only exons 9 and 20 hot spot mutations in PIK3CA were analyzed by DNA sequencing. Moreover, the association between HER2 expression and PIK3CA mutations in gastric cancer has not been reported.

A significant correlation has been found between Epstein-Barr virus (EBV) and the methylation of multiple genes in gastric cancers^[18-20]. EBV infection reportedly induces PTEN expression loss through CpG island methylation of its promoter, leading to activation of the PI3K-Akt signaling pathway, in EBV-associated gastric cancer^[21].

The aim of our present study was to systematically characterize HER2 expression, PIK3CA mutations, and EBV infection, all of which are involved in the PI3K-Akt signaling pathway, in a large cohort of gastric cancers ($n = 231$). We wished to determine the prevalence of each of these factors with a high precision and thereby correlate them with clinicopathological and molecular features of gastric lesions, including microsatellite instability (MSI) and phospho Akt (pAkt) expression.

MATERIALS AND METHODS

Tissue samples

A total of 231 formalin-fixed, paraffin-embedded (FFPE) gastric cancer tissue specimens from Japanese patients who had undergone surgical treatment was analyzed in

Table 1 Clinicopathological characteristics of patients with gastric cancer

Variables (n = 231)		n (%)
Sex	Male	157 (68)
	Female	74 (32)
Age (yr)	Median (range)	71 (25-91)
Location	Cardias	82 (35)
	Body	62 (27)
	Antrum	83 (36)
	Unknown	4 (2)
Depth of invasion	T2	125 (54)
	T3	92 (40)
	T4	14 (6)
Lymph node metastasis	N0	65 (28)
	N+	158 (68)
	N1	73 (32)
	N2	56 (24)
	N3	29 (13)
Stage	Unknown	8 (3)
	I B	49 (21)
	II	45 (19)
	III A + III B	82 (35)
	IV	51 (22)
	Unknown	4 (2)
Lauren histotype	Intestinal	113 (49)
	Diffuse	112 (48)
	Others	6 (3)

this study. The patients' age, sex, tumor location, depth of invasion, pathological type, lymph node metastasis, and pathological stage were determined by a review of their medical records. Clinicopathological findings were determined according to the criteria of the Japanese Research Society for Gastric Cancer (Table 1). Our institutional review committee approved the study.

Immunohistochemistry

HER2 expression was analyzed using the HercepTest™ kit (DAKO, Carpinteria, CA) by manual sample processing in accordance with the manufacturer's instructions. Standard criteria for HER2 positivity (0, 1+, 2+ and 3+) were used. Tumors that scored 3+ were considered HER2-positive. For the immunohistochemical analysis of pAkt, FFPE specimens were processed using SignalStain Boost Detection Reagent (Cell Signaling Technology, Beverly, MA). Briefly, 5- μ m-thick sections were dewaxed in xylene, rehydrated in ethanol, and heated with target retrieval solution (DAKO) in an autoclave for antigen retrieval. Endogenous peroxidase was blocked by incubation with 0.3% hydrogen peroxide in methanol for 10 min. The tissue sections were then washed twice with tris-buffered saline (TBS) and preblocked with 10% goat serum in TBS for 60 min. After washing with TBS, the sections were incubated with an anti-phospho-Akt (Ser473) polyclonal antibody (D9E, Cell Signaling Technology) at a dilution of 1:100 for 30 h at 4 °C. The sections were washed three times in TBS and incubated with SignalStain Boost Detection Reagent for 45 min. After three further washes in TBS, a diamino-benzidine tetrahydrochloride working solution was applied. Finally, the sections were

counterstained with hematoxylin. Tumors were considered pAkt-positive when the percentage of positive tumor cells was 10% or more^[22]. Only clear staining of the tumor cell nucleus and/or cytoplasm was considered positive.

Mutation analysis of the PIK3CA gene by pyrosequencing

Genomic DNA was extracted from tumor specimens and mutations in exon9 and exon20 of the *PIK3CA* gene were analyzed by pyrosequencing as described previously^[23,24]. We also developed a pyrosequencing assay to detect PIK3CA exon1 mutations using the primer sets exon1-RS1 (5'-GGGAAGAATTTTTTGGATGAAACA-3' for the biotinylated forward primer and 5'-GGTTGCCTACTGGTTCAATTACTT-3' for the reverse primer) and exon1-RS2 (5'-CGGCTTTTTCAACCCTTTTT-3' for the forward primer and 5'-ATTTCTCGATTGAGGATCTTTTCT-3' for the biotinylated reverse primer). Each polymerase chain reaction (PCR) mix contained the forward and reverse primers (each 10 μ mol/L), a 25 mmol/L dNTP mix with dUTP, 75 mmol/L MgCl₂, 1 \times PCR buffer, 1.0 U of exTaq, and 2 μ L of template DNA in a total volume of 25 μ L. PCR conditions were as follows: initial denaturing at 95 °C for 5 min; 50 cycles of 94 °C for 20 s, 50 °C for 20 s and 74 °C for 40 s; and a final extension at 72 °C for 1 min. The PCR products (each 25 μ L) were sequenced using the PyroMark kit and Pyrosequencing PSQ96 HS System (Qiagen, Valencia, CA).

In situ hybridization for EBER1

The presence of EBV in the carcinoma tissues was evaluated by *in situ* hybridization (ISH) targeting of EBV-encoded small RNA (EBER-ISH) with an EBER-RNA probe (Dako Cytomation).

Microsatellite instability analysis

MSI was analyzed by PCR using the mononucleotide markers (BAT25 and BAT26). Based on the number of markers showing instability per tumor sample, cancers were divided into two groups; those with one or more of the two markers displaying MSI and those with no instability (microsatellite stable).

Statistical analysis

For all statistical analysis, the JMP program was used. All *P* values were two-sided and statistical significance was set at *P* \leq 0.05. For categorical data, the χ^2 test was used. For survival analysis, Kaplan-Meier method and log-rank test were used. For analysis of cancer-specific mortality, we excluded surgery-related deaths (deaths within one month of surgery).

RESULTS

HER2 expression in gastric cancer tissues

HER2 expression levels of 0, 1+, 2+ and 3+ were found in 167 (72%), 32 (14%), 12 (5%) and 20 (8.7%) samples, respectively (Figure 1). HER2 overexpression (IHC 3+) significantly correlated with intestinal histological type

Table 2 Clinicopathological characteristics of patients with gastric cancer based on human epidermal growth factor receptor 2 expression, phosphatidylinositol 3-kinase, catalytic, alpha polypeptide mutations and Epstein-Barr virus infection *n* (%)

		HER2		<i>P</i> value	Mutation (<i>n</i> = 20)	PIK3CA		<i>P</i> value	EBV		<i>P</i> value
		Positive (<i>n</i> = 20)	Negative (<i>n</i> = 211)			Wild type (<i>n</i> = 211)	Positive (<i>n</i> = 18)		Negative (<i>n</i> = 204)		
Sex	Male	15 (75)	142 (67)	0.48	13 (65)	144 (68)	0.77	14 (78)	138 (68)	0.38	
	Female	5 (25)	69 (33)		7 (35)	70 (32)		4 (22)	66 (32)		
Age	Median	69 (50-84)	71 (25-91)	0.26	71 (25-85)	70 (38-91)	0.40	72 (48-90)	70 (38-91)	0.41	
Location	Cardias	10 (50)	72 (34)	0.49	5 (25)	77 (36)	0.31	8 (44)	73 (36)	0.70	
	Body	5 (25)	57 (27)		4 (20)	58 (27)		5 (28)	55 (27)		
	Antrum	5 (25)	78 (37)		10 (50)	73 (35)		5 (28)	75 (37)		
	Unknown	0	4 (2)		1 (5)	2 (1)		0	1 (0)		
Depth	T2	12 (60)	113 (54)	0.48	8 (40)	117 (55)	0.15	12 (67)	106 (52)	0.35	
	T3	8 (40)	84 (40)		9 (45)	83 (39)		6 (33)	85 (42)		
	T4	0	14 (6)		3 (15)	11 (5)		0	13 (6)		
L/N meta	N0	5 (25)	60 (28)	0.71	4 (20)	61 (29)	0.37	3 (17)	57 (28)	0.28	
	N+	14 (70)	144 (68)		16 (80)	142 (67)		14 (77)	140 (69)		
	N1	5 (25)	68 (32)		8 (40)	65 (31)		8 (44)	63 (31)		
	N2	6 (30)	50 (24)		6 (30)	50 (24)		2 (11)	53 (26)		
	N3	3 (15)	26 (12)		2 (10)	27 (13)		4 (22)	24 (12)		
	Unknown	1 (5)	7 (3)		0	8 (4)		1 (6)	7 (3)		
Stage	I	5 (25)	44 (21)	0.89	1 (5)	48 (23)	0.14	3 (17)	41 (20)	0.98	
	II	3 (15)	42 (20)		7 (35)	38 (18)		4 (22)	39 (19)		
	III	6 (30)	76 (36)		8 (40)	74 (35)		6 (33)	75 (37)		
	IV	5 (25)	46 (22)		4 (20)	47 (22)		4 (22)	46 (23)		
	Unknown	1 (5)	3 (1)		0	4 (2)		1 (6)	3 (1)		
Lauren histotype	Intestinal	15 (75)	98 (46)	0.05	14 (70)	99 (47)	0.13	5 (28)	105 (51)	0.04	
	Diffuse	5 (25)	107 (51)		6 (30)	106 (50)		13 (72)	93 (46)		
	LELC	0	6 (3)		2 (10)	4 (2)		5 (28)	0		
	Others	0	6 (3)		0	6 (3)		0	6 (3)		
MSI		2 (10)	28 (13)	0.72	10 (50)	20 (9)	< 0.01	1 (6)	26 (13)	0.36	
pAkt		16 (84)	103 (51)	< 0.01	12 (63)	107 (53)	0.37	8 (47)	103 (52)	0.69	
3 yr OS (%)		29.4	59.2	0.24	57.3	56.8	0.59	57.4	57.3	0.98	

MSI: Microsatellite instability; LELC: Lymphoepithelioma-like carcinoma; HER2: Human epidermal growth factor receptor 2; PIK3CA: Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide mutations; EBV: Epstein-Barr virus; pAkt: Phospho Akt; OS: Overall survival.

Table 3 Frequencies of phosphatidylinositol 3-kinase, catalytic, alpha polypeptide mutations detected in gastric cancer tissues

	Mutation	Overall frequency	Percent/total cases	Percent/mutated cases	Microsatellite instability
Exon1	R88Q	1	0.4	5	1
	R108H	4	1.7	20	1
	Total	5	2.2		2
Exon9	E542K	5	2.2	25	1
	E545K	2	0.9	10	1
	E545G	2	0.9	10	1
	Total	9	4.0		3
Exon20	H1047Y	1	0.4	5	1
	H1047R	6	2.6	30	4
	Total	7	3.0		5

(15/20 *vs* 98/205, *P* = 0.05, Table 2). Three-year survival rates were 29% in patients with HER2 overexpression and 59% in cases without HER2 overexpression, respectively [hazard ratio (HR) 1.73; 95%CI: 0.87-3.14, *P* = 0.24].

Mutations of the PIK3CA gene in gastric cancer tissues

PIK3CA mutations were present in 20 cases (8.7%) (Table 2 and Figure 2). The mutation frequency was high (21%)

in T4 cancers and low (6%) in T2 cancers. Mutations in exons 1, 9 and 20 of PIK3CA were detected in 5 (2%), 9 (4%) and 7 (3%) cases, respectively (Table 3). One case had multiple PIK3CA mutations (R108H and E542K). The exon20/exon9 prevalence ratio was 0.78 (7/9). Two new types of PIK3CA mutations, R88Q and R108H, were detected in exon1. All mutations were heterozygous missense single-base substitutions and the most common mutation was H1047R (6/20; 30%) in exon20. PIK3CA mutations were also found to significantly correlate with MSI (10/20 *vs* 9/211, *P* < 0.01) but not with other clinicopathological characteristics. The three-year survival rates were 57% in patients with PIK3CA mutations and 57% in cases without PIK3CA mutations, respectively (HR 1.37; 95%CI: 0.68-3.26, *P* = 0.59).

EBV infection

Eighteen samples in our cohort (8%) were EBV-positive and this positivity significantly correlated with diffuse histological type (13/18 *vs* 93/198, *P* = 0.04) (Table 2 and Figure 3). There were 7 cases of LELC and 6 of those cases were EBV-positive (percent/EBV: 6/18, 33%; percent/all LELC: 6/7, 86%). The three-year survival rates were 57% in patients with EBV infection and 57% in those without EBV infection (HR 0.81; 95%CI:

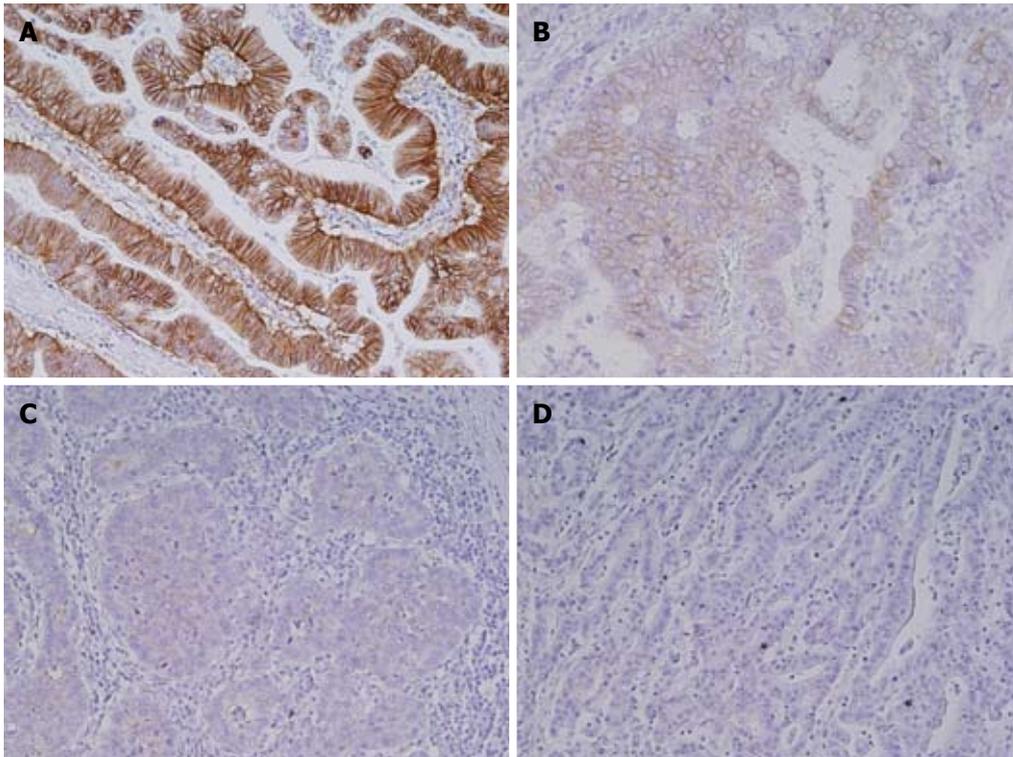


Figure 1 Immunohistochemical analysis of human epidermal growth factor receptor 2 in gastric cancer tissues. A: Human epidermal growth factor receptor 2 (HER2) 3+; B: HER2 2+; C: HER2 1+; D: HER2 0. Original magnification, $\times 200$.

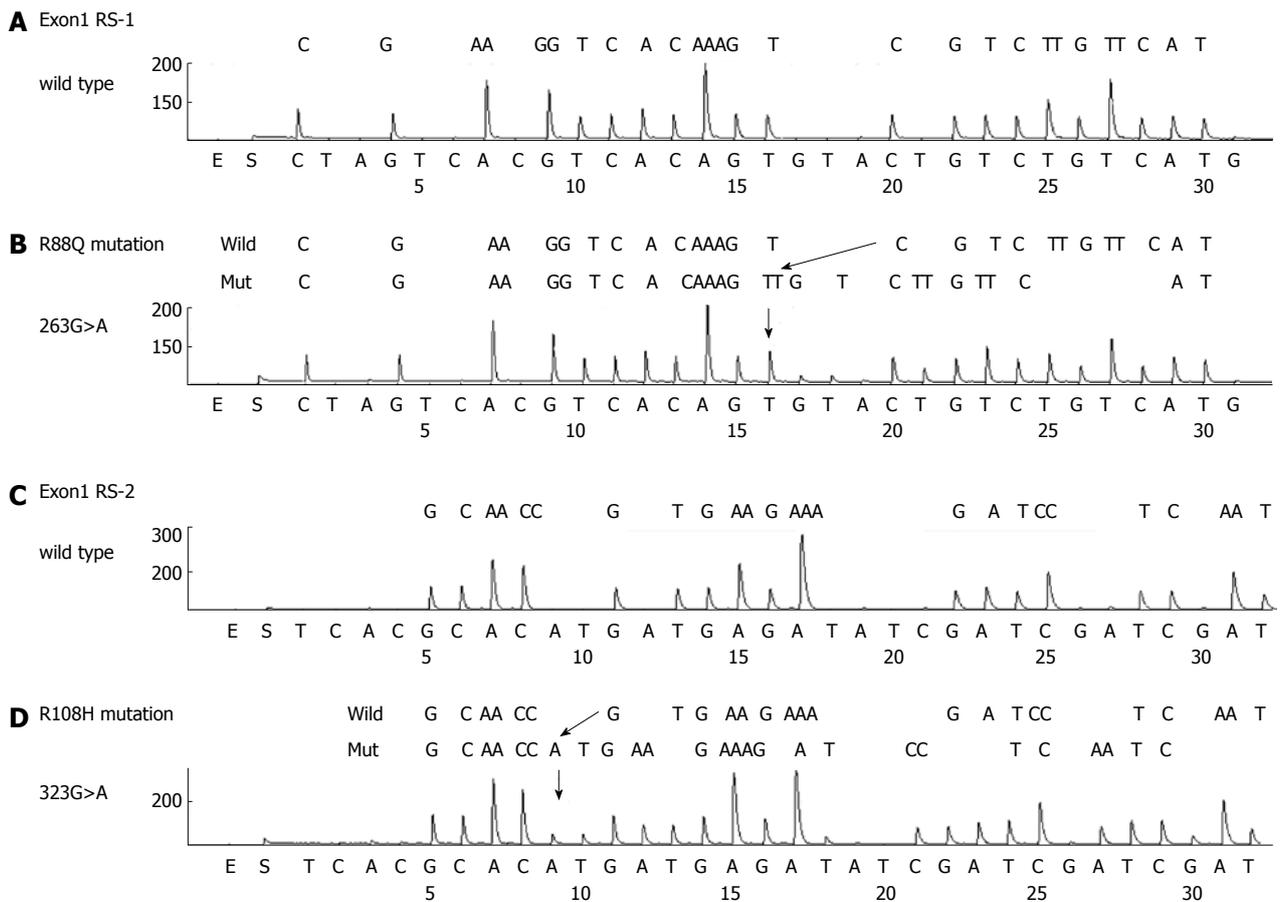


Figure 2 Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide mutations detected by pyrosequencing in gastric cancer tissues. A: Exon1 RS1 wild type; B: 263G>A (R88Q) mutation; C: Exon1 RS2 wild type; D: 323G>A (R108H) mutation.

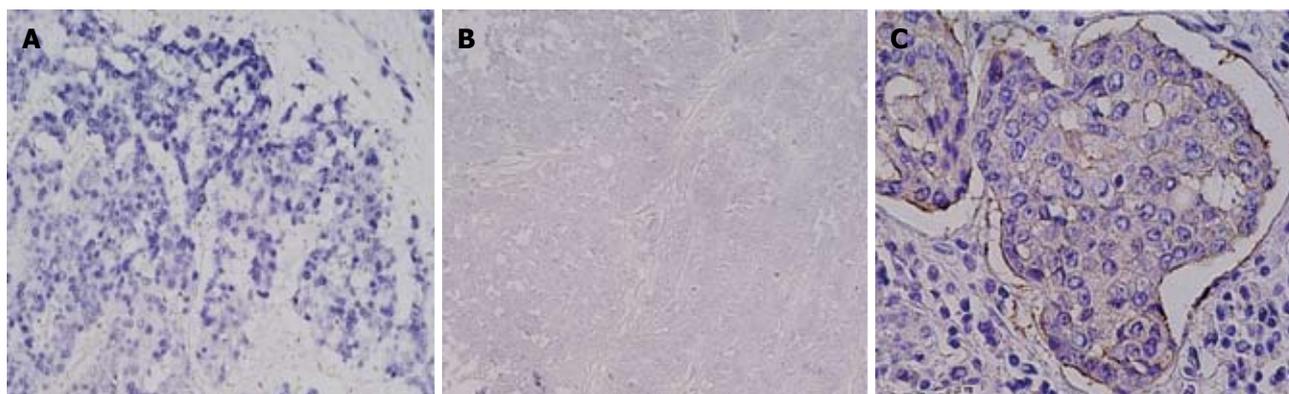


Figure 3 *In situ* hybridization analysis of Epstein-Barr virus-encoded small RNA-1 and human epidermal growth factor receptor 2 immunohistochemical expression in gastric cancer tissues. A: Gastric adenocarcinoma positive for Epstein-Barr virus-encoded small RNA-1 (EBER-1); B: Gastric adenocarcinoma negative for EBER-1; C: Immunohistochemical analysis of human epidermal growth factor receptor 2 (HER2) in an Epstein-Barr virus-positive and HER2-positive case. Original magnification, $\times 200$.

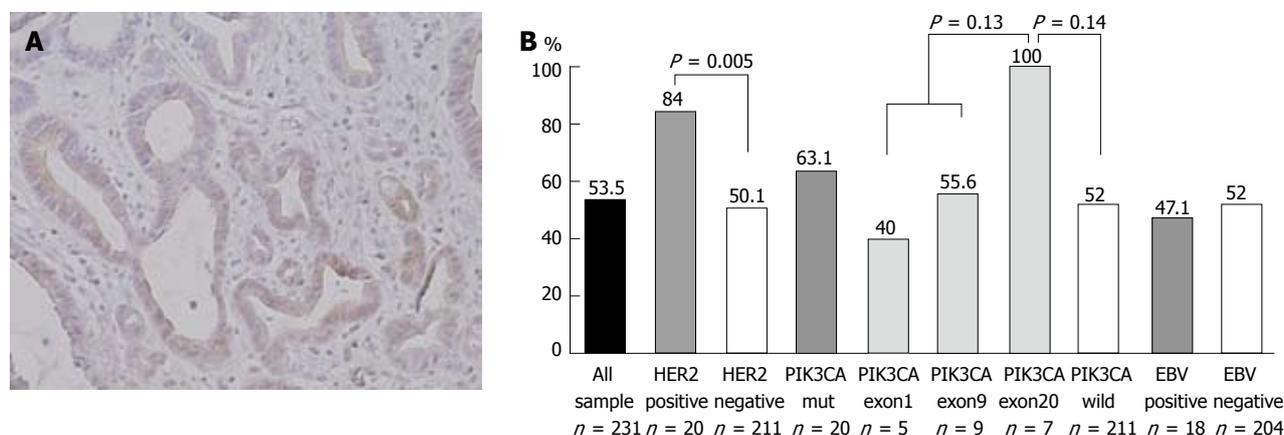


Figure 4 Immunohistochemical analysis and assessment of phospho Akt positivity based on molecular alterations in gastric cancer tissues. A: Gastric adenocarcinoma showing phospho Akt (pAkt) positivity. Original magnification, $\times 200$; B: pAkt expression significantly correlates with human epidermal growth factor receptor 2 (HER2) overexpression ($P < 0.01$) but not with phosphatidylinositol 3-kinase, catalytic, alpha polypeptide (PIK3CA) mutations ($P = 0.37$) or Epstein-Barr virus (EBV) infection ($P = 0.69$).

0.36-2.31, $P = 0.98$).

Association of HER2 overexpression, PIK3CA mutations and EBV infection

One of our cases showed both HER2 overexpression and EBV infection and 3 cases showed both PIK3CA mutations and EBV infection. However there were no cases showing both PIK3CA mutations and HER2 overexpression. Three of the 4 cases were positive also for pAkt expression. PIK3CA mutations were present in 3 EBV-positive cancers, including 2 cases of LELC (2/5, 40%). One EBV-positive cancer with a PIK3CA mutation (H1047R) was MSI-positive.

pAkt expression

pAkt expression was positive in 119 (53%) of our cases but this showed no correlation with clinicopathological characteristics (Figure 4A). On the other hand, pAkt expression was found to be significantly correlated with HER2 overexpression (16/19 *vs* 103/204, $P < 0.01$) but not with PIK3CA mutations (12/19 *vs* 107/204, $P = 0.37$)

or EBV infection (8/17 *vs* 103/198, $P = 0.69$) (Table 2). The frequency of pAkt expression was higher in cancers with exon20 mutations (100%) than in those with exon1 (40%) or exon9 (56%) mutations of PIK3CA, although this difference did not reach statistical significance (Figure 4B). The five-year survival rates were 37% in patients with pAkt expression and 59% in those without pAkt expression (HR 1.75; 95%CI: 1.12-2.80, $P = 0.02$) (Figure 5). Hence, pAkt expression significantly correlates with a poor prognosis in gastric cancer.

DISCUSSION

In our present study, we systematically characterized HER2 expression, PIK3CA mutations and EBV infection, all of which are involved in the PI3K-Akt signaling pathway, in a large cohort of patients with gastric cancer ($n = 231$). We aimed to determine the prevalence of these characteristics with a high level of precision and to correlate them with clinicopathological and molecular features, such as MSI and pAkt expression.

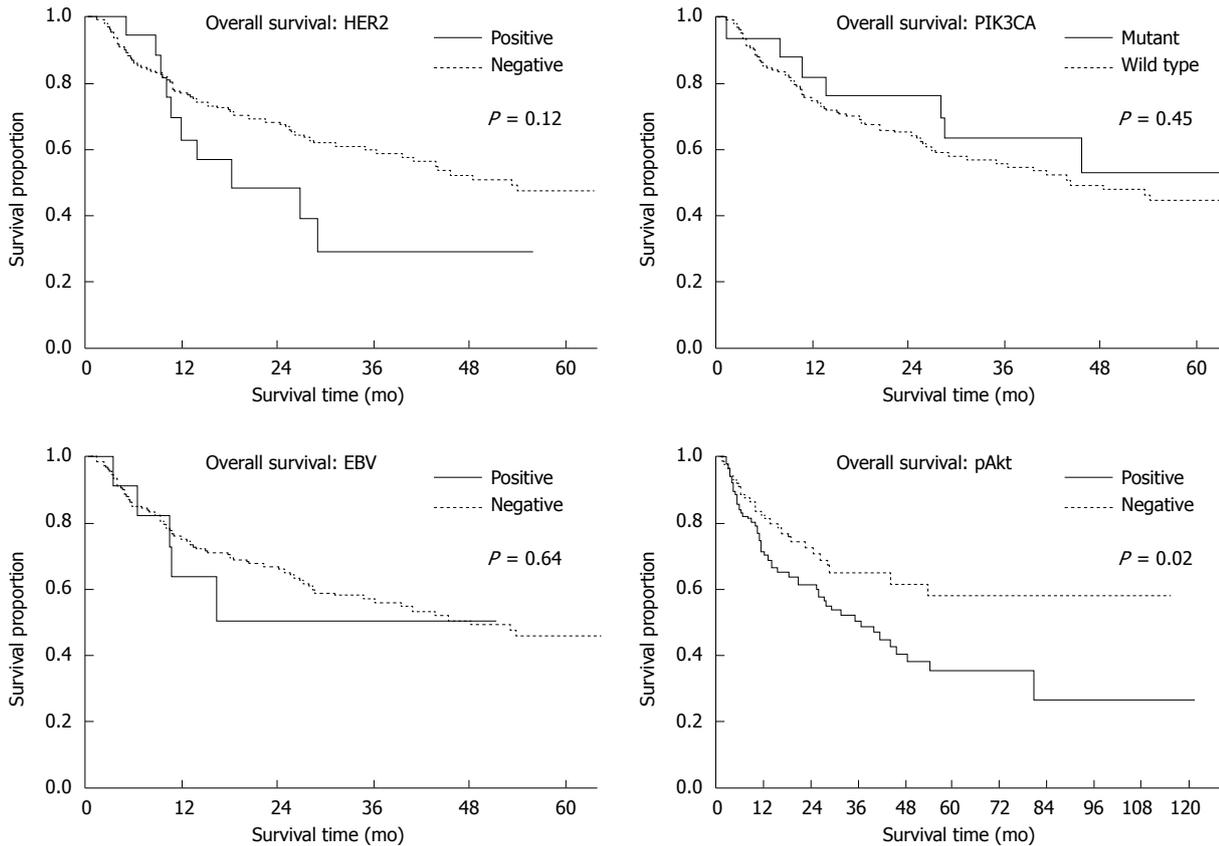


Figure 5 Survival analysis of gastric cancer patients. Three year survival of human epidermal growth factor receptor 2 (HER2)-positive vs HER2-negative, 29.1 mo vs 59.4 mo; Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide (PIK3CA) mutation vs wild type, 63.7 mo vs 56.3 mo; Epstein-Barr virus (EBV)-positive vs EBV-negative, 51.3 mo vs 57.6 mo; And phospho Akt (pAkt)-positive vs pAkt-negative, 50.7 mo vs 64.8 mo. Five year survival of pAkt-positive vs pAkt-negative cases, 35.5 mo vs 58.1 mo.

HER2 overexpression (IHC 3+) was present in 20 samples (8.4%), a value that is within the range (7%-34%) reported in the current literature^[5-9]. HER2 overexpression was found to significantly correlate with the intestinal histological type. Hence, the frequency of HER2 expression may depend on, at least in part, the distribution of histology in a cohort of gastric cancer samples. Some studies have suggested that HER2 positivity in gastric cancer is associated with poor outcomes and aggressive disease, but the results are conflicting. We found for the first time in our present analyses that HER2 overexpression significantly correlates with pAkt expression in gastric cancer tissues. Moreover, pAkt expression correlated with a poor prognosis in these patients. Thus, the HER2-Akt axis may play an important role in gastric cancer.

Pyrosequencing-based methods facilitate the identification of low-frequency tumor mutations and allow a more accurate assessment of tumor mutation burden^[17,23,24]. We characterized PIK3CA mutations in gastric cancer tissues using pyrosequencing for the first time. The overall prevalence of PIK3CA mutations was found in our analysis to be 8.7%, a value that is within the previously reported range (4% to 25%)^[10,12-15]. The mutation frequency was found to be high (21.4%) in T4 cancers and low (6.4%) in T2 cancers in our sample cohort. Thus, PIK3CA mutations appear to be late events in gastric carcinogenesis,

leading to tumor progression. These patients might therefore be appropriate for targeted therapies directed against the PI3K pathway.

The most common PIK3CA mutation found in our analysis was H1047R, which was also found previously^[15]. Importantly, two new types of mutations were found in exon1. To our knowledge, PIK3CA mutations involving residues 88 and 108 (R88Q and R108H) have been never reported previously in gastric cancer, nor described in the COSMIC database, despite the large number of previous studies in which this region was investigated. These mutations have been detected in several other types of cancer tissues^[25]. Importantly also, these mutations have been reported to be gain-of-function^[26-28]. Our present results thus have potential clinical implications since the mutational status of PIK3CA could stratify patients for genotype-based molecular therapies targeting the PI3K pathway. Hence, exon1 of PIK3CA should be analyzed in gastric cancer patients in these clinical settings.

PIK3CA mutations were found to be significantly associated with the MSI phenotype in our experiments. An association between PIK3CA mutations and MSI has been reported, or at least suggested, for both gastric and colon cancers^[12,13,29]. We found in our present study that PIK3CA mutations in cancers with MSI are distributed in exon1, exon9 and exon20. These results further sup-

port the notion that PIK3CA is one of the most important oncogenes activated by missense mutations in MSI-positive gastric cancers.

The frequency of pAkt expression was found to be higher in cancers with exon20 mutations (100%) than in those with exon1 (40%) or exon9 (56%) mutations in PIK3CA. These results further support the notion that the functional significance of PIK3CA mutations depends on the mutation type and that the H1047R hotspot mutation has high oncogenic activity.

The previous ToGA study has shown that the addition of trastuzumab to the chemotherapeutic regimen improves survival in patients with advanced gastric or gastroesophageal junction cancer^[5,6]. PIK3CA mutation is one of the mechanisms underlying the resistance to trastuzumab in breast cancer^[30]. Trastuzumab is likely to be effective for HER2-overexpressing breast cancers with no PIK3CA mutations, with possible rescue using HER2-TKIs in cases of relapse^[31]. For HER2-overexpressing breast cancer with PIK3CA mutations, inhibitors against molecules of the PI3K pathway are possibly more effective than anti-HER2 agents, which are unlikely to be beneficial^[32]. In our present study, PIK3CA mutations were not found in gastric cancers with HER2 overexpression. Thus, it is unlikely that PIK3CA mutation is a major mechanism underlying the resistance to trastuzumab in gastric cancer.

HER2 overexpression was found in only one of the 18 EBV-positive gastric cancers in our sample cohort. This result can be explained, at least in part, by the fact that HER2 overexpression and EBV infection significantly correlate with intestinal and diffuse histological types, respectively. On the other hand, PIK3CA mutations were identified in 3 EBV-positive cancers, including 2 cases of LELC (2/5, 40%). Although not analyzed in our current study, EBV infection reportedly inactivates PTEN through the CpG island methylation of its promoter in EBV-associated gastric cancer^[21]. Thus, alterations in the PI3K-Akt signaling pathway in EBV-positive gastric cancers may differ from those in EBV-negative cancers.

Finally, pAkt expression was found to correlate with a poor prognosis in gastric cancer. A significant association between increased pAkt expression and poor prognosis has been reported previously in patients with T3/T4 gastric cancer but not in those with T1/T2 cancer^[33]. It has been reported also that pAkt expression is associated with increased resistance to multiple chemotherapeutic agents in gastric cancer patients, when chemotherapeutic sensitivities were tested using MTT assays^[34]. Thus, Akt activation appears to lead to a poor prognosis and resistance to chemotherapeutic agents in gastric cancer. A positive correlation between a decrease in the pAkt levels after gefitinib administration and tumor apoptotic index in gastric cancer has also been reported^[35]. Further analyses regarding the pAkt status in cancer tissues before and after chemotherapy and molecular targeted therapy will be necessary. Not all Akt activation events can be

explained by HER2 expression, PIK3CA mutations, and EBV infection in gastric cancer. We have reported previously that a dominant negative insulin-like growth factor (IGF)-1 receptor blocks the Akt-1 activation induced by IGF-1 and IGF-2 in gastric cancer cell lines^[36]. Thus, molecular alterations, such as the overexpression of IGF-1 receptor, might be involved in the activation of Akt in gastric cancer and this issue needs to be clarified in the near future.

COMMENTS

Background

Personalized therapy has begun also in advanced gastric cancer through the use of trastuzumab, an anti-human epidermal growth factor receptor 2 (HER2) antibody. Many drugs targeting the phosphatidylinositol 3-kinase (PI3K)-Akt pathway have now been developed and clinical trials are ongoing. An appropriate biomarker is necessary for successful molecular targeted therapy. The alterations of molecules in the PI3K-Akt pathway could be a good biomarker for such drugs.

Research frontiers

Various alterations, such as activation of growth factor receptors, PI3K, catalytic, alpha polypeptide (PIK3CA) mutations and Epstein-Barr virus (EBV) infection lead to activation of the PI3K-Akt signaling pathway. However, clinicopathological and molecular correlates among such alterations have not been clearly addressed. In the present study, the authors identify new clinicopathological and molecular correlations between HER2 expression, PIK3CA mutations, EBV infection and phospho Akt (pAkt) expression in gastric cancer.

Innovations and breakthroughs

This is the first study to systematically characterize HER2 expression, PIK3CA mutations and EBV infection, all of which are involved in the PI3K-Akt signaling pathway, in a large cohort of patients with gastric cancer. The prevalence of these characteristics was thereby determined with a high level of precision and correlations with the clinicopathological and molecular features of gastric cancers, such as microsatellite instability and pAkt expression, could be assessed accurately for the first time.

Applications

The results have potentially important clinical implications since the mutational status of PIK3CA can be used to stratify cancer patients for genotype-based molecular therapies that target the HERs-PI3K pathway.

Terminology

PI3K-Akt pathway: Akt is believed to transduce the major downstream PI3K signals in cancer. Akt regulates cell growth and survival pathways by phosphorylating substrates such as GSK3, forkhead transcription factors, and the TSC2 tumor suppressor protein; PIK3CA: PIK3CA encodes a key enzymatic subunit of PI3K. Gain of function mutations in PIK3CA occur frequently in several cancer types. Hotspots of PIK3CA mutations are located in exons 9 and 20.

Peer review

The authors investigated HER2 expression, PIK3CA mutations and EBV infection in patients with gastric cancer. The results demonstrated that pAkt expression significantly correlates with the prognosis and the HER2 expression status in gastric cancer. This article is important for the further development of molecular targeted therapy in patients with advanced gastric cancer.

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Tumour seeding after percutaneous cryoablation for hepatocellular carcinoma

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Abstract

AIM: To assess the rate and risk factors for tumour seeding in a large cohort of patients.

METHODS: Over an 8-year period, 1436 hepatocellular carcinoma (HCC) patients with 2423 tumour nodules underwent 3015 image-guided percutaneous cryoablation sessions [1215 guided by ultrasonography and 221 by spiral computed tomography (CT)]. Follow-up CT or magnetic resonance imaging was performed every 3 mo. The detailed clinical data were recorded to analyse the risk factors for seeding.

RESULTS: The median follow-up time was 18 (range

1-90) mo. Seeding was detected in 11 patients (0.76%) at 1-24 (median 6.0) mo after cryoablation. Seeding occurred along the needle tract in 10 patients and at a distant location in 1 patient. Seeded tumours usually showed similar imaging and histopathological features to the primary HCCs. Univariate analyses identified subcapsular tumour location and direct subcapsular needle insertion as risk factors for seeding. Multivariate analysis showed that only direct subcapsular needle insertion was an independent risk factor for seeding ($P = 0.017$; odds ratio 2.57; 95%CI: 1.47-3.65). Seeding after cryoablation occurred earlier in patients with poorly differentiated HCC than those with well or moderately differentiated HCC [1.33 ± 0.577 mo vs 11.12 ± 6.896 mo; $P = 0.042$; 95%CI: (-19.115)-(-0.468)].

CONCLUSION: The risk of seeding after cryoablation for HCC is small. Direct puncture of subcapsular tumours should be avoided to minimise seeding.

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Key words: Cryoablation; Hepatocellular carcinoma; Tumour seeding; Clinical feature; Risk factor

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INTRODUCTION

In patients with hepatocellular carcinoma (HCC) superimposed on cirrhosis, orthotopic liver transplantation, surgical resection and percutaneous ablation are considered radical treatments as they provide better survival rates compared with no treatment^[1]. Because of the poor acceptance of surgery and a severe shortage of donor organs, image-guided percutaneous ablation therapies play an important role in the management of HCC. Various local ablation therapies such as percutaneous ethanol injection (PEI), radiofrequency ablation (RFA), microwave (MW) ablation and cryoablation have been developed for the treatment of unresectable HCC. All these procedures require the insertion of long, sharp needles into the liver parenchyma and tumours, which may cause various complications, although the complication rates are low. Tumour seeding is one of the most serious complications, especially in patients who are waiting for liver transplantation^[2]. The reported incidence of seeding after other ablation procedures varies widely: 0.2%-1.4% following PEI^[3,4], 0.005%-12.5% following RFA^[5,6] and 0.75% following MW ablation^[7].

Certain factors have been found to increase the likelihood of needle-tract seeding, including a superficial or subcapsular tumour^[8], high number of needle insertions^[9,10], large needle bore^[9,11], end-cutting needle^[8,9], absent or thin layer of normal liver parenchyma surrounding the needle tract^[9,10], high-grade HCC (moderately or poorly differentiated^[3,8,9], high serum alpha-fetoprotein (AFP) level^[2], tumour volumes > 2 cm³ and immunosuppression^[12].

Argon-helium cryoablation is a new local ablation modality. At one time, this technology caused some authors to question its use in HCC. Most of the bias against this percutaneous setting is based on a theoretical risk of post-procedure haemorrhage. However, the gradual downsizing of cryoprobes has fueled interest in percutaneous use, which offers several potential advantages versus the heat-based ablation modalities^[13]. First, multiple cryoprobes can be used simultaneously to generate a large zone of ablation. Second, the size and shape of the developing ice ball can be readily visualized using intra-procedural computed tomography (CT), magnetic resonance imaging (MRI) or ultrasound (US). Third, in contrast to heat-based ablation, percutaneous cryoablation is a relatively painless procedure. Recently, many studies have reported that imaging-guided percutaneous cryoablation is safe and effective for the treatment of HCC^[14-16]. In our previous study, the 1-, 2- and 3-year survival rates in patients with HCC < 5 cm in diameter who were treated with cryoablation were 92%, 82% and 64%, respectively, and the rate of serious complications was low^[15]. To our knowledge, tumour seeding after percutaneous cryoablation for HCC has not been described to date. The present study was conducted to evaluate the incidence and possible risk factors for seeding after percutaneous cryoablation, by reviewing the prospective database of HCC patients treated by cryoablation in our department.

MATERIALS AND METHODS

Patients

This study included 1436 consecutive patients with HCC who were treated at the Center of Therapeutic Research for Hepatocellular Carcinoma, 302 Hospital of PLA, between April 2003 and June 2011. HCC was diagnosed based on typical findings on MRI or CT (hyperattenuation in the arterial phase and hypoattenuation in the portal-venous phase) and serum AFP level. The diagnosis was confirmed by histopathological examination of US- or CT-guided biopsy specimens in 736 patients. Until 2007, we biopsied almost all tumours before treatment, and after 2007 we only biopsied cases in which we could not make a definite diagnosis using dynamic CT or MRI. Biopsy specimens for histological examination were obtained with 1-2 passes of a 19.5-gauge end-cutting needle (Auto-Vac; Angiomed, Karlsruhe, Germany). Histopathological grading of tumour differentiation was performed using the criteria described by Edmondson *et al*^[17]. Tumour stage was defined according to the Barcelona Clinic Liver Cancer (BCLC) classification^[18]. Performance status (PS) was defined according to the Eastern Cooperative Oncology Group criteria (ECOG). The 1436 patients had a total of 2423 tumours with a diameter of 1.2-15.0 cm (mean 4.5 ± 2.3 cm). The clinical characteristics of the patients are shown in Table 1. All cryoablation treatments were approved by the Research Ethics Committee at 302 Hospital of PLA. Written informed consent was obtained from all patients who met the inclusion criteria, before blood and tumour specimens were obtained, and before data were collected and analysed.

Inclusion and exclusion criteria

Inclusion criteria for cryoablation were as follows: contraindications to surgical resection or orthotopic liver transplantation, Child-Pugh class A or B liver function, total serum bilirubin level < 51.3 µmol/L, platelet count ≥ 20 × 10⁹/L and performance status ≤ 2. Ascites was controlled before the procedure with diuretics. Patients with early HCC who were reluctant to undergo hepatic resection or transplantation were included. Patients were excluded for the following reasons: BCLC stage D (ECOG PS > 2, Child-Pugh C), tumour thrombosis at the main branch of the portal vein and the size of the thrombosis exceeded 50% of the diameter of the portal vein, extra-hepatic metastasis, tumours which were not accessible percutaneously, or a history of other ablation therapies. We generally performed percutaneous cryoablation in patients with up to three lesions, all of which were ≤ 5 cm in diameter, but we performed a combination of repeated cryoablation and transarterial chemoembolisation (TACE) in some patients who did not meet these criteria.

Technical terms

We defined a session as a single treatment consisting of one or more ablations performed on one or more tumours. To assess tumour depth, we categorised tumours

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

Baseline characteristics	Value
Age (yr) ¹	55.9 ± 9.2
Sex	
Male	1176 (81.9)
Female	260 (18.1)
Aetiology	
HBs-Ag positive only	1229 (85.6)
HCV-Ab positive only	168 (11.7)
Both positive	19 (1.3)
Both negative	20 (1.4)
Child-Pugh score	
Class A	874 (60.9)
Class B	562 (39.1)
Tumour size (cm)	
≤ 3	411 (28.6)
3-5	656 (45.7)
≥ 5	369 (25.7)
Tumour number	
Single	1213 (84.5)
2-3	223 (15.5)
Tumour location	
Subcapsular	484 (33.7)
No subcapsular	952 (66.3)
Route of needle insertion	
Direct subcapsular insertion	213 (14.8)
Deep	1223 (85.2)
BCLC staging	
Stage A	787 (54.8)
Stage B	453 (31.5)
Stage C	196 (13.7)
Completed ablation	
Yes	1168 (81.3)
No	268 (18.7)
Tumour differentiation ²	
Well or moderate	490 (66.6)
Poorly	246 (33.4)
Biopsy performed prior to cryoablation ²	736 (51.3)
Number of sessions	
Single	336 (23.4)
2	743 (51.7)
> 2	357 (24.9)
AFP (ng/mL) ¹	575 ± 2039

¹Values are expressed as mean ± SD, *n* = 1436; ²Of 736 cases in which biopsy was performed. HBs-Ag: Hepatitis B surface antigen; HCV-Ab: Hepatitis C virus antibody; BCLC: The Barcelona Clinic Liver Cancer classification; AFP: Alpha-fetoprotein.

as subcapsular or deep. Tumours were defined as subcapsular when they were located adjacent to the surface of the liver, less than 0.5 cm of parenchyma between the tumour and the liver capsule, otherwise, they were defined as deep. Direct subcapsular needle insertion was defined as puncture of subcapsular tumours without traversing a sufficient portion of normal hepatic parenchyma. The number of needle insertions was defined as the total number of needle positions in all sessions.

Argon-helium cryoablation procedure

Argon-helium cryoablation was performed as described in our previous report^[16]. Briefly, an argon-helium gas-based CRYOcare system (EndoCare, Irvine, CA, United States) and cryoprobes were used to freeze the tumour with a dual freeze-thaw cycle under US or CT guidance.

After determining the most favourable percutaneous approach, we inserted the 3-mm cryoprobe into the tumour through the sheath introducer system under US or CT guidance, and advanced the tip to the distal margin of the targeted lesion. The number of probes used depended on the location and size of the lesions to be ablated. The dual freeze-thaw cycle comprised a 20-min freeze, a 10-min thaw and a further 15-min freeze. The dimensions of the frozen tissue were monitored by US or CT. The cryoprobe temperatures were reduced to $-135\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ within 1 min. After removal of the probes, all tracts were packed with Surgicel (Johnson and Johnson, Inc., Arlington, TX, United States) through the sheath introducer to control bleeding, and the sheath introducer was removed. We aimed to perform curative ablation of all tumours in each session by single or multiple cryoablation, particularly for tumours < 5 cm in diameter. Dynamic CT or MRI was performed 2-3 d after treatment to evaluate treatment efficacy. Complete ablation was defined as non-enhancement of the entire lesion on CT or MRI with a safety margin in the surrounding liver parenchyma. Patients underwent additional ablation sessions until complete ablation was confirmed in all nodules, to a maximum of three sessions. If ablation was incomplete after three sessions, we performed TACE. The cryoablation procedure was performed under conscious sedation. Echocardiography, ventilation and oxygen saturation levels were monitored throughout the procedure. Patients were kept warm during cryoablation with warming mats.

Follow-up and tumour seeding

All patients underwent MRI and CT at 1 mo after cryoablation. Patients were then assessed every 2-3 mo, including measurements of liver biochemistry and AFP level, and by CT or MRI. A newly detected tumour attached to the peritoneum or pleura was considered to be seeded, and the diagnosis was confirmed by biopsy and histopathological examination. Seeded tumours were treated with repeat cryoablation, PEI or TACE when feasible. The seeding rate was calculated based on the number of patients.

Statistical analysis

Potential risk factors for seeding were analysed. The following variables were recorded: age, sex, viral markers, tumour size, number of tumour nodules, tumour location, direct subcapsular needle insertion, tumour differentiation, number of cryoablation sessions, number of needle insertions, percutaneous biopsy prior to cryoablation and serum AFP level. Continuous variables were compared between patients with and without seeding using the Student's *t* test. The χ^2 test or Fisher's exact test was used to compare categorical variables between the groups. Variables with *P* < 0.1 were entered into a multivariate logistic regression model using stepwise selection of variables. Variables with *P* < 0.05 were considered statistically significant. All analysis were conducted using SPSS software version 13 (SPSS Inc., Chicago, IL, United States).

Table 2 Characteristics of the 11 hepatocellular carcinoma patients who had tumour seeding after cryoablation

Case No.	1	2	3	4	5	6	7	8	9	10	11
Age (yr)	66	51	47	51	65	43	49	61	58	58	72
Sex	M	F	M	M	F	M	M	M	M	F	M
Child-Pugh class	A	A	A	A	B	A	A	B	A	A	B
BCLC Stage	A	B	B	B	B	C	A	B	A	A	B
AFP (ng/mL)	7	9	3550	368	8589	16	75	33	23	48	294
No. of tumours	1	1	1	1	1	1	1	3	1	2	1
Tumour size (cm)	6	3	5.4	6	3.2	8	2.4	4.8	2.6	2	5.6
No. of sessions	2	1	4	3	1	3	1	2	1	4	2
No. of needle insertions	4	2	6	4	2	6	1	3	1	4	3
Completed ablation	Yes	Yes	No	No	Yes	No	Yes	Yes	Yes	Yes	Yes
Biopsy	No	Yes	Yes	Yes	Yes	No	No	No	No	Yes	Yes
Tumour differentiation											
Primary HCC	-	Poor	Mod	Mod	Poor	-	-	-	-	Well	Poor
Seeding HCC	Mod	Poor	Mod	Mod	-	Mod	Mod	Mod	Mod	Well	-
Subcapsular location	Yes	No	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	No
Direct subcapsular insertion	Yes	No	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	No
Seeding location	Ip	Ip	Sf	Ip	Pleura	Ip and Im	Ip	Ip	Ip	Ip	Pc
Seeding time (mo)	24	2	5	5	1	6	12	7	12	18	1
Overall survival (mo)	36	25	9	19	18	12	18	26	36	60	5
Prognosis	Died	Alive	Died	Died	Died	Alive	Alive	Died	Alive	Alive	Alive

M: Male; F: Female; Ip: Intraperitoneal; Sf: Subcutaneous fat; Im: Intercostal muscle; Pc: Peritoneal cavity; HCC: Hepatocellular carcinoma; BCLC: The Barcelona Clinic Liver Cancer classification; AFP: Alpha-fetoprotein; Mod: Moderate.

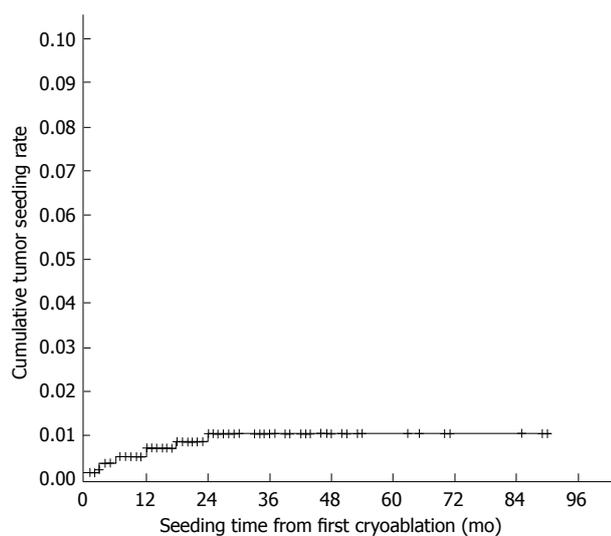


Figure 1 Cumulative tumour seeding rate. The cumulative rate was 0.49% at 1 year and 1.0% at 2 years.

RESULTS

Clinical features of patients with tumour seeding

A total of 1436 HCC patients underwent 3015 cryoablation sessions (1215 guided by ultrasonography and 221 guided by CT; average 2.1 sessions per patient) for 2423 nodules. When a patient underwent more than one treatment session, the data from the initial session were used. During the follow-up period (median 18 mo; range 1-90 mo), seeding was diagnosed in 11 patients at an interval of 1-24 (median 6.0) mo after the first cryoablation. The seeding rate was 0.76% per patient (11/1436). The longest interval between the first cryoablation session and detection of seeding was 2 years. The cumulative seeding rates

were analysed using Kaplan-Meier estimates, and were 0.49% at 1 year and 1.0% at 2 years (Figure 1). Table 2 shows the baseline characteristics of the 11 patients with seeding. Eight of these patients were male, and the mean patient age was 56.5 ± 9.0 years. Ten patients were hepatitis B surface antigen positive and one patient was hepatitis C virus antibody positive. Eight patients were Child-Pugh class A and three were Child-Pugh class B. The mean tumour size was 4.5 ± 1.9 cm, and the mean number of tumours was 1.3 ± 0.6 . The mean number of needle insertions was 3.3 ± 1.7 . Direct subcapsular needle insertions were performed in eight patients with subcapsular tumours. Six patients underwent biopsy prior to cryoablation, of which three had poorly differentiated HCC. Four patients were classified as BCLC stage A, six as stage B, and one as stage C. The tumours were completely ablated in eight patients. The mean serum AFP level was 1182.9 ± 2668.6 ng/mL.

Seeding occurred along the cryoablation needle tract in 10 patients, and at a distant location in 1 patient (Figure 2). The seeding was intraperitoneal in seven patients, intraperitoneal and in the intercostal muscles in one patient, pleural in one patient, and in the abdominal wall (subcutaneous fat) in one patient. One patient had distant seeding in the peritoneal cavity. In ten patients, the seeded tumours were < 3 cm in diameter, and in one patient the tumour was 3 cm in diameter. Nine (81.8%) patients had a single seeded tumour, and the other two (18.2%) patients had two and three seeded tumours, respectively, indicating that multiple seeding was not uncommon. One patient developed treatment-related liver haemorrhage 5 mo before the seeding was detected.

CT and MRI are the preferred imaging modalities for detecting needle-tract seeding. The seeded tumours are usually detected as one or a few round or oval-shaped

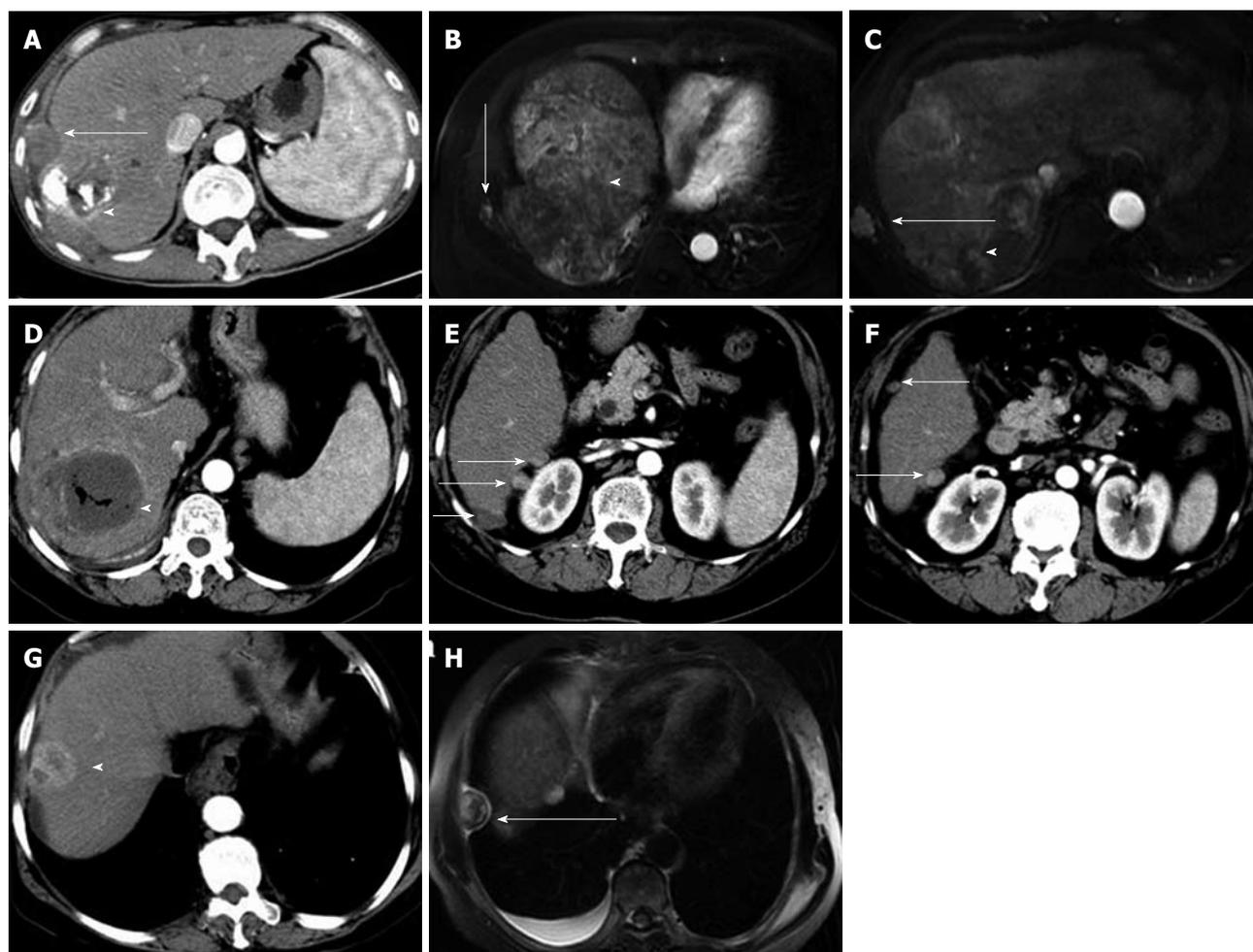


Figure 2 Locations of hepatocellular carcinoma and of seeding after cryoablation. A: Intraperitoneal seeding (case 4); B: Intraperitoneal and intercostal muscle seeding (case 6); C: Seeding in subcutaneous fat (case 3); D: Complete ablation of primary hepatocellular carcinoma (HCC) (case 11); E and F (case 11): Multiple, small, intraperitoneal seeding nodules 1 mo later; G: Complete ablation of primary HCC (case 5); H: Pleural seeding nodules 1 mo later (case 5). Arrowheads indicate primary HCCs, arrows indicate seeding.

enhancing nodules along the needle tract, with a few occurring at a distant location. Seeded tumours showed a similar imaging pattern to primary HCCs, with arterial phase hyperattenuation followed by portal-venous phase hypoattenuation (Figure 3).

Tumour biopsies performed before cryoablation in six patients who developed seeding showed that one patient had a well differentiated tumour, two patients had moderately differentiated tumours, and three patients had poorly differentiated tumours. The seeded tumours in the nine patients without distant or pleural seeding were confirmed by biopsy and histopathological examination. The seeded tumour showed similar differentiation features to the primary HCC in four of these patients (Figure 4).

Seeding was treated by PEI in four patients, resection in one patient, cryoablation in two patients, cryoablation plus sorafenib in two patients, and conservative treatment in two patients. Of the nine patients with seeding who were treated, recurrence of seeding after treatment occurred in five (55.6%), including three treated with PEI and two treated with cryoablation plus sorafenib. Three

of the patients with recurrent seeding were treated with RFA plus radiation and did not have further recurrence, and the other two patients were treated with sorafenib plus cryoablation and TACE (Figure 5).

At the end of the follow-up period, five patients with seeding had died. The causes of death were intrahepatic HCC progression and liver failure. No patient died of their seeded tumour nodules. In patients with seeding, the cumulative survival rates were 90% at 1 year, 68% at 2 years, 53% at 3 years, 32% at 4 years and 32% at 5 years. In patients without seeding, the cumulative survival rates were 86% at 1 year, 61% at 2 years, 51% at 3 years, 43% at 4 years and 34% at 5 years. There were no significant differences in survival rates between the two patient groups ($P = 0.942$) (Figure 6).

Risk factors for tumour seeding

Table 3 shows the results of the univariate analysis to identify risk factors for seeding. Direct subcapsular needle insertion ($P = 0.0043$) and subcapsular tumour location ($P = 0.0152$) were associated with seeding. There were no significant associations between seeding and age, sex,



Figure 3 Computed tomography showing tumour seeding in case 2 after cryoablation for hepatocellular carcinoma, with a history of transcatheter arterial chemoembolisation. A: Contrast-enhanced computed tomography (CT) image showing a 3-cm diameter hepatocellular carcinoma (HCC) in segment II before cryoablation (black arrow); B and C: Contrast-enhanced CT images during the arterial phase (B) and portal-venous phase (C) showing intraperitoneal seeding (white arrow) at 2 mo after biopsy and percutaneous cryoablation. The tumour showed hyperattenuation during the arterial phase and hypoattenuation during the portal-venous phase, similar to the primary HCC. Histopathological examination of the seeded tumour showed a poorly differentiated HCC. Note that the intrahepatic tumour was completely ablated.

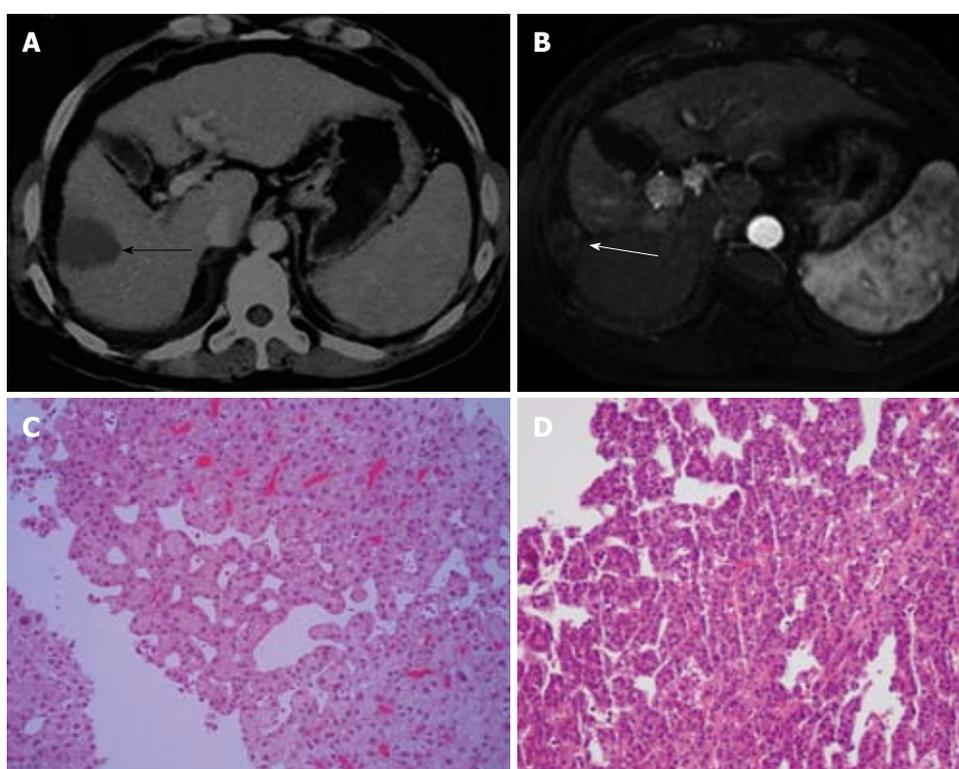


Figure 4 Computed tomography image and histopathology of primary and seeded tumours (case 10). The 58-year-old woman underwent biopsy and percutaneous cryoablation for a 2-cm diameter subcapsular hepatocellular carcinoma (HCC). A: The tumour was completely ablated (black arrow); B: Intraperitoneal seeding at 18 mo after treatment (white arrow); C: Histopathological examination of the biopsy specimen from the primary tumour showed a well differentiated HCC; D: The seeded tumour was resected, and histopathological examination showed a well differentiated HCC. The patient was alive and tumour-free at 60 mo after cryoablation.

viral markers, Child-Pugh class, tumour size, number of nodules, number of sessions, number of needle insertions, tumour differentiation, biopsy prior to cryoablation, BCLC stage, incomplete ablation, or serum AFP level. Even though there was no significant association between seeding and tumour differentiation, seeding was detected earlier in patients with poorly differentiated HCC than in those with well or moderately differentiated HCC [1.33 ± 0.577 mo *vs* 11.12 ± 6.896 mo; 95%CI: (-19.115)-(-0.468); $P = 0.042$]. Multivariate analysis showed that the only significant risk factor for seeding was direct subcapsular needle insertion (odds ratio 2.57; 95%CI: 1.47-7.65; $P = 0.017$).

DISCUSSION

The new modality of imaging-guided percutaneous argon-helium cryoablation has been widely developed in China. Many studies have reported the safety and efficacy of this technique in the treatment of HCC^[14-16]. Although many complications have been reported, the majority are minor and can be treated conservatively. In carefully selected patients, the rate of serious complications is low^[16]. Because of its minimal invasiveness and resulting large ablation zone, percutaneous cryoablation is a useful treatment modality for HCC^[19].

However, occasional tumour seeding after percuta-

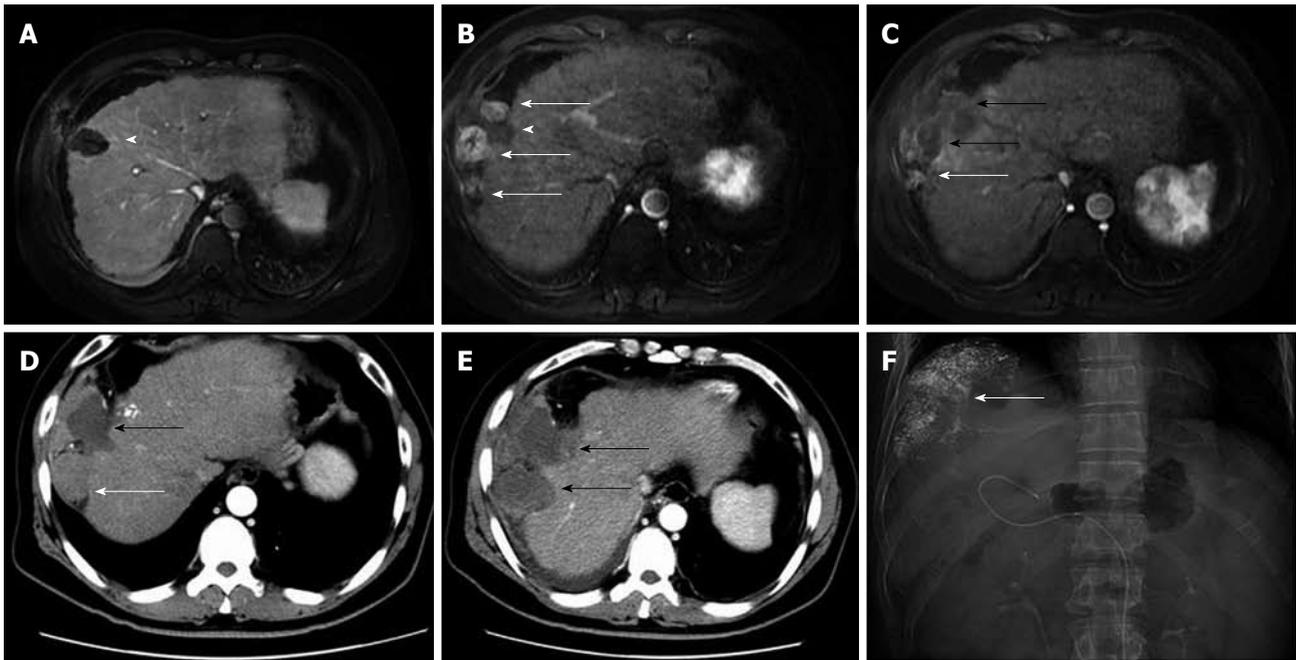


Figure 5 Patient treated with sorafenib plus cryoablation and transcatheter arterial chemoembolisation for hepatocellular carcinoma seeding (case 7). A: Complete ablation of the subcapsular hepatocellular carcinoma (arrowhead); B: Multiple, small, intraperitoneal seeded nodules (white arrows); C: Seeded nodules were treated with cryoablation (black arrows) plus sorafenib; D: Recurrence of seeding (white arrow); E: The recurrent seeding was treated with cryoablation (black arrows); F: The seeding was also treated with transarterial chemoembolisation (white arrow). Six months later, there was no further recurrence.

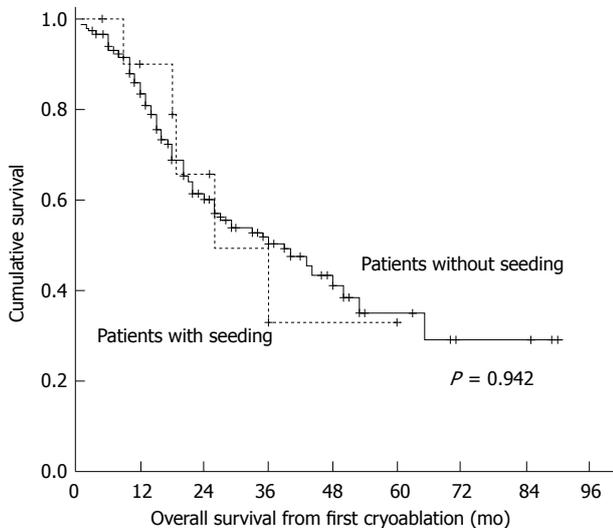


Figure 6 Cumulative overall survival in patients with and without tumour seeding. Survival curves are drawn according to the Kaplan-Meier method, using the log-rank test.

neous cryoablation for HCC remains unavoidable, as in other local ablation treatments such as PEI, RFA and MW ablation. In this study, we systematically searched for evidence of seeding using state-of-the-art imaging over a long follow-up period, and found seeding in 11 of 1436 patients treated with 3015 cryoablation sessions. The seeding rate was 0.76% per patient. The median interval between the first cryoablation session and detection of seeding was 6 (range 1-24) mo, with a median follow-up time of 18 (range 1-90) mo. At the end of the follow-up

period, 580 patients had died, and these patients had a median survival time of 15 (range 1 to 65) mo. All patients were under close observation, and no patients were lost to follow-up. Because of the duration and quality of follow-up, the likelihood of having missed a seeded tumour in this study is minimal.

Similar to other percutaneous interventions such as biopsy, PEI and RFA, the sites of seeding after cryoablation were the thoracic wall, abdominal wall, diaphragm and peritoneal cavity^[20-22]. Seeding usually occurred along the needle tract, but a few cases were at a distant location, with pleural and peritoneal cavity seeding in one patient each. Regular follow-up with contrast-enhanced CT or MRI from the chest to the pelvis is therefore very important.

The median time to diagnosis of seeding has been reported to be 13 (range 1-58) mo after biopsy^[21], 6 (range 2-48) mo after PEI^[21] and 28.5 (range 8.6-60.7) mo after RFA^[22]. In the present study, the median time to diagnosis was 6.0 (range 1-24) mo after cryoablation. The longest interval from the first cryoablation session to the diagnosis of seeding was 2 years. It can therefore be concluded that it is necessary to carefully monitor patients for at least 2 years after cryoablation for HCC. The reason for the longest interval to the diagnosis of seeding in the present study being shorter than in previous studies on biopsy, PEI and RFA is unknown. There are no reports of growth rates for seeded tumours after cryoablation, but it has been reported that the growth rate of needle-tract seeding after biopsy varies depending on the initial number of implanted tumour cells and the doubling time of the tumour, as well as the microenvironment surrounding the seeded tumour. The doubling time of seeded tumours after bi-

Table 3 Characteristics of patients with and without tumour seeding

Variable	Seeding was identified (n = 11)	Seeding was not identified (n = 1425)	P value
Age (yr) ¹	56.5 ± 9.0	55.5 ± 9.3	0.8970
Gender (male/female)	8/3	1168/257	0.6895
HBs-Ag positive only	10	1219	0.9412
HCV-Ab positive only	1	167	1.0000
Both positive	0	19	1.0000
Both negative	0	20	1.0000
Child-Pugh class (A/B)	8/3	866/559	0.6176
Tumour size (cm) ¹	4.5 ± 1.9	4.6 ± 3.0	0.8800
Number of tumours ¹	1.3 ± 0.6	1.3 ± 0.7	0.9520
Number of sessions ¹	2.2 ± 1.2	2.3 ± 1.1	0.8764
Number of needle insertions ¹	3.3 ± 1.7	3.8 ± 1.1	0.3430
Direct subcapsular insertion	8	205	0.0043
Subcapsular location	8	476	0.0152
Biopsy performed ²	6	730	0.8264
Poorly differentiated tumour	3	243	0.6674
BCLC stage (A/B/C)	4/6/1	783/447/195	0.2869
Completed ablation (yes/no)	8/3	1161/264	0.7235
AFP (ng/mL) ¹	1182.9 ± 2668.6	577.0 ± 2038.1	0.3270

¹Values are expressed as mean ± SD; ²Of 736 cases in which biopsy was performed. HBs-Ag: Hepatitis B surface antigen; HCV-Ab: Hepatitis C virus antibody; BCLC: The Barcelona Clinic Liver Cancer classification; AFP: Alpha-fetoprotein.

opsy is 112 (range 22-415) d^[8], which is comparable to that of primary HCC. Regarding differentiation features, Matsukuma *et al*^[23] reported that peritoneal seeding can infrequently proliferate aggressively with more differentiated features. In the current study, four patients with seeding showed similar tumour differentiation features to the primary HCC, and only three patients had poorly differentiated HCC, so it is difficult to explain the shorter interval in terms of tumour differentiation. The available data report a median HCC diameter of 32.5 mm in patients who underwent PEI and 30 mm in patients who underwent RFA, and the use of 14- to 22-gauge needles for biopsies, 21- to 22-gauge needles for PEI and 14- to 17-gauge needles for RFA^[20,21]. The shorter interval to detection of seeding after cryoablation may be related to the use of larger needles (a 3-mm cryoprobe correlates to an 11-gauge needle) and larger nodule size (mean 45 ± 19 mm).

HCC is particularly prone to seeding, with higher seeding rates after biopsy (0%-5.1%)^[24] than other solid tumours such as pancreatic tumours (0.003%-0.017%) and other abdominal tumours (0%-0.03%)^[9,25].

In a recent review, several factors were suggested to contribute to seeding after percutaneous interventional procedures, which were listed as follows: poorly differentiated tumour, high serum AFP level, subcapsular tumour location, biopsy prior to RFA, high number of sessions and high number of electrode placements^[19]. However, only Shirai *et al*^[22] and Imamura *et al*^[26] have reported multivariate analyses of these factors. Imamura *et al*^[26] reported that only poor tumour differentiation was an independent risk factor for seeding. Shirai *et al*^[22] reported that only RFA was an independent risk factor. In



Figure 7 The sheath introducer system. The cryoablation needle (black arrow) is inserted and removed through the sheath introducer system (white arrow).

the present study, univariate analyses identified subcapsular tumour location and direct subcapsular needle insertion as risk factors. There were no significant associations between seeding and age, sex, viral markers, Child-Pugh class, tumour size, number of nodules, number of sessions, number of needle insertions, tumour differentiation, biopsy prior to cryoablation, BCLC stage, incomplete ablation or serum AFP level. Multivariate analysis showed that only direct subcapsular needle insertion was an independent risk factor for seeding.

Several studies have reported that subcapsular tumour location was a risk factor for seeding^[2,27-29]. In a study reporting a 12.5% seeding rate after RFA, all patients with seeding had a subcapsular tumour^[2]. This is consistent with the results of our univariate analysis. In our initial experience, percutaneous cryoablation of subcapsular tumours was also associated with liver haemorrhage^[16]. In the present study, treatment-related liver haemorrhage occurred in one patient with seeding. We therefore insert our cryoprobe across a portion of normal hepatic parenchyma, and avoid direct subcapsular needle insertion for subcapsular tumours whenever possible. This minimises both liver haemorrhage and needle-tract seeding. This may explain why multivariate analysis only identified direct subcapsular needle insertion as an independent risk factor.

There is still controversy regarding whether tumour biopsy prior to treatment or a poorly differentiated tumour increase the risk of seeding^[21-23,30]. In this study, biopsy and a poorly differentiated tumour were not associated with a higher rate of seeding. The current study also did not show a significant association between seeding and tumour size or incomplete cryoablation, which is consistent with the findings of other studies^[21,26]. Although the 3-mm cryoprobe was large, the risk of seeding after cryoablation was small. The risk of seeding may be reduced by the use of the sheath introducer system (Figure 7), through which cryoablation needles are inserted and removed. Similarly, Maturen *et al*^[31] reported that no seeding occurred when they used a needle introducer that remained in position during multiple passes of a coaxial cutting needle for biopsies, which may protect the tissue along the needle tract and reduce seeding. Further studies should be conducted

to assess the effects of the sheath introducer system on the risk of seeding in percutaneous cryoablation for HCC. In addition, the low risk of cryoablation may be related to the mechanisms of cryoablation. Cryotherapy is believed to kill cells by several mechanisms, including intracellular ice formation, solute-solvent shifts that cause cell dehydration and rupture, small vessel obliteration causing hypoxia and specific anti-tumour immunoreactions that limit tumour growth^[32,33]. Several studies found that cryoablation resulted in both local tumour necrosis and necrosis and shrinkage of the tissues adjacent to the tumour, which was thought to indicate ectopic tumour suppression^[33]. Preclinical evidence of a cryo-immunologic response as well as some clinical data indicate that cryoablation may generate an anti-tumour response^[34,35]. Our previous study indicated that cryoablation not only directly destroys malignant tissues, but also has effects on the adjacent tissues^[36]. Cryotherapy resulted in reduced numbers of peripheral Treg cells and a lowering of the CD8-FoxP3+/CD8+FoxP3- ratio in malignant tissues^[37]. We therefore speculate that anti-tumour immunoreactions induced by cryoablation may limit seeding. This concept deserves further study.

Although poorly differentiated tumour did not increase the risk of seeding, we found that seeding occurred earlier in patients with poorly differentiated HCC than in those with well or moderately differentiated HCC. It is possible that poorly differentiated HCC lacks cohesiveness^[17] and grows more rapidly, allowing the seeding to be identified earlier.

It is not clear whether seeding affects prognosis. Shirai *et al*^[22] and Imamura *et al*^[26] investigated the prognosis of HCC patients with seeding after RFA. They reported that the survival rate was not particularly low in patients with seeding, and that seeding itself did not directly affect survival. The present study also did not find significant differences in the cumulative survival rates of patients with and without seeding. By the end of the follow-up period, five patients with seeding had died of intrahepatic HCC progression and liver failure. No patient died due to the growth of seeded nodules. Nevertheless, the survival rates of patients with seeding tended to be lower from the second year onwards. The reasons for the lack of significant differences in survival rates may be as follows: First, the number of patients with seeding was very small compared with the number without seeding. Second, seeded tumours were treated radically, which may improve outcome^[7,38]. It is therefore impossible to claim that seeding does not affect prognosis, and seeded tumours should be treated with the aim of achieving local cure. It is essential to recognise that seeding is difficult to treat successfully. In the present study, the recurrence rate after local radical treatment of seeding was 55.6%.

In conclusion, the relatively low rate of tumour seeding after cryoablation for HCC is considered an acceptable clinical risk. Direct puncture of subcapsular tumours was found to be a risk factor for seeding. Although seeding is sometimes unavoidable, strict attention to detail

and knowledge of seeding and its risk factors are helpful for minimising its occurrence.

COMMENTS

Background

Imaging-guided percutaneous argon-helium cryoablation is widely used in China, and this technique has been found to be safe and effective for the treatment of hepatocellular carcinoma (HCC). However, details of tumour seeding after this procedure have not been reported to date, even though seeding is one of the most important complications.

Research frontiers

This study reports the rate of tumour seeding after percutaneous cryoablation and analyses the risk factors for seeding in a large cohort of HCC patients who were treated with cryoablation sessions over an 8-year period.

Innovations and breakthroughs

Seeding occurred in 11 (0.76%) of 1436 patients treated with percutaneous cryoablation in this study. Only direct puncture of a subcapsular tumour was an independent risk factor for seeding.

Applications

This study indicates that the risk of seeding after percutaneous cryoablation for HCC is small and is considered an acceptable clinical risk. This procedure is minimally invasive and results in a large ablation zone, making it a useful treatment modality for HCC. However, direct puncture of a subcapsular tumour should be avoided. The small risk of seeding may be due to the use of an introducer sheath, or to the mechanisms of cryoablation, and further research is warranted.

Terminology

Percutaneous cryoablation requires the insertion of needle into the liver parenchyma and tumour, which may cause tumour seeding. However, the incidence of HCC seeding after the procedure is low.

Peer review

The authors analyzed the incidence of HCC tumour seeding after percutaneous cryoablation. It is very interesting study and has a great scientific value for physicians who take care of patients with this pathology. The study is well designed and data is convincing.

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Clinical significance of human kallikrein 12 gene expression in gastric cancer

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Abstract

AIM: To investigate whether the expression of kallikrein 12 (KLK12) is related to the development of gastric cancer (GC) and to determine the role of KLK12 in gastric cancer cells growth, invasion and migration.

METHODS: Between September 2007 and March 2008, 133 patients with histologically confirmed GC were recruited for the study. Expression of KLK12 was detected in samples from GC patients by quantitative real-time reverse transcription polymerase chain reaction and immunohistochemistry. The relationship between KLK12 protein expression and clinicopathological features of GC was analyzed. The difference in 5-year survival rates between the high KLK12 protein expression group and the low KLK12 expression group was compared. Additionally, the expression of KLK12 was examined in various human GC cell lines, including MKN-28, SGC-7901 and MKN-45. Small interfering RNA (siRNA) was used

to inhibit KLK12 expression in MKN-45 cells. Cell clones stably transfected with KLK12 siRNA were tested for KLK12 expression by quantitative real-time reverse transcription-polymerase chain reaction and Western blotting. Furthermore, a series of functional assays were performed in this study to assess the biological features of transfected cells. Cell proliferation was assessed using the methylthiazolyltetrazolium assay. Finally, cell migration and invasion were assessed using transwell chamber assays.

RESULTS: Of the 133 GC patients included in the study, 126 (94.7%) showed a higher expression level of KLK12 mRNA when compared to noncancerous tissue specimens. Expression of KLK12 mRNA was significantly higher in GC tissues than in normal tissue ($P < 0.001$). KLK12 protein expression was detected in 96 of 133 (72.2%) GC samples with moderate or strong staining primarily in the cytoplasm. In contrast, negative immunostaining for KLK12 protein was observed in the corresponding normal gastric mucosal tissue. Overexpression of KLK12 protein was significantly associated with lymph node metastasis ($P = 0.001$), histological type ($P < 0.001$) and tumor-node-metastasis stage ($P = 0.005$), while no significant correlation was observed between expression of KLK12 protein and sex, age, depth of invasion, tumor size or lymphatic invasion. Furthermore, patients with high KLK12 expression had a significantly poorer 5-year survival rate than those with low KLK12 expression ($P = 0.002$). Expression of KLK12 mRNA was significantly higher in MKN-45 GC cells compared to normal mucosal cells or two other GC cell lines ($P < 0.01$). Expression of KLK12 in MKN-45 cells was downregulated after transfection with siRNA. Knockdown of KLK12 markedly decreased the proliferation of MKN-45 cells when compared with parent or mock-transfected cells ($P = 0.001$), especially from the 3rd to the 5th day of the assay. In migration assays, fewer KLK12 siRNA cells migrated through the chambers (22.00 ± 1.81) when compared to the parent (46.47 ± 2.42) or mock-transfected cells (45.40 ± 1.99); these differences were statistically sig-

nificant ($P < 0.001$). However, in the invasion assay, the number of KLK12 siRNA cells that invaded the chambers was 18.40 ± 1.12 , closely similar to both the parent (18.67 ± 0.98) and mock-transfected cells (18.53 ± 0.92). There was no significant difference between the three groups in the invasion assay ($P = 0.054$).

CONCLUSION: The *KLK12* gene is markedly overexpressed in GC tissue, and its expression status may be a powerful prognostic indicator for patients with GC. KLK12 might serve as a novel diagnosis and prognosis biomarker in GC.

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Key words: Gastric cancer; Human kallikrein 12; Immunohistochemistry; Prognosis; Small interfering RNA; Migration; Invasion

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INTRODUCTION

Gastric cancer (GC) is the fourth most common malignancy, and the second most common cause of cancer mortality worldwide^[1]. Although morbidity and mortality rates for GC are steadily decreasing steadily in many countries, the overall outcomes for patients with GC have not changed significantly in recent decades^[2]. Additionally, none of the potential biomarkers proposed throughout the years for GC have presented the desired properties to be incorporated into routine clinical practice. The human tissue kallikrein (*KLK*) genes are a newly identified subgroup of putative serine proteases, consisting of 15 genes located within approximately 256 kb on chromosome 19q13.3-4^[3-5]. Due to their protease activity and expression in many tissues and cell types, KLKs have been implicated in a wide range of physiological processes and the pathogenesis of human diseases^[6,7]. Over the last several decades, a steadily increasing number of studies have suggested that human KLKs are involved in human carcinogenesis and that several KLKs may be promising biomarkers of prostate, ovarian, testicular, and breast cancers^[8-10]. The most notable kallikrein protein biomarker is KLK3, also known as prostate specific antigen (PSA)^[11]. PSA is currently the only biochemical test for prostate cancer, although the specificity of the test is not optimal^[12]. Recently, a considerable amount of work has focused on identifying novel KLK-derived molecular markers of GC^[13-16]. The human *KLK12* gene is a member

of the KLK family, encoding human kallikrein 12 protein (hK12). Similar to other kallikreins, KLK12 is an enzyme with serine protease activity that participates in several biological processes^[17]. Moreover, some researchers have shown that KLK12 might also play a role in human carcinogenesis^[17-19]. However, no information is available regarding KLK12 expression in human GC.

To explore the vital role of KLK12 in the tumorigenesis and progression of GC, we examined expression patterns of KLK12 in GC tissues, analyzed the relationship between hK12 expression and clinicopathological factors of GC. Furthermore, a series of function assays utilizing small interfering RNA (siRNA)-mediated downregulation of KLK12 expression were performed.

MATERIALS AND METHODS

Patients and samples

Prior to operation, no patient had received any type of treatment. All research examinations were approved by the Ethics Committee Board of Renji Hospital. Moreover, participants in this study signed an informed consent form so that their samples could be used for research purposes from September 2007 to March 2008. A computerized database with the medical history of each patient was created for an extensive statistical analysis. Selection criteria included confirmation of GC diagnosis by histopathology and the availability of sufficient tumor tissue for RNA extraction. Tumor stage was defined according to the 7th edition of International Union Against Cancer tumor-node-metastasis classification. All specimens were snap-frozen in liquid nitrogen immediately after surgery, and then stored at -80°C until analysis.

Cell lines and cell culture

The human GC cell lines MKN-28, SGC-7901 and MKN-45 and the normal gastric mucosal cell line GES-1 were obtained from Shanghai Institute of Digestive Disease (Shanghai, China). Cell lines were cultured in Dulbecco's modified Eagle's medium (Gibco, United States) with 10% fetal bovine serum (FBS, Gibco, United States).

Total RNA extraction and cDNA synthesis

Total RNA was extracted from the human tissues and GC cells using TRIzol Reagent (Invitrogen, United States) according to the manufacturer's instructions. The RNA concentration and purity were determined using the absorbance ratio at 260/280 acquired by a spectrophotometer. cDNA synthesis from 4 μg of total RNA was performed with a reverse transcription system kit (Promega, United States) according to the manufacturer's protocol. Briefly, samples were preincubated at 70°C for 10 min, cooled on ice, then added to a reaction mixture consisting of 3 μL dNTP mixture, 2 μL M-MLV reverse transcriptase, 10 μL reverse transcription 5 \times buffer, 1 μL Rnasin and 2 μL oligo-(dT)15 primer in a final volume of 20 μL . The reaction mixture was sequentially incubated at 95°C for 15 min, 99°C for 15 s and 62°C for 40 s. The cDNA was

stored at -20 °C before use.

Quantitative real-time reverse transcription polymerase chain reaction assay

Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) was carried out in 96-well polypropylene microplates on an ABI Prism 7500 sequence detection PCR system (Applied Biosystems, United States) with the following primers: KLK12: 5'-GCCTCAACCTCTCCATC-GTC-3' (forward) and 5'-CTTGAAGGACTCCCCCA-CAC-3' (reverse); β -actin: 5'-CATGTACGTTGCTATC-CAGGC-3' (forward) and 5'-CTCCTTAATGTCACG-CACGAT-3' (reverse). The 20 μ L PCR reaction consisted of (2 \times) SYBR[®] Premix Ex Tag[™] (Takara, Japan), 2 μ L cDNA and 0.4 μ L of each gene-specific primer. The thermal cycling protocol included an initial denaturation step at 95 °C for 30 s followed by 45 cycles at 95 °C for 5 s for denaturation and 62 °C for 40 s for primer annealing and extension. After the last cycle, a melting curve analysis was performed. All PCR reactions were run in triplicate. KLK12 mRNA expression was calculated from the standard curve, and quantitative normalization in each sample was performed using β -actin gene expression as an internal control. Relative quantification was performed using the $2^{-\Delta\Delta Ct}$ method.

Immunohistochemistry analysis

Immunohistochemistry (IHC) studies of hK12 were performed on surgical specimens from patients with GC using the avidin-biotin-peroxidase method (KIT-9702, Maixin Bio, China) on formalin-fixed, paraffin-embedded tissue specimens. All sections were incubated with polyclonal sheep anti-human KLK12 antibody (AF3095, R and D Systems, United States) at a dilution of 1:50. Slides were counterstained with hematoxylin. All sections were evaluated independently and blinded to outcome data by a pathologist three times. A cutoff of more than 30% of the tumor cells with moderate or strong KLK12 cytoplasmic staining in a gastric tumor section was considered to be positive.

siRNA transfection

The expression vector pBSKH1 (SIBS, China) was used for expression of siRNA. The siRNA designed to target the *KLK12* gene (sense strand: 5'-AAACAGUGACAGC-CACGUATT-3', anti-sense strand: 5'-UACGUGGCUG UCACUGUUUGG-3') was inserted into the pBSKH1 vector according to the manufacturer's instructions, and then, the vector was transfected into the GC cells by the Lipofectamine[™] 2000 (Invitrogen, United States). A mock vector-transfected clone of the cell line was used as a control. Stably transfected cell clones were tested for KLK12 expression by quantitative real-time RT-PCR and Western blotting.

Western blotting analysis

Cells were harvested 72 h after transfection, and whole-cell lysates were prepared for protein extraction. The

protein concentrations of the samples were determined by the bicinchoninic acid protein assay. Proteins were separated in 10% sodium dodecyl sulphate polyacrylamide gels, electrophoretically transferred to polyvinylidene difluoride membranes and incubated in a blocking solution of 5% skim milk powder for 1 h at room temperature. Membranes were then incubated with polyclonal sheep anti-human KLK12 antibody (1:500, AF3095, R and D Systems, United States) and anti- β -actin antibody (1:1000, sc-47778, Santa Cruz, United States) overnight at 4 °C. The membranes were washed 3 times in tris-buffered saline tween-20 (TBST) and incubated for 1 h at room temperature with a 1:1000 dilution of secondary antibody conjugated to horseradish peroxidase (Invitrogen, United States). After incubation with a secondary antibody, the membranes were washed in TBST and developed using electrochemiluminescence according to the manufacturer's instructions.

In vitro cell proliferation assay

Cell proliferation was evaluated using the methylthiazolyltetrazolium (MTT) assay. Cells were grown in a monolayer culture and plated at a density of 4×10^3 cells/well into separate wells of a 96-well culture plate. The cells were incubated with MTT after 1, 2, 3, 4 or 5 d. Absorbance was measured at 570 nm using a microplate reader (SpectraMax 250, Molecular devices, United States). The experiments were performed in triplicate.

In vitro migration and invasion assays

The motility and invasiveness of plasmid-transfected cells were evaluated in 24-well transwell chambers with upper and lower culture compartments separated by polycarbonate membranes with 8- μ m pores (CytoSelect[™] 24 Well Cell Migration and Invasion Assay Combo Kit, 8- μ m, CBA100-C, Cell Biolab, United States). Prior to plating cells into the transwells, the 24-well migration plate was allowed to warm up to room temperature for 10 min. A cell suspension containing 1.0×10^6 cells/mL in serum-free media was plated into the top chamber. Five hundred microliters of media containing 10% FBS was placed into the bottom chamber to act as a chemoattractant. Then, 300 μ L of the cell suspension solution was added to the inside of each insert and incubated 24 h in a cell culture incubator. The cells that migrated through the 8- μ m pores and adhered to the lower surface of the membrane were transferred to a clean well containing 400 μ L of 0.09% crystal violet as a cell staining solution and washed several times in a beaker with water. The migratory cells were counted using light microscopy under high magnification, with 5 individual fields per insert. In a similar fashion, the invasiveness of plasmid-transfected cells was also evaluated using this kit (Invasion Chamber Plate, Cell Biolab, United States). Cells, media, experimental conditions, and the analysis performed were similar to those for the migration assays. Triplicate assays were performed for each group of cells in both the migration and invasion assays.

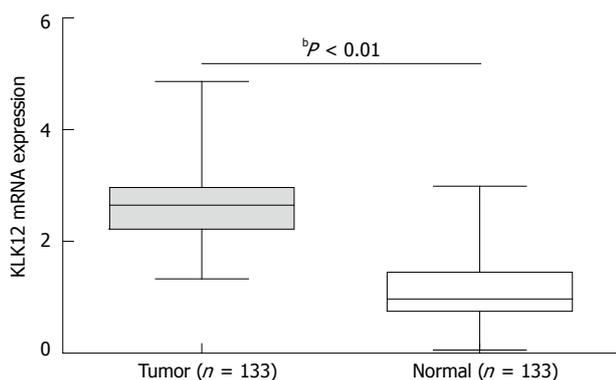


Figure 1 Upregulation of kallikrein 12 mRNA expression in gastric cancer. Quantitative real-time reverse transcription polymerase chain reaction showed that the mean expression value of kallikrein 12 (KLK12) mRNA in cancer tissues was significantly higher than the value in relevant normal tissues. Data are shown as mean ± SD, using the Student's *t* test (^b*P* < 0.01 between tumor and normal group, horizontal bars represent medians).

Statistical analysis

Statistical analysis were performed using SPSS 11.0 software (Shanghai Jiaotong University, China). The data are expressed as the mean ± SD. The Student's *t* test and one-way analysis of variance test were used to compare data between the different groups. The χ^2 test was used to assess the relationship between hK12 expression levels and clinicopathological characteristics of GC. Survival curves were drawn according to the Kaplan-Meier method, and the log-rank test was applied to compare the survival curves. Differences were considered significant at the *P* < 0.05 level.

RESULTS

KLK12 mRNA level is upregulated in GC patients

There were 133 GC patients included in the study. The age of the patients ranged from 18 to 87 years (median 61 years). Overall, 126 of the 133 patients (94.7%) showed a higher expression level of KLK12 mRNA in GC tissue specimens compared to noncancerous tissue specimens. The mean expression value of KLK12 mRNA in cancer tissues was significantly higher than the value in relevant normal tissues (2.75 ± 0.78 vs 1.10 ± 0.56 , *P* < 0.001, Figure 1).

Immunostaining demonstrates hK12 overexpression in GC tissues

To further validate the expression and localization of hK12 in surgical specimens, IHC was performed on paraffin-fixed GC tissues and matched noncancerous mucosa of 133 patients. Dark-brown immunostaining was most prevalent in cancer cells, whereas the staining levels were lower in stromal cells or fibroblasts of GC tissues. hK12 expression was detected in 72.2% (96/133) GC samples with moderate or strong staining (Figure 2A), primarily located in the cytoplasm (Figure 2A and B). In contrast, negative immunostaining for hK12 was observed in normal gastric mucosal sections (Figure 2C and D).

Table 1 Relationship between human kallikrein 12 protein expression and clinicopathological features in 133 patients with gastric cancer

Feature	n	hK12 expression		P value ¹
		High (n = 96)	Low (n = 37)	
Sex				
Male	83	59	24	0.716
Female	50	37	13	
Age (yr)				
≥ 61	69	47	22	0.277
< 61	64	49	15	
Depth of invasion (T)				
T1 + T2	21	14	7	0.539
T3 + T4	112	82	30	
Tumor size (cm)				
≥ 5	77	59	18	0.180
< 5	56	37	19	
Lymph node metastasis				
Positive	95	76	19	0.001
Negative	38	20	18	
Lymphatic invasion				
Absent	47	33	14	0.708
Present	86	63	23	
Histological type				
Undifferentiated	94	77	17	< 0.001
Differentiated	39	19	20	
Stage				
I + II	44	25	19	0.005
III + IV	89	71	18	

¹*P* value was determined by the χ^2 test. hK12: Human kallikrein 12 protein.

Clinicopathological significance of hK12 expression in GC

The clinicopathological factors analyzed in relation to hK12 expression in tumor tissues are shown in Table 1. The incidence of lymph node metastasis was significantly higher (*P* = 0.001) in the high-expression group (76 of 95, 80.0%) than in the low-expression group (20 of 38, 52.6%). The histological type and pathological stage also correlated with the groups. However, no significant difference was observed with regard to sex, age, depth of invasion, tumor size or lymphatic invasion. The 5-year actuarial overall survival rates in patients with high hK12 levels and patients with low hK12 levels were 62.2% and 31.3%, respectively (Figure 3). The difference in survival rates between these two groups was statistically significant (*P* = 0.002).

Expression of KLK12 mRNA in GC cell lines

Furthermore, three well-characterized GC cell lines, MKN-28, SGC-7901 and MKN-45, and the normal gastric mucosal cell line GES-1 were chosen to examine KLK12 mRNA expression due to their diverse differentiation features. MKN-45 cells expressed the highest level of KLK12 across the four cell lines (Figure 4). Therefore, MKN-45 cells were chosen to do a series of functional experiments involving knockdown of KLK12 expression.

KLK12 siRNA-transfected GC cell line stably suppresses both KLK12 mRNA and hK12

As shown in Figure 5A, MKN-45 cells transfected with siRNA targeting KLK12 showed a remarkable decrease

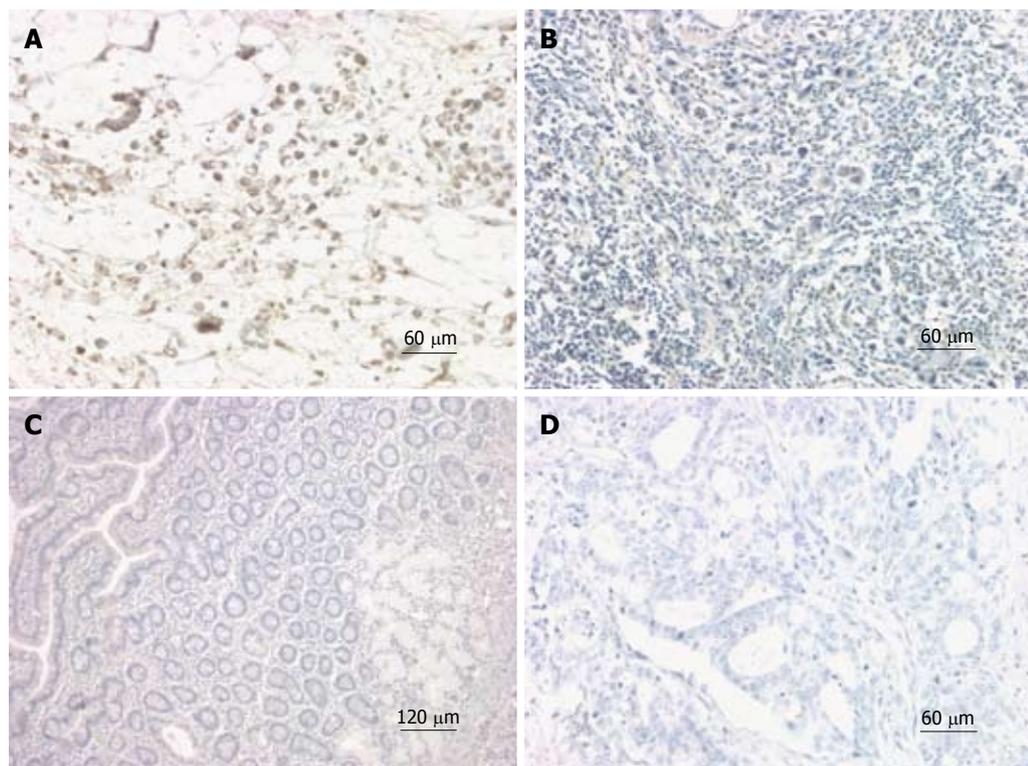


Figure 2 Expression of human kallikrein 12 protein in gastric cancer and non-cancerous mucosal tissues detected by immunohistochemistry. A: Strong positive human kallikrein 12 protein (hK12) immunostaining in gastric cancer (GC) tissues, hK12 staining was observed in the cytoplasm of cancer cells; B: Weak positive hK12 immunostaining in GC tissues; C: Negative hK12 immunostaining in relevant normal tissues; D: Negative hK12 immunostaining in GC tissues (original magnification A, C $\times 200$, B, D $\times 100$).

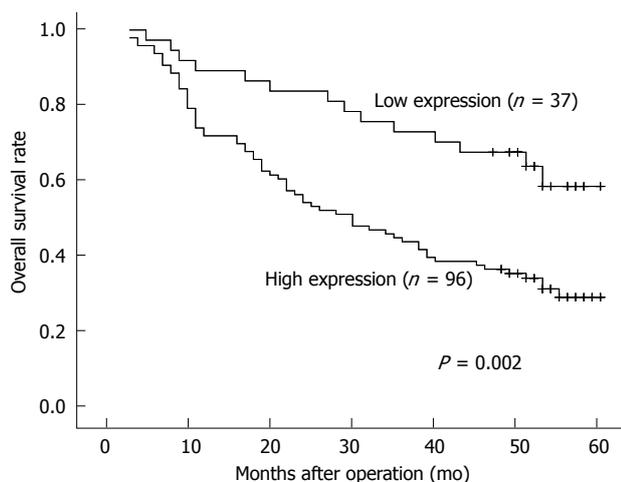


Figure 3 Overall survival of patients with gastric cancer according to human kallikrein 12 protein expression in the cancer tissues. Patients in the high human kallikrein 12 protein (hK12) expression group had a significantly poorer prognosis than those in the low hK12 expression group. Survival curves are drawn according to the Kaplan-Meier method, using the log-rank test to compare the survival rates ($P = 0.002$).

in the level of KLK12 mRNA compared to mock-transfected or parent MKN-45 cells, as determined by quantitative real-time RT-PCR. Furthermore, we performed Western blotting analysis to verify the efficiency of the KLK12 siRNA. The stable KLK12-suppressed clone was confirmed to express markedly lower levels (about one fourth) of hK12 than the MKN-45 parental cells (Figure 5B), confirming that KLK12 siRNA decreased KLK12 expression, consistent with the quantitative real-time RT-PCR results.

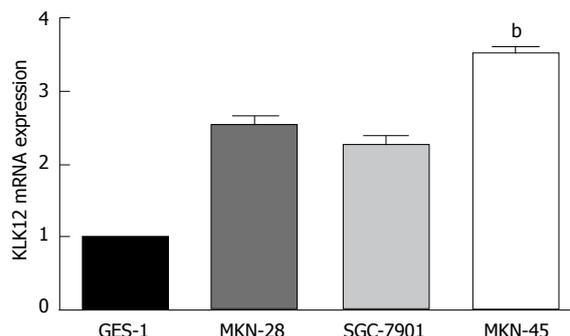


Figure 4 Expression of kallikrein 12 mRNA in gastric cancer cell lines and normal gastric mucosal cell line. Gastric cancer cell lines show higher levels of kallikrein 12 (KLK12) mRNA expression than normal gastric mucosal cell line, while MKN-45 cells expressed the highest level of KLK12 across the four cell lines. Data are shown as mean \pm SD, using the one-way analysis of variance test (^b $P < 0.01$ vs other cell lines).

Knockdown of KLK12 affects the proliferative ability of MKN-45 GC cells

We analyzed whether suppressing the KLK12 expression would alter the growth rate of MKN-45 GC cells. As shown in Figure 6, transfection with KLK12 siRNA remarkably decreased the proliferative ability of MKN-45 cells when compared with the parent and mock-transfected cells ($P = 0.001$), especially from the 3rd to 5th days of the assay.

Knockdown of KLK12 affects the migratory ability of MKN-45 GC cells

We evaluated whether suppression of KLK12 expression could alter the ability of *in vitro* migration and invasion

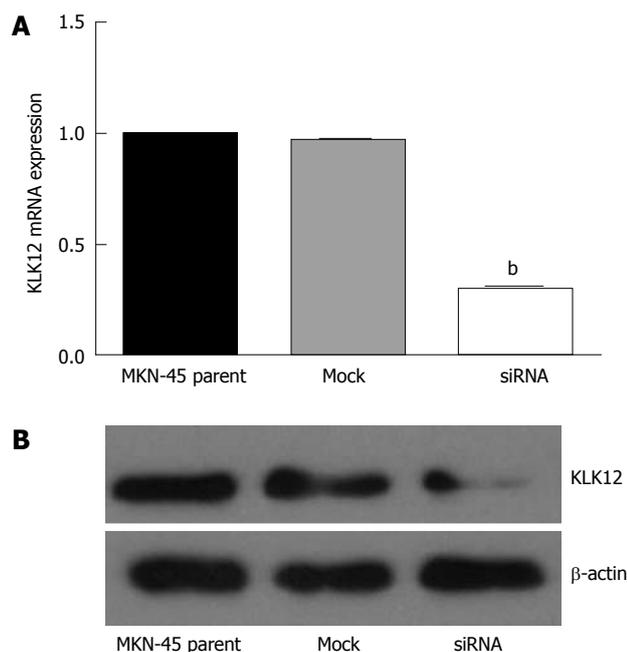


Figure 5 Efficiency of small interfering RNA in silencing the kallikrein 12 mRNA, and protein expression in MKN-45 cells. A: MKN-45 cells transfected with small interfering RNA targeting human kallikrein 12 (*KLK12*) gene showed a remarkable decrease in the level of KLK12 mRNA compared to mock-transfected or parent MKN-45 cells. Data are shown as mean \pm SD, using the one-way analysis of variance test ($^bP < 0.01$ vs other cell lines); B: Western blotting analysis showed a reduced protein expression in KLK12 small interfering RNA (siRNA) transfected cells. The protein levels are measured by Image J software (National Institutes of Health, United States) with β -actin protein normalization.

of MKN-45 cells. As shown in Figure 7, fewer KLK12 siRNA-transfected cells migrated through the chambers (22.00 ± 1.81) when compared to the parent (46.47 ± 2.42) or mock-transfected (45.40 ± 1.99); these differences were statistically significant ($P < 0.001$, Figure 7A and B). However, in the invasion test, the number of KLK12 siRNA-transfected cells that invaded the chambers in 18.40 ± 1.12 , closely similar to both the parent (18.67 ± 0.98) and mock-transfected cells (18.53 ± 0.92). There was no significant difference between the three groups in the invasion assay ($P = 0.054$).

DISCUSSION

The *KLK12* gene is a new member of the *KLK* gene family, some members of which are implicated in the initiation and progression of cancer^[20-22]. *KLK12* is encoded by 5 coding exons, and is structurally similar to serine proteases and other known *KLKs*. *KLK12* is expressed in a variety of tissues including the salivary gland, stomach, uterus, lung, thymus, prostate, colon, brain, breast, thyroid, and trachea. Initially, it was reported that expression of *KLK12* is downregulated at the mRNA level in breast cancer tissues and is upregulated by steroid hormones in breast and prostate cancer cell lines^[17]. Memari *et al*^[19] demonstrated that more than 95% of prostate cancers were *KLK12* positive in a tissue microarray study. To the best of our knowledge, this study is the first investiga-

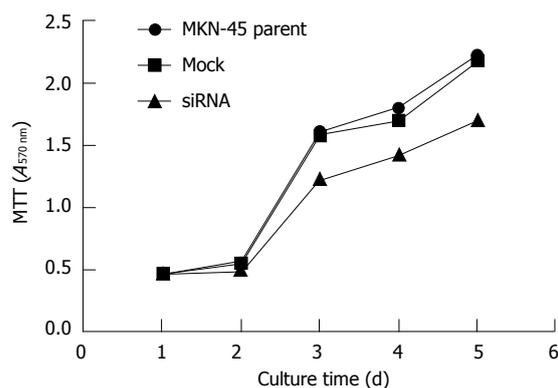


Figure 6 Reduction of cell proliferation by methylthiazolyltetrazolium assay after silencing the kallikrein 12. The proliferative ability significantly decreased in MKN-45 cells after transfection with kallikrein 12 small interfering RNA (siRNA), especially from the 3rd to 5th days of the assay. Data are shown as mean \pm SD, using the one-way analysis of variance test ($P = 0.001$). MTT: Methylthiazolyltetrazolium.

tion to analyze *KLK12* expression in GC. We performed quantitative real time RT-PCR, IHC and a series of functional assays utilizing siRNA-mediated downregulation of *KLK12* expression to determine the role and explore the mechanism of *KLK12* in GC. Our results showed a drastic difference in *KLK12* expression between GC and normal mucosal tissues. Substantially elevated expression of the *KLK6* gene has been observed in clinical tissue samples and several GC cell lines^[13], while other authors have reported that cancerous stomach tissues were found to present significantly decreased levels of *KLK11* and *KLK13* mRNA transcripts in comparison with their normal counterparts^[15,16]. Moreover, our study showed that high hK12 expression was significantly correlated with the lymph node metastasis ($P = 0.001$), histological type ($P < 0.001$) and pathological stage ($P = 0.005$) of GC. High expression of hK12 was also associated with a poor prognosis for patients with GC. These findings suggest that enhanced expression of *KLK12* might play an important role in various pathological processes of GC. MKN-45, a poorly differentiated GC cell line, was chosen for functional experiments because *KLK12* mRNA expression was higher in MKN-45 cells compared to other cell lines. Additionally, hK12 expression was significantly higher in undifferentiated GC. In our experiments, we established clones with suppressed *KLK12* expression by gene silencing using RNA interference (RNAi) techniques. RNAi is mediated by siRNAs that are produced from long double stranded RNAs of exogenous or endogenous origin by an endonuclease of the RNase-III type called Dicer^[23]; this technique has emerged as a powerful tool for understanding gene functioning. As quantitative real-time RT-PCR and Western blotting revealed, *KLK12* siRNA remarkably reduced *KLK12* expression of MKN-45 cells. MTT proliferation assays showed with *KLK12* siRNA dramatically decreased the proliferation of MKN-45 cells when compared with MKN-45 parent and mock-transfected cells. The differences were significant, especially from the 3rd day to the 5th day after transfection, which is in accor-

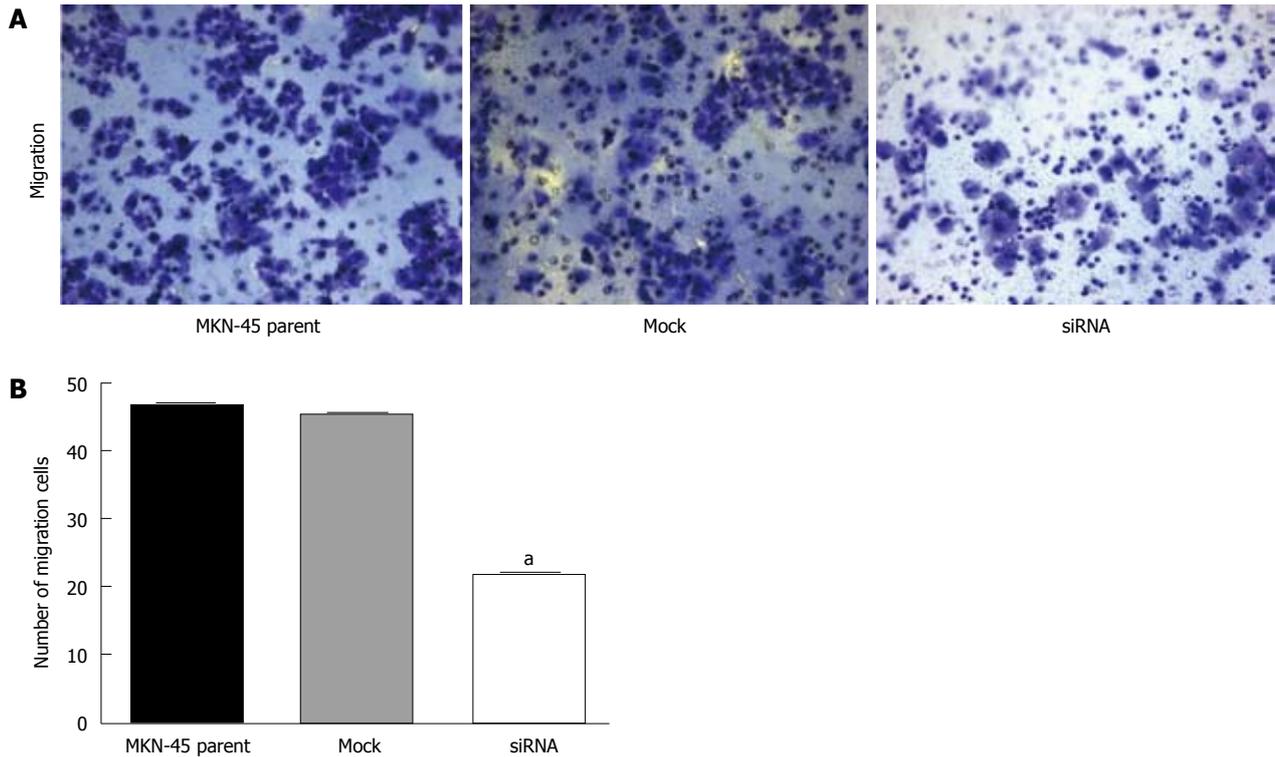


Figure 7 Effect of cell migration by kallikrein 12 knockdown. A: 24-well transwell chambers with upper and lower culture compartments separated by polycarbonate membranes with 8- μ m pores were used for migration or invasion assay. The chambers were stained by 0.09% crystal violet and cells were counted using light microscopy under high magnification (magnification $\times 10$); B: The migration or invasion cells were counted in 5 individual fields per insert. Values were the number of cells. Data are shown as mean \pm SD, using the one-way analysis of variance test ($^*P < 0.05$ vs other cell lines).

dance with the Western blotting data showing knockdown of KLK12 expression was greatest 72 h after transfection. Metastasis and local recurrence are known to be the primary causes of death after radical surgery of GC patients. Tumor metastasis is a complicated process and involves many steps including migration of cancer cells to, and invasion through, the basement membrane^[24]. In our experiments, significantly fewer KLK12-suppressed cells migrated across polycarbonate membranes when compared to MKN-45 parent and mock-transfected cells ($P < 0.001$). The invasiveness of all three cell lines was comparable, and the KLK12-suppressed cells displayed no difference in the invasion assay compared to parent or mock-transfected cells. Other KLKs have been shown to degrade components of the extracellular matrix. Magklara *et al.*^[25] reported that hK6 can degrade fibrinogen and collagen type I, basic constituents of the extracellular matrix, as well as collagen type IV, a major component of the basement membrane *in vitro*. The lysis of certain components of the extracellular matrix is linked with an altered regulation of tumor metastasis. However, Memari *et al.*^[26] reported that KLK12 is secreted as an inactive proenzyme, which is able to autoactivate to gain enzymatic activity. However, active KLK12 quickly loses its activity due to autodegradation, and its activity can also be rapidly inhibited by zinc ions and by alpha2-antiplasmin through covalent complex formation. According to these results, it is reasonable to conclude that the increased KLK12 expression may not play an important role in metastasis. In

conclusion, we present an early report that KLK12 was remarkably overexpressed in GC tissues and that high KLK12 expression levels were associated with the lymph node metastasis, histological type, pathological stage and poor patient prognosis. Our findings also demonstrate that knockdown of KLK12 expression leads to reduced proliferation and migratory ability with little effect on invasiveness in MKN-45 GC cells. Consequently, KLK12 might serve as a novel diagnostic and prognostic biomarker, as well as a potential therapeutic target, in GC.

COMMENTS

Background

Gastric cancer (GC) is the fourth most common malignancy and the second most common cause of cancer mortality worldwide. Although morbidity and mortality rates for GC are decreasing steadily in many countries, the overall outcome for patients with GC has not changed significantly in recent decades. It is necessary to find a reliable biomarker for GC to be incorporated into routine clinical practice.

Research frontiers

The human kallikrein 12 (KLK12) gene is a new member of the KLK gene family. Similar to other kallikreins, KLK12 is a proteolytic enzyme with serine protease activity, and participates in several biological processes. Moreover, KLK12 may also play a role in human carcinogenesis of cancers such as breast and prostate cancer. However, little is known about KLK12 in human GC.

Innovations and breakthroughs

The results of this study provide strong evidence suggesting that KLK12 expression is upregulated in GC tissue and correlated with lymph node metastasis, poor histological type, advanced clinical stage and decreased overall survival rate. Furthermore, the authors found that knockdown of KLK12 markedly

decreased the proliferative and migratory abilities of MKN-45 GC cells.

Applications

These results demonstrate that KLK12 might serve as a novel diagnostic and prognostic biomarker in GC. This study may represent a future strategy for therapeutic intervention in the treatment of patients with GC.

Terminology

The *KLK* genes are a newly identified subgroup of putative serine proteases, consisting of 15 genes located within approximately 256 kb on chromosome 19q13.3-4.

Peer review

This is a good descriptive study in which the authors investigate expression of KLK12 in GC tissues. The biological effects of KLK12 knockdown in the GC cell line MKN-45 were also studied. The results are interesting and suggest knockdown of KLK12 overexpression in GC may be a potential therapeutic target. The study was well designed and the data are convincing.

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Hepatoprotective effects of baicalein against CCl₄-induced acute liver injury in mice

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Abstract

AIM: To investigate the hepatoprotective effect of baicalein against carbon tetrachloride (CCl₄)-induced liver damage in mice.

METHODS: Mice were orally administered with baicalein after CCl₄ injection, and therapeutic baicalein was given twice a day for 4 d. The anti-inflammation effects of baicalein were assessed directly by hepatic histology and serum alanine aminotransferase and aspartate aminotransferase measurement. Proliferating cell nuclear antigen was used to evaluate the effect of

baicalein in promoting hepatocyte proliferation. Serum interleukin (IL)-6, IL-1 β and tumor necrosis factor- α (TNF- α) levels were measured by enzyme-linked immunosorbent assay and liver *IL-6*, *TNF- α* , transforming growth factor- α (*TGF- α*), hepatocyte growth factor (*HGF*) and epidermal growth factor (*EGF*) genes expression were determined by quantitative real-time polymerase chain reaction.

RESULTS: CCl₄-induced acute liver failure model offers a survival benefit in baicalein-treated mice. The data indicated that the mRNA levels of IL-6 and TNF- α significantly increased within 12 h after CCl₄ treatment in baicalein administration groups, but at 24, 48 and 72 h, the expression of IL-6 and TNF- α was kept at lower levels compared with the control. The expression of TGF- α , HGF and EGF was enhanced dramatically in baicalein administration group at 12, 24, 48 and 72 h. Furthermore, we found that baicalein significantly elevated the serum level of TNF- α and IL-6 at the early phase, which indicated that baicalein could facilitate the initiating events in liver regeneration.

CONCLUSION: Baicalein may be a therapeutic candidate for acute liver injury. Baicalein accelerates liver regeneration by regulating TNF- α and IL-6 mediated pathways.

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Key words: Baicalein; Carbon tetrachloride; Liver injury; Liver regeneration; Hepatocyte proliferation

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INTRODUCTION

Liver is an important organ which plays a central role in metabolic homeostasis^[1]. It also has an amazing regenerative capability after liver mass loss, as demonstrated by Higgins *et al.*^[2] in 1931. Carbon tetrachloride (CCl₄)-induced hepatic injury is a very classic model widely used for hepatoprotective drug screening^[3,4]. The acute hepatotoxicity of CCl₄ lies in its biotransformation to trichloromethyl free radical (CCl₃) or trichloroperoxy radical (CCl₃O₂) produced by the mixed-function cytochrome P450 oxygenase system of the endoplasmic reticulum, which causes oxidative stress and membrane damage^[5]. These free radicals cause lipid peroxidation which results in hepatocellular damage and enhances formation of inflamed tissues. The advantage of this model is that CCl₄ can fulminate hepatitis within a few hours, which specifically leads to necrosis and fatty liver, in a similar way as what happens in the cases of acute hepatitis. Meanwhile, following an inflammatory response launched by resident inflammatory cells, CCl₄-induced acute liver injury also involves an intricately regulated process of hepatocyte regeneration when the dosage of CCl₄ is below lethal level which would lead to irreversible liver damage^[6,7].

Baicalein (5, 6, 7-trihydroxyflavone, BAE, C₁₅H₁₀O₅) is a flavonoid extract from the root of *Scutellaria baicalensis* Georgi, a plant used in traditional Chinese medicine. Previous studies reported that baicalein has multiple functions. It acts as an anti-bacteria and anti-inflammation agent, inhibits the aggregation of blood platelets, decreases the production of endotoxin, and alleviates the reperfusion injury in ischemic tissues^[8,9]. Baicalein was indicated to suppress the growth of human hepatoblastoma cells^[10,11], human breast cancer cells^[12,13], human lung fibroblasts and peripheral lymphocytes^[14] and human leukemia HL-60 cells^[15]. Baicalein has beneficial effects against the cytotoxicity and genotoxicity to hepatocytes by tert-butylhydroperoxide *via* quench free radicals. Moreover, baicalein could protect animals from *D*-galactosamine/lipopolysaccharides induced acute liver failure in murine models, and especially reduce apoptosis (even hepatic necrosis) *via* cellular FLICE-like inhibitory protein and mitogen-activated protein kinase pathway^[16,17]. However, the antihepatotoxic mechanism of baicalein remains vague so far. The aforementioned investigations for liver diseases on the role of baicalein in selectively inducing apoptosis of cancer cells and inhibiting normal hepatocyte apoptosis, prompted us to study whether baicalein would increase the secretion of various inflammatory cytokines and facilitate regeneration of liver cells. The aim of this study is to assess whether baicalein could prevent acute liver injury induced by CCl₄ in mice and to investigate the possible mechanism of its protective role.

MATERIALS AND METHODS

Animals and chemicals

Specific pathogen-free male C57 BL/6 mice (8 wk old) were obtained from Shanghai Slac Laboratory Animal Corporation. The mice were maintained in a conventional clean facility in accordance with the National Animal Care and Use Committee. CCl₄ and baicalein were purchased from Sigma-Aldrich Biotechnology (St Louis, MO, United States). Assays kits for the detection of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were purchased from Jiancheng Biological Technology, Inc. (Nanjing, China). Mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA) and the SABC Staining Kit were from Boster Biological Technology (Wuhan, China). Serum levels of interleukin (IL)-1 β , IL-6 and tumor necrosis factor- α (TNF- α) were measured by enzyme-linked immunosorbent assay (ELISA) kits for IL-1 β , IL-6 and TNF- α from R and D system (Minneapolis, MN, United States). All other chemicals were of the highest grade commercially available.

Induction of liver injury and baicalein administration

Acute liver injury in mice was induced by intraperitoneal injection of CCl₄ at a dose of 1 mL/kg body weight (1:3 diluted in corn oil). A lethal dose was administered by intraperitoneal injection of CCl₄ at 2.6 mL/kg (1:1 diluted in corn oil). At the indicated time points, serum and liver specimens were collected. Mice were orally administered with baicalein (80 mg/kg) dissolved in CMC-Na to 200 mg/L 1 h after CCl₄ injection, and the same dose of baicalein was given twice a day for 4 d, and control mice were treated with same dosage CMC-Na.

Serum AST and ALT

Serum AST and ALT levels were determined with a commercial assay kit (Nanjing Jiancheng Biological Technology, Inc., China). Enzyme activities were presented in international unit per liter (IU/L).

Histology-injury grading

Formalin-fixed, paraffin-embedded liver sections were stained with hematoxylin-eosin for the histological studies. To evaluate the degree of necrosis after acute liver injury, we created an injury grading score (Grades I-IV) based on severity of necrotic lesions in the liver parenchyma (Table 1).

Proliferating cell nuclear antigen staining

For PCNA immunohistochemical staining, de-paraffinized sections of liver blocks were used. Liver tissues were fixed for 24 h in neutral buffered formalin, processed routinely and embedded in wax. Immunohistochemical staining was performed as previously described^[18]. The sectioned liver tissues were stained using a mouse monoclonal antibody against PCNA and the SABC Staining Kit (Wuhan Boster Biological Technology, Wuhan, China) according to the

Table 1 Liver injury grading system

No. of mice	Day 2 ¹	Day 3 ¹	Day 5 ¹	Day 7 ¹
Baicalein				
1	III	I	I	0
2	III-IV	II	0	0
3	III	I	0	0
4	III	II	0	0
5	III-IV	I	I	0
6	III	I	0	0
Control				
1	IV	II-III	I-II	0
2	III-IV	III	II	I
3	III-IV	III	II	II
4	IV	III	II	0
5	IV	II	II	I
6	IV	III	I	I

¹Days after CCl₄ treatment at the sacrifice point. Injury grading with respect to severity of necrosis in liver parenchyma. Grade 0: Normal histology; Grade I: Presence of degenerated hepatocytes with only rare foci of necrosis; Grade II: Mild centrilobular necrosis around the central vein, occupying only a part of Rappaport's zone III; Grade III: Established necrosis limited to zone III; Grade IV: Extensive, confluent centrilobular necrosis involving Rappaport's zone III and II.

manufacturer's protocol, then subjected to photomicroscopic observation (NIS-Elements Basic Research, Nikon Eclipse 50i, Kanagawa, Japan).

ELISA

Serum IL-1 β , IL-6 and TNF- α levels were measured by ELISA kit (RD system, Minneapolis, MN, United States) according to the manufacturer's instructions. Cell lysates were generated by adding 1 mL fresh medium to 100 mg liver specimen or 1×10^7 cells followed by three freeze-thaw cycles. Transforming growth factor- α (TGF- α), hepatocyte growth factor (HGF) and epidermal growth factor (EGF) and ELISA kits were used to determine protein concentrations^[19,20]. ELISA was performed in triplicate for each sample of lysate.

Real-time quantitative polymerase chain reaction

Total RNA was obtained from the liver of mice and was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA, United States). The quantification and qualification of RNA were performed using ultraviolet absorbance assay and electrophoresis in 1.2% agarose. RNA quality was satisfactory for the 28s rRNA band on gel and had twice the intensity of the 18s rRNA band without significant smearing of rRNA. Real-time quantitative polymerase chain reactions (PCRs) were performed with the MJ chromo 4 reverse transcription-PCR detection system (Bio-Rad Laboratories, Hercules, CA, United States). Specific primers were designed using Primer 5.0 software (Premier Biosoft International, Palo Alto, CA, United States) and their sequences are listed in Table 2. As an internal control, the expression of the housekeeping gene β -actin was measured and remained constant at all the experimental conditions studied.

Table 2 Primer sequences used for real-time quantitative polymerase chain reaction

Gene	Sense	Anti-sense
IL-6	CCACTCCCAACAGACCT-GTCTATAC	CACAACCTTTTCTCATTTT-CACGA
TNF- α	AAGCCGTAGCCACGTC-GT	CGTAGTCGGGGCAGCCTT-GTC
HGF	GTGCTGGGCATTACTAT-GATGG	CTGCATCTCCCTTCACAGG
TGF- α	GGCGGCTGCAGTGGT-GTCTC	AGCCACCACAGCCAGGAG-GTHGF
EGF	CGGACAGCTACACGGAATG	CGAGGCAGACACAAATA-ACCC
β -actin	AGCCTTCCTTCTTGGGTATG	GTGTTGGCATAGAGGTCTT-TAC

TNF- α : Tumor necrosis factor- α ; IL: Interleukin; TGF- α : Transforming growth factor- α ; HGF: Hepatocyte growth factor; EGF: Epidermal growth factor.

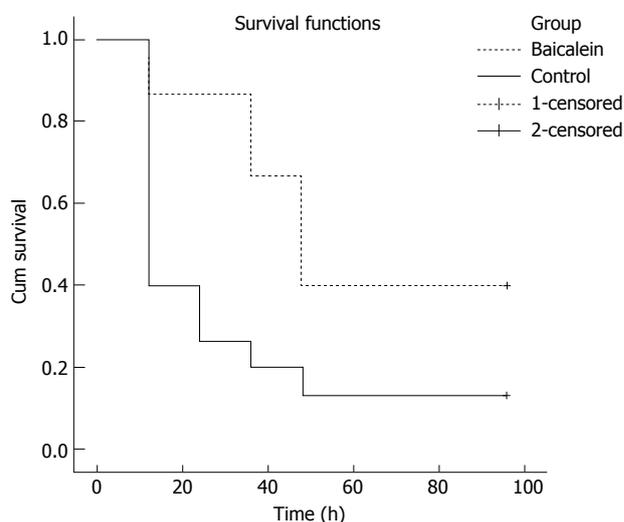


Figure 1 Baicalein increased probability of survival after a lethal dose of carbon tetrachloride (2.6 mL/kg). Mice ($n = 15$) were administered with or without baicalein twice a day for 5 d. Survivals were scored twice a day, and the results were analyzed using the log-rank test and expressed as the Kaplan-Meier survival curves. $P = 0.009$ between control and baicalein groups.

Statistical analysis

Student's t test (unpaired, two-tailed) was used for comparisons between data from specified different conditions. Results from survival experiments were analyzed using the log-rank test and presented as Kaplan-Meier survival curves.

RESULTS

Baicalein reduces mortality after a lethal dose performance

In a previous experiment to observe the dosage-dependent effect of CCl₄, we found that 2.6 mL/kg CCl₄ was a median lethal dose (a mortality of 50%, data not shown) within 24 h. Oral baicalein administration offers a survival

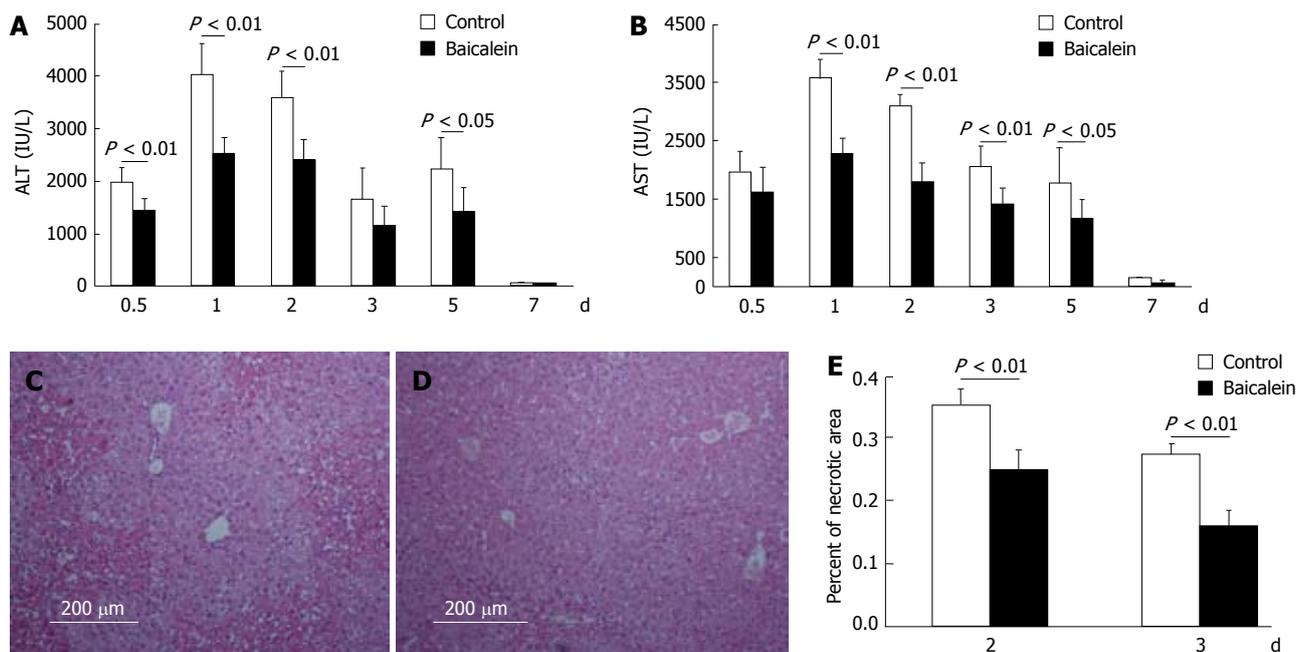


Figure 2 Baicalein protects liver against carbon tetrachloride induced acute liver injury. A: Serum alanine aminotransferase (ALT); B: Serum aspartate aminotransferase (AST); C: Hematoxylin and eosin (HE) stained liver sections of control group 3 d after carbon tetrachloride (CCl₄) treatment; D: HE stained liver sections of baicalein group 3 d after CCl₄ treatment; E: Percent of necrotic areas in control group and baicalein group 2 and 3 d after CCl₄ treatment. Mice received intraperitoneal CCl₄ at the dosage of 1 mL/kg body weight (1:3 diluted in corn oil). Mice in baicalein group were orally administered baicalein (80 mg/kg) 1 h after CCl₄ injection, twice a day for 4 d (original magnification, ×100). Necrosis with clusters of inflammatory cells around central vein was seen in control group; and histological recovery with only inconspicuous necrosis remaining around central vein, and very few inflammatory cells were present in the baicalein group. Control mice were treated with an equal volume of CMC-Na. Values represent mean ± SE (n = 6). P < 0.05, P < 0.01 between control and baicalein groups.

benefit for mice, increasing the probability of survival significantly one d after CCl₄ injection (P = 0.009, Figure 1).

Baicalein protects mice from acute hepatocellular damage

To confirm the effect of baicalein in protecting mice from hepatic damage, we used serum ALT and AST levels as indicators for liver injury. In the control group, the serum level of ALT and AST rapidly reached the peak level at day 1, and decreased thereafter, while baicalein significantly inhibited the elevated ALT and AST from day 1 to day 5 (n = 6) (Figure 2A and B). The attenuated increase of serum AST and ALT indicated that baicalein plays a direct protective role in hepatocytes. To evaluate the effect of baicalein on hepatocellular necrosis and inflammation, histological changes in the liver after CCl₄ administration with or without baicalein treatment were examined by histology-injury grading (Table 1). Liver sections from the baicalein-treated mice demonstrated only moderate necrosis involving the centrilobular areas, maintaining a rather normal architecture. The necrotic areas were significantly diminished around the central vein and centrilobular regions in baicalein-treated mice at day 3 (Figure 2C-E). These findings indicated that baicalein has potential anti-hepatotoxic activity.

Baicalein promotes hepatocyte proliferation from an early phase

To confirm whether baicalein has the potent advantage

of accelerating hepatocyte proliferation from an early phase, we investigated the proliferation of hepatocytes using immunostaining of PCNA in sections of liver tissue at days 2 and 3. The PCNA staining confirmed that baicalein administration increased the number of positive staining cells more significantly at day 2 compared with the control group (Figure 3A and B). A great number of PCNA⁺ hepatocytes could be detected in the liver sections of baicalein-treated mice at day 3 (Figure 3C and D), which demonstrated that baicalein significantly increased the number of PCNA⁺ cells. Numbers of PCNA⁺ cells in at least 12 mm² tissue sections were counted for each mouse, and data showed that baicalein could accelerate hepatocyte proliferation (Figure 3E).

Serum levels of IL-1β, IL-6 and TNF-α

To evaluate the hepatoprotective mechanism of baicalein, serum IL-1β, TNF-α and IL-6 levels were determined by ELISA kit. Serum IL-1β was found to be elevated after CCl₄ treatment^[21], whereas baicalein administration resulted in significant attenuation of the elevation (Figure 4A). CCl₄-induced acute liver injury could activate hepatic non-parenchymal cells (including Kupffer cells and stellate cells) and increase the production of TNF-α and IL-6^[22]. In our study, we found that serum TNF-α and IL-6 were rapidly increased and reached the peak level within 12 h in baicalein administration group as compared with the control group, and then decreased within 24 h (Figure 4B and C).

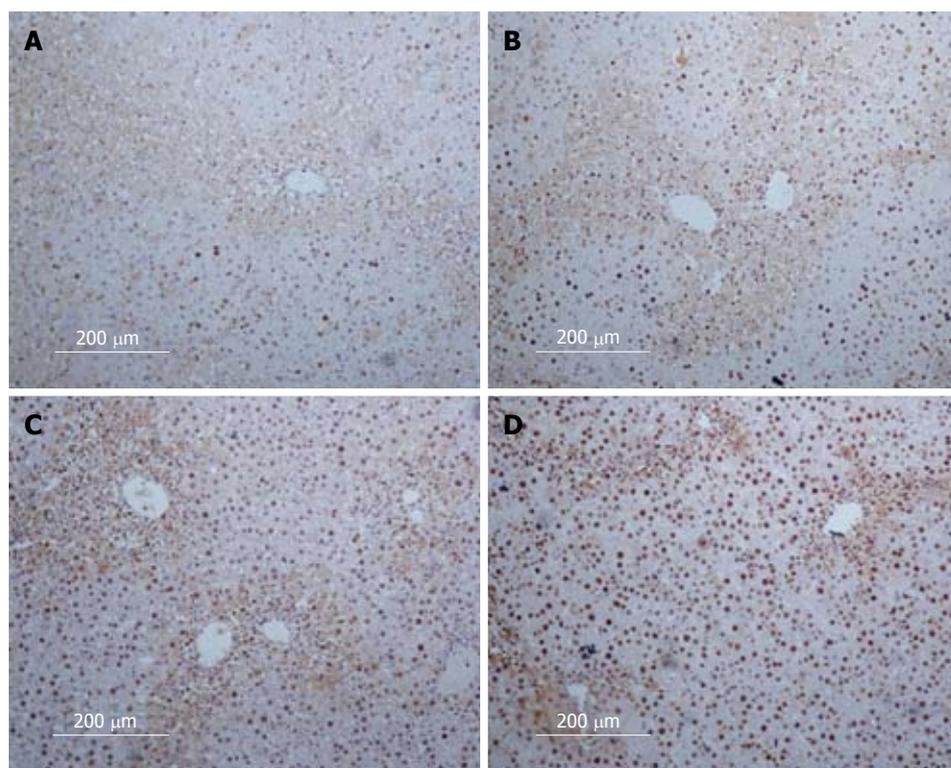
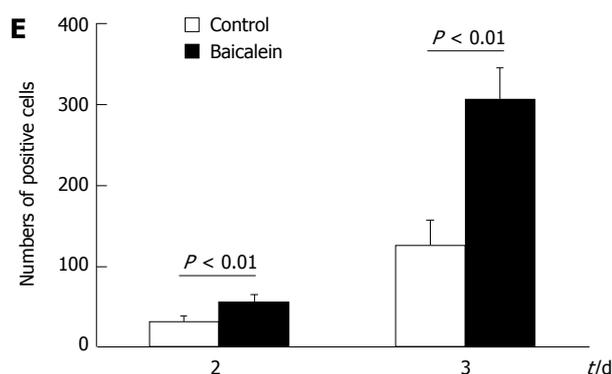


Figure 3 Proliferation status of carbon tetrachloride induced mice after treated with or without baicalein. A, B: Immunostaining of proliferating cell nuclear antigen (PCNA) in liver sections from control (A) and baicalein (B) groups 2 d after carbon tetrachloride (CCl₄) treatment; C, D: Immunostaining of PCNA in liver sections from control (C) and baicalein (D) groups 3 d after CCl₄ treatment; E: Numbers of PCNA⁺ cells in CCl₄ induced mice after treated with or without baicalein. At least six 12-mm² tissue sections were counted for each mouse. Values represent mean \pm SE ($n = 6$). $P < 0.01$ between control and baicalein groups.



Expression of TNF- α and IL-6 in liver

Real-time quantitative PCR was used to quantify the expression of TNF- α and IL-6 in mouse liver. Data showed that in baicalein administration group, the production of TNF- α and IL-6 mRNA reached a peak level, which was even higher than in the control group, and then decreased rapidly in 24 h (Figure 5A and B).

Expression of TGF- α , HGF and EGF in liver

Real-time quantitative PCR was used to quantify the levels of TGF- α , HGF and EGF mRNA in liver. Data showed that the production of TGF- α , HGF and EGF mRNA was upregulated more rapidly in the baicalein administration group during the early phase and kept at a generally higher level within the process of liver regeneration (Figure 5C-E).

DISCUSSION

The model of acute intoxication with CCl₄ has been used

for decades to investigate the response of acute liver injury, because the elementary lesions caused by this hepatotoxin replicate those seen in most cases of human liver diseases, which makes it a good model to study both signal transduction and cell cycle events *in vivo*^[23,24]. Using this delicate model, we have identified the protective effect of baicalein against the typical acute liver injury.

Oral administration of baicalein to mice which have received a LD₅₀ dosage of CCl₄ resulted in a significantly reduced mortality rate. Since the pathological effect of CCl₄ in the animals has been proved to be mainly restricted to the liver and lethality of high-dose CCl₄ is mostly related with organ failure following acute liver failure instead of direct injury to other organs, it is reasonable to hypothesize that administration of baicalein can reduce animal mortality mainly through attenuating acute liver damage by CCl₄, and facilitating the preservation and restoration of liver functions.

It has been proved that baicalein administration indeed attenuated acute liver damage. The indicators for

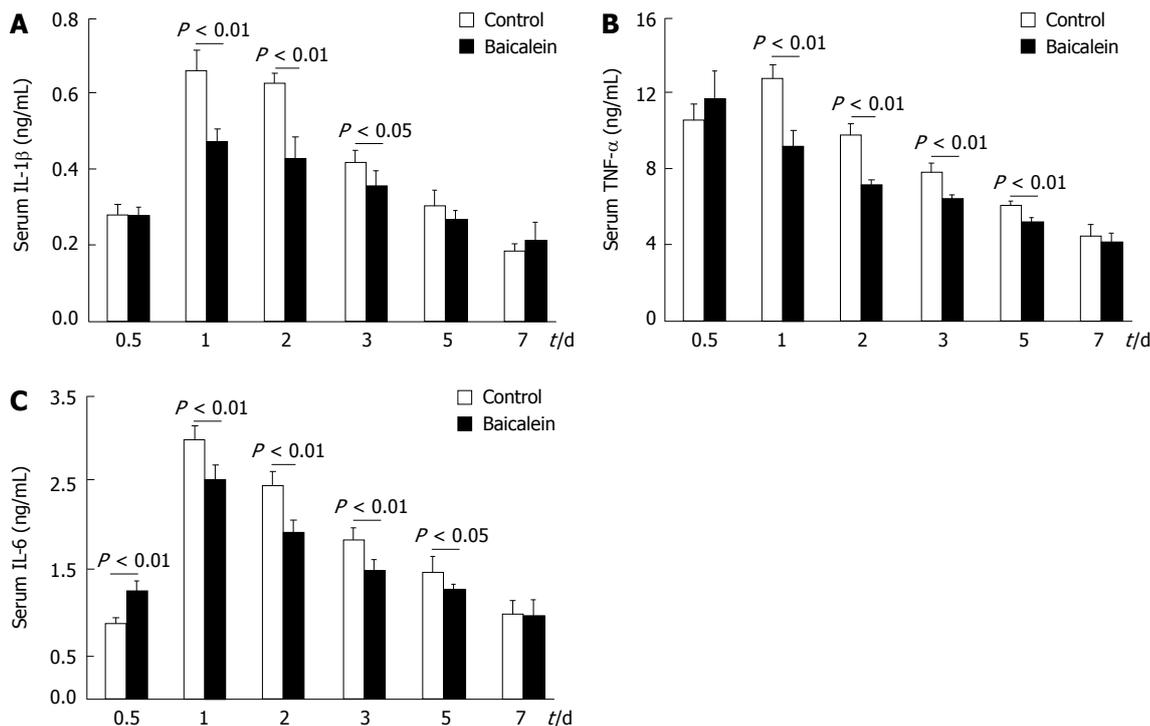


Figure 4 Levels of interleukin-1β, interleukin-6 and tumor necrosis factor-α in serum in control and baicalein groups after CCl₄ (1 mL/kg) treatment. A: Serum interleukin (IL)-1β; B: Serum tumor necrosis factor-α (TNF-α); C: Serum IL-6. A, B and C were determined by enzyme-linked immunosorbent assay kit. Values represent mean ± SE (n = 6). P < 0.05, P < 0.01 between control and baicalein groups.

the liver damage we have utilized are serum aminotransferase activities, including AST and ALT. They are commonly referred to as “liver enzymes”, because the levels of these enzymes are released from damaged hepatocytes into the blood, and their levels in the serum have been widely recognized as a very important indicator to judge the severity of acute hepatic injury^[25]. In our experiment, administration of baicalein attenuated the elevated serum ALT and AST induced by CCl₄ in mice, which indicated that the proportion of damaged hepatocytes was reduced as a direct result of baicalein administration. Elevated ALT level was found to be significantly attenuated 12 h after CCl₄ treatment, while similar phenomenon appeared at 24 h after CCl₄ treatment for AST. Both time points are defined as the early-stage liver damage in which cell apoptosis and necrosis dominate the process. When the liver damage progresses over time, the speed of cell damage as a result of either cell apoptosis or necrosis is reduced, as indicated by the relative decrease of AST/ALT levels at later time points of days 3 to 5. On the other hand, regeneration of liver gradually took place from the middle to late stages of liver damage, during which cell proliferation rate would naturally increase till the original weight and shape of the liver and its functions, is restored. We used another statistical index to measure the possible role of baicalein in the regeneration of liver tissue. It is the density of positive cells in a certain area of tissue section immunostained with PCNA antibody. The index strongly indicated that baicalein treatment contributes to a faster liver recovery after CCl₄-induced liver injury by promoting the endogenous regeneration

process from the middle stage of the entire liver damage process. We also used histological methods as supportive means to reveal the degree of cell necrosis and inflammation. Data also showed that oral baicalein administration inhibited inflammation, necrosis, and destruction of liver architecture.

To investigate the underlying mechanism, we evaluated the effects of baicalein treatment on the serum level of certain key cytokines tightly related to inflammation and cell proliferation. IL-1β, IL-6 and TNF-α, as acute-phase proteins, are considered to be the special biomarkers that reflect inflammatory status^[26]. IL-1β plays a key role in inflammation, usually leading to tissue destruction. Furthermore, IL-1β has been previously shown to antagonize hepatocyte proliferation^[27,28]. Serum IL-1β can increase dramatically during different inflammatory and non-inflammatory processes. In the present study, we observed that baicalein administrated mice demonstrated a significantly lower serum level of IL-1β at days 1, 2, 3 and 5, compared with the control group. The decreased level of inflammatory cytokines may explain the accelerated liver regeneration observed in baicalein administrated mice. IL-6 and TNF-α expression has been identified as attractive targets for liver regeneration. The release of TNF-α, as a pro-inflammatory mediator in liver apoptosis, is also linked to cytotoxicity induced by CCl₄^[17,29]. Kupffer cells (macrophages in liver) produce TNF-α in rapid response to tissue injury, which then up-regulates the expression of IL-6. TNF-α and IL-6 together activate the neighboring hepatocytes, leading to signal transducer and activator of transcription STAT3 activation and

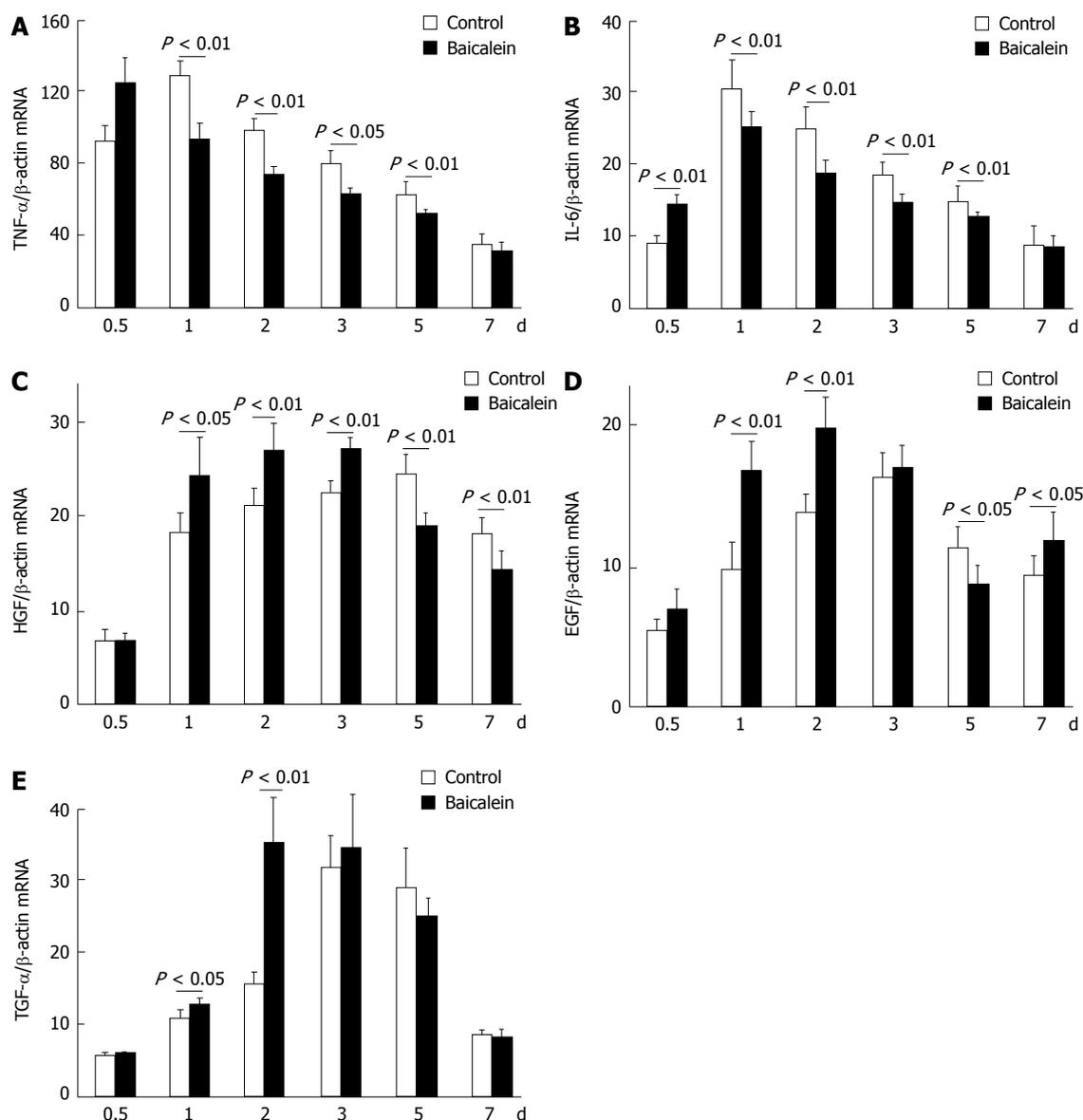


Figure 5 The microRNA levels of tumor necrosis factor- α , interleukin-6, transforming growth factor- α , hepatocyte growth factor and epidermal growth factor in liver of control and baicalein groups after carbon tetrachloride (1 mL/kg) treatment. Total RNA was isolated from liver tissue using TRIzol methods and quantified spectrophotometrically at 260 nm. The mRNA levels of tumor necrosis factor- α (*TNF- α*) (A), interleukin-6 (*IL-6*) (B), hepatocyte growth factor (*HGF*) (C), epidermal growth factor (*EGF*) (D) and transforming growth factor- α (*TGF- α*) (E) genes were quantified using reverse transcription polymerase chain reaction and normalized to β -actin housekeeping gene. Values represent mean \pm SE ($n = 6$). $P < 0.05$, $P < 0.01$ between control and baicalein groups.

the production of several other proteins that are shared within the growth-factor-mediated pathway network. In previous studies, pretreatment with IL-6 before CCl₄ reduces acute CCl₄-mediated cell apoptosis, and accelerates regeneration in both wild-type and IL-6^{-/-} livers^[30]. The mechanism of IL-6 and TNF- α in protecting the liver against injury has not been fully clarified^[31-33]. Previous studies showed that liver regeneration and hepatoprotection require the cytokine IL-6 immediately after liver injury^[34,35]. But overexpression of IL-6 inhibits hepatocyte growth and causes liver injury^[36,37]. In the present study, the expression of TNF- α and IL-6 in baicalein administrated mice reached a high level at day 0.5 and then was kept at a relatively lower level at days 1, 2, 3 and 5 compared with the control. We consider that the lower

levels of TNF- α and IL-6 which are cell death mediators from days 1 to 5 may facilitate liver regeneration. In term of the mechanisms, we found that gene expression of *IL-6* and *TNF- α* in treated liver was enhanced in a similar pattern as the level of corresponding proteins, leading to the conclusion that baicalein could indeed alter the expression of certain cytokines to affect the liver damage process.

Another group of molecules we have investigated are growth factors such as HGF, TGF- α and EGF. They promote hepatic survival by stimulating liver regeneration and providing hepatoprotection in various models of liver injury, such as toxic damage caused by CCl₄^[38]. It has been proven that HGF, TGF- α and EGF are the main growth factors secreted after hepatic injury^[39]. HGF is the most

potent mitogen for mature hepatocytes and acts as a hepatotropic factor. HGF level is increased markedly in mouse liver after various liver injuries such as hepatitis, ischemia, physical crush and partial hepatectomy. HGF acts as a trigger for liver regeneration and strongly enhances EGF expression. Previous studies indicated that the liver regenerative response is blocked if antibodies to HGF are administered at the same time as CCl₄ treatment^[40]. HGF administration to rodents was confirmed to reduce the level of CCl₄-induced injury. HGF has been shown to regulate DNA synthesis partially through upregulation of other growth factors in hepatocytes *in vivo* and *in vitro*, which indicates that all of them are crucial for liver regeneration^[41,42]. In our study, a significant increase of HGF, EGF and TNF- α expression occurred in livers from baicalein-treated groups during the proliferation phase (from days 1 to 3). Such expression reached a lower level in baicalein-treated mice at day 5 compared with the control, which indicated that the liver regeneration was terminated at an earlier phase.

In conclusion, we found baicalein from the Chinese herbal medicine possesses strong beneficial effects in a mouse model against acute liver injury caused by CCl₄. The expression of inflammatory cytokines IL-6 and TNF- α are markedly increased at the very early stage, which activate crucial signal transducers, including signal transducer and activator of transcription 3 and trigger certain signal cascades related to liver regeneration. During the middle stage, the expression level of such cytokines was significantly lowered to reduce inflammation cell apoptosis. The subsequent elevation of HGF, TGF- α and EGF may promote hepatic survival by stimulating hepatocyte regeneration. The protective effect of baicalein represents a clinical potential in the development of novel therapeutic agents for acute liver injury.

COMMENTS

Background

Baicalein is one of the bioactive compounds of *Scutellaria baicalensis* Georgi which has been shown to have anti-inflammatory, anti-bacteria and anti-hepatotoxic effects. However, the underlying mechanisms by which baicalein protects the liver from drug-induced injury still remain speculative.

Research frontiers

Previous investigations of liver diseases on the role of baicalein in selectively inducing apoptosis of cancer cells and inhibiting normal hepatocyte apoptosis, have prompted studies whether baicalein would increase the secretion of various inflammatory cytokines and facilitate regeneration of liver cells. This study assessed whether baicalein administration could prevent acute liver injury induced by carbon tetrachloride in mice and investigated the possible mechanism of its protective role.

Innovations and breakthroughs

The authors found that baicalein significantly elevated the serum level of tumor necrosis factor- α (TNF- α) and interleukin (IL)-6 at the early phase, which indicated that baicalein could facilitate the initiating events in liver regeneration. This study supports the possibility that baicalein may be a therapeutic candidate for acute liver injury, and indicates that baicalein could accelerate liver regeneration by regulating the TNF- α and IL-6 mediated pathways.

Applications

All these results support the possibility of baicalein being a therapeutic candidate for acute liver injury, and indicate that baicalein accelerates liver regeneration by regulating the TNF- α and IL-6 mediated pathways.

Peer review

The authors concluded that baicalein could facilitate the initiating events in liver regeneration. The experiments were well done and the results were clearly shown. This study is well designed and performed, and is of great interest for its novelty and impact in the field.

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Is proliferative colonic disease presentation changing?

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colorectal cancer (CRC) and polyps in a single referral center in Rome, Italy, during two periods.

METHODS: CRC data were collected from surgery/pathology registers, and polyp data from colonoscopy reports. Patients who met the criteria for familial adenomatous polyposis, hereditary non-polyposis colorectal cancer syndrome or inflammatory bowel disease were excluded from the study. Overlap of patients between the two groups (cancers and polyps) was carefully avoided. The χ^2 statistical test and a regression analysis were performed.

RESULTS: Data from a total of 768 patients (352 and 416 patients, respectively, in periods A and B) who underwent surgery for cancer were collected. During the same time periods, a total of 1693 polyps were analyzed from 978 patients with complete colonoscopies (428 polyps from 273 patients during period A and 1265 polyps from 705 patients during period B). A proximal shift in cancer occurred during the latter years for both sexes, but particularly in males. Proximal cancer increased > 3-fold in period B compared to period A in males [odds ratio (OR) 3.31, 95%CI: 2.00-5.47; $P < 0.0001$]. A similar proximal shift was observed for polyps, particularly in males (OR 1.87, 95%CI: 1.23-2.87; $P < 0.0038$), but also in females (OR 1.62, 95%CI: 0.96-2.73; $P < 0.07$).

CONCLUSION: The prevalence of proximal proliferative colonic lesions seems to have increased over the last decade, particularly in males.

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Key words: Colorectal cancer; Polyp; Location; Colonoscopy; Surgery

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Abstract

AIM: To compare the site, age and gender of cases of

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies worldwide, the etiology of CRC involves environmental and genetic factors. In most cases, the cancer develops according to the classic adenoma-carcinoma sequence^[1], as supported by epidemiological, clinical-pathological and molecular genetic studies. Thus, early detection and removal of adenomatous polyps are essential for cancer prevention. In fact, the risk of developing CRC within six years of a polypectomy is reduced by 75%-90%. However, not all CRC cases are preceded by adenomatous polyps, and some cancers have been shown to develop directly from aberrant crypts or flat lesions^[2]. These alternative carcinogenetic pathways are relatively more frequent on the right side of the colon, making the efficacy of preventative strategies very challenging. In this regard, one of the topics of particular interest in CRC is the possible change in site distribution observed in recent decades. In fact, recent data from different studies report a change in the site distribution of CRC (proximal shift) related to gender, race^[3,4] and older age^[5]. Many factors are potentially involved in this phenomenon, and most of them are not easily evaluated. However, the crucial issue is to establish if and how much of these changes are due to a real biological event or related to multiple diagnostic biases. Indeed, the question is not theoretical, but rather implies important decisions related to strategies for screening, surveying and treating millions of people worldwide, with health and economic implications. In fact, proximal colon cancer represents a great challenge for physicians, both due to the technical limitations of screening strategies in the detection of right-sided colon lesions and to the peculiar behavior of these tumors^[6]. Data on CRC location have been reported from different sources, such as cancer registries, colonoscopy reports, retrospective clinical analyses or autptic data^[5,7-9]. All of these sources have biases that could potentially under- or over-estimate the specific issue of the cancer location. However, data concerning a possible increase over time of right-sided colon cancers have been reported recently in large population studies^[10,11]. The possible changes in the location of polyps over time have been less investigated, but a possible proximal shift in these lesions has been described by some studies^[12-14]. Few studies have addressed the possible "right shift" of CRC in the Italian popula-

tion^[15,16], and only two studies have analyzed the changing distribution of both CRC and polyps over time^[17,18], thus data are scarce and not conclusive.

The present study aims to address this issue retrospectively by analyzing records from a large set of patients, either operated on for CRC or diagnosed with colon polyps by colonoscopy, during two distinct periods of time at an Italian single referral center.

MATERIALS AND METHODS

We performed a retrospective, observational study of CRC and polyps at a single referral center ("Sapienza" University Hospital - Rome, Italy) for two periods of time: from 1989 to 1993 (period A) and from 2003 to 2007 (period B). The aim of the study was to compare the location of CRC and polyps and to study the differences in the age and gender distributions between the two periods.

The age and gender of the patients and the location, histology, morphology and dimensions of their lesions were recorded. For discrimination between the proximal and distal colon, the boundary was situated at the juncture of the splenic flexure, as was performed in previous studies^[16,19].

Overlap of patients in the two groups (CRC and polyps) was carefully avoided. The study was approved by the institutional University review board; because this study was a retrospective analysis of an existing data set, written informed consent was not obtained from the participating subjects.

During the two periods, endoscopic examinations were performed using Olympus videocolonscopes (CF100I in period A, CFQ145I in period B).

Colorectal cancer data

CRC data were obtained from surgery registries, and the diagnoses were all confirmed by histological examination of surgical resections. Overall, 768 consecutive patients diagnosed with cancer who underwent surgery were analyzed. Of these, 352 were operated on from 1989-1993 (period A) and 416 from 2003-2007 (period B).

Polyp data

Polyp data were obtained from colonoscopies. Only complete colonoscopy examinations with adequate bowel preparation were considered. Subjects with uncompleted examinations or unsatisfactory cleansing were excluded, unless a second complete colonoscopy was performed within three months. Only patients with sporadic polyps were included, and patients who met the criteria for familial adenomatous polyposis, hereditary non-polyposis colorectal cancer syndrome or other polyposis syndromes, or who had been diagnosed with or suspected to have inflammatory bowel disease (ulcerative colitis or Crohn's disease), were excluded from the study.

Four senior gastroenterologists, each with more than 10 years of endoscopic experience, performed 4176 colonoscopies (1030 and 3146 for periods A and B, re-

Table 1 Characteristics of the 768 patients with colorectal cancer *n* (%)

	Period A (1989-1993)	Period B (2003-2007)	<i>P</i> value
	352 patients	416 patients	
Male	202 (57.4)	253 (60.8)	0.335
Female	150 (42.6)	163 (39.2)	
Age (yr)			
< 50	49 (13.9)	27 (6.5)	< 0.0001
50-59	70 (19.9)	55 (13.2)	
60-69	121 (34.4)	95 (22.8)	
70-79	90 (25.6)	180 (43.3)	
≥ 80	22 (6.2)	59 (14.2)	

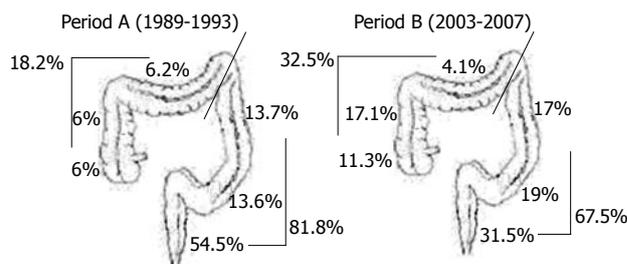


Figure 1 Relative distribution of colorectal cancer according to colon segment in periods A and B. The relative percentage of colorectal cancer cases are indicated next to the corresponding tract of the colon. Total proximal and distal colorectal cancer percentages, with the splenic flexure as the boundary, are also reported.

spectively). Polyps were detected in 27% and 23% of colonoscopies in periods A and B, respectively.

A total of 978 patients were analyzed, and 1693 polyps were found.

The data obtained from each polyp were included in the descriptive analysis. For patients with more than one polyp, the most advanced lesion, either in the proximal or in the distal segment of the colon, was taken into consideration in the multivariate analysis.

Statistical analysis

Proportions were calculated for the categorical data, and means and standard deviations were calculated for the quantitative data. χ^2 and *t* tests were used to assess the differences between periods A and B. Multivariate logistic regression was used to estimate the relative risk of finding a proximal CRC and polyp, adjusting for age, sex and the diagnosis period (A *vs* B) as independent variables. The limit of statistical significance for all tests was set at 0.05.

RESULTS

Colon cancer

As shown in Table 1, a higher percentage of cancers was recorded in men than in women, and there was no statistically significant difference between the periods. Patients were older in period B than in period A. In particular, there were fewer patients with CRCs in period B than in period A in all age groups less than 70 years.

From period A to B, proximal CRC incidence increased by an absolute 14.3% (from 18.2% to 32.5%, *P* < 0.0001). In particular, the increase was observed in the cecum and in the ascending colon (12.0% *vs* 28.4% in periods A and B, respectively), whereas in distal CRC cases, a consistent reduction was noted in the rectum, with a decrease from 54.5% to 31.5% in periods A and B, respectively (*P* < 0.0001) (Figure 1).

In the multivariate analysis, the risk of finding a proximal CRC, after adjusting for age and sex, showed a statistically significant interaction term between period B and gender. Thus, two regression equations were run. For men, the risk of developing a proximal cancer in period B was more than 3 times greater than that in period A, adjusting for age [odds ratio (OR) 3.31, 95%CI: 2.00-5.47; *P* = 0.0001], whereas for females, there was an

increased risk, but this increase was not statistically significant (OR 1.21, 95%CI: 0.72-2.04; *P* = 0.4637). There was no significant evidence of an effect of age in males (OR 1.34, 95%CI: 0.86-2.11; *P* = 0.1999) or in females (OR 1.23, 95%CI: 0.74-2.07; *P* = 0.4272).

Polyps

As shown in Table 2, polyps were more frequently found in males than in females, with no statistically significant difference between the periods (*P* = 0.0892). We evaluated 428 polyps from 273 patients in period A and 1265 polyps from 705 patients in period B. The mean number of polyps per patient increased from 1.6 in period A to 1.8 in period B (*P* = 0.01).

No univocal trend in age distribution between the two periods was observed. With regard to the percentage of patients with polyps in period A to period B, a decrease was observed for age groups < 50 years and 60-69 years, whereas an increase was observed for the age groups 50-59, 70-79 and ≥ 80 years (*P* < 0.0015). A similar trend was observed in both males and females (data not shown).

From period A to B, the incidence of proximal polyps increased by an absolute 12.7% (from 22.8% to 35.5%, *P* < 0.00005). In analyzing the anatomical segments separately, an increase in incidence of proximal polyps was observed in the ascending colon (from 7.9% to 13.2%) and in the transverse colon (from 7.2% to 14.7%). In the distal colon, a reduction in polyps was observed in the descending colon (from 21.6% to 9.1%) and in the rectum (from 32% to 25.4%), whereas an increase was noted in the sigmoid colon (from 23.6% to 30%) (*P* < 0.00005) (Figure 2).

In the multivariate logistic regression analysis, after adjusting for age, a male's risk of developing a proximal polyp in period B was almost 90% greater than his risk in period A (OR 1.87, 95%CI: 1.23-2.87; *P* = 0.004), whereas for females, there was an increase of more than 60% in the risk, which was close to statistical significance (OR 1.62, 95%CI: 0.96-2.73; *P* = 0.07). When considering age groups stratified by greater or less than 70 years, no differences in proximal polyp detection was demonstrated for either gender.

The size and histopathological pattern of the polyps were also analyzed.

Table 2 Characteristics of the 978 patients with polyps *n* (%)

	Period A (1989-1993) 273 patients	Period B (2003-2007) 705 patients	<i>P</i> value
Male	178 (65.2)	418 (59.3)	0.0892
Female	95 (34.8)	287 (40.7)	
Age (yr)			
< 50	50 (19.2)	91 (12.9)	< 0.0015
50-59	44 (16.9)	154 (21.9)	
60-69	103 (39.5)	226 (32.1)	
70-79	57 (21.8)	185 (26.3)	
≥ 80	7 (2.7)	48 (6.8)	
Total No. of polyps	428	1265	
No. of polyps, mean ± SD	1.6 ± 1	1.8 ± 1.3	0.0102
No. of polyps, median	1	1	
Range	1-6	1-14	
Dimensions			
< 5 mm	114 (26.6)	530 (41.9)	< 0.00005
5-9 mm	228 (53.3)	454 (35.9)	
10-19 mm	44 (10.3)	191 (15.1)	
20-29 mm	23 (5.4)	52 (4.1)	
30-39 mm	9 (2.1)	25 (2)	
40+ mm	10 (2.3)	13 (1)	
Histopathological pattern			
Hyperplastic	91 (33.6)	438 (38.7)	< 0.00005
Mild/moderate dysplasia ¹	151 (55.7)	597 (52.7)	
Severe dysplasia ²	27 (10)	87 (7.7)	
Others ³	2 (0.7)	10 (0.9)	

¹Tubular, mixed or villous adenoma with mild or low grade dysplasia;

²Tubular, mixed or villous adenoma with severe dysplasia or cancer in situ or serrated adenoma; ³Lymphatic lump, hamartoma, leiomyoma, anal human papilloma virus.

There was a statistically significant increase in the percentages of micropolyps (< 5 mm) from 26.6% to 41.9% and from 10.3% to 15.1% for polyps of 10-19 mm in size, whereas the percentage of large polyps (40 mm) diminished from 2.3% to 1.0% in period B *vs* A ($P < 0.00005$).

Histopathology data were available for 63.3% and 89.5% of polyps in period A and B, respectively. No statistically significant differences in the overall number of hyperplastic polyps and adenomas with mild/moderate and severe dysplasia were observed between periods A and B. Nonetheless, when the histopathological pattern was analyzed according to polyp location, from period A to B, adenomas with mild/moderate dysplasia in the proximal colon increased significantly from 21.8% to 41.2% ($P < 0.001$), whereas adenomas with severe dysplasia decreased from 37% to 23%, which was not statistically significant.

DISCUSSION

In recent decades, screening strategies for early diagnosis and/or prevention of CRC have been consistently implemented. Nonetheless, colon malignancies still remain the third most common cancer and an important cause of death in Western countries^[20]. Thus, many efforts have been made in order to improve the efficacy of screening strategies, which often differ even regionally in the same country. Colonoscopy is considered the “gold standard” for the diagnosis and removal of pre-malignant colon

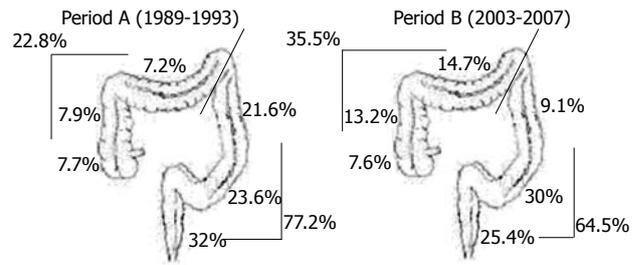


Figure 2 Relative distribution of polyps according to colon segment in periods A and B. Relative percentage of polyp detection are indicated next to the corresponding tract of the colon. Total proximal and distal colonic polyp percentages, with the splenic flexure as the boundary, are also reported.

lesions, even though a careful risk stratification strategy is required in order to optimize resources for screening purposes. In this setting, the presumed right-side increase in pre-malignant lesions and CRC may represent a further stimulus to perform high-quality endoscopic examination of the right side of the colon, which is often difficult to explore carefully (especially the cecum)^[6]. Moreover, even though in the last year colonoscopies have increased in number and quality, it has been demonstrated that a relatively high proportion of cases of CRC may develop without macroscopic evidence of pre-malignant lesions, introducing further challenges to prevention strategies. Despite the consistent number of studies that analyze differences in the location of colon CRC and polyps, data are still not univocal and indeed remain difficult to interpret. As already mentioned, results are often difficult to compare due to the different sources from which the data are collected. Another important reason concerns the length of the observation, which varies from a few years to decades according to different studies.

This study retrospectively evaluated the differences in the site distribution of CRC and polyps between two 5-year periods over a period of 10 years, analyzing data from surgical registries and from endoscopic reports in a single referral center. The relatively short interval time between the two periods (10 years) could have, at least in part, influenced the observed differences, which may be more striking with a wider interval time.

Bearing in mind the aforementioned limitations for data interpretation, many recent large studies have reported a trend for “proximalization” of CRC in different geographic areas^[10,15,21,22]. Conversely, other studies have questioned the possible “right shift” in CRC location^[23,24] or have observed the phenomenon only in specific sub-groups^[3,25,26]. Moreover, some other authors have explained that the putative increase in proximal CRCs is mainly consequent to the decrease in rectal cancer cases^[8,16]. In this study, we confirmed the proximal shift in CRC over time and observed a 3-fold increase in the risk of finding proximal cancer in males in period B *vs* period A. In line with previous findings in Italian populations^[16], the single anatomical segments analysis emphasized that the relative increase in proximal CRC cases over time was partly due to a reduction in the number of rectal cancer cases (54.5% *vs*

31.5% in periods A and B, respectively). In fact, excluding rectal cancers, the trend for a proximal shift in CRCs over time, although maintained, showed less of a difference (data not shown). This relatively small, but homogeneous study from a single referral center, confirmed the trend of a proximal shift in CRC location during recent years, and is thus further confirmation of the phenomenon previously described in large cohorts of patients from different areas.

Published data on polyp prevalence are scarce and less consistent than are data for CRC, and the related studies mainly concern advanced adenomas^[27]. However, some studies have suggested a proximal shift in those lesions over time^[12-14,17,18]. In this study, we observed a proximal shift in polyps between periods A and B, albeit less consistent than that observed for CRC. As already observed for CRC, the proximalization of lesions was more evident in males (90% increase in period B *vs* A). The increase in total polyps, and in particular in proximal lesions, refers mainly to micropolyps and low-grade dysplastic polyps, that could be partially explained by the increase in colonoscopies for cancer prevention in period B *vs* period A, and to the “see and sampling” strategy that has become more popular in recent years. Notably, the present data on polyp dimensions and histopathological patterns need to be interpreted with caution, both due to the high rate of missing histological data [157 (36.7%) and 133 (10.5%) polyps with missing histological reports in periods A and B, respectively] and due to the fact that the two variables (size and histology) are not independent.

Besides possible biological explanations of increased proliferative right-sided colon lesions over time, many confounding factors related to the global technical and behavioral medical changes throughout the years could have partially contributed to this location shift. With regard to the latter, the most important consideration concerns the impact of increased sensibilization for CRC prevention in the last decade that could have potentially influenced either CRC or polyp presentation in our population during period B. In fact, the older age of CRC patients in that period could be at least partially due to the preventive effect of the screening approach, and the same “proximal shift” could be an effect of better prevention of distal lesions (which are more easily detected by screening methods such as sigmoidoscopy). Considering polyps, the modifications of colonoscopy indications, particularly due to an increased trend to cancer prevention, may have influenced the different findings in the two periods, even though only a slight decrease in the proportion of colonoscopies with polyp detection was found between the two periods (23% in period B *vs* 27% in period A). Regarding technical progress, the right-sided CRC increase could be a result of the recent different surgical treatment options for right-sided CRC, in particular the laparoscopic approach, that in many centers has made surgery much more possible in elderly patients compared to previous years. This detail is particularly true considering that for CRC evaluation, we included

exclusively surgical registry data without considering the surgical approach. Moreover, procedural improvements (i.e., standardization of retraction time) and the amelioration of bowel cleansing could have potentially influenced the observed difference in polyp detection between the two time periods. Nonetheless, no substantial improvements in technical equipment occurred between the two periods, since high definition endoscopes were not available in both periods.

Nonetheless, even if the precise amount and specific causes of the right shift in pre-malignant and malignant colon lesions remain to be established, the present retrospective analysis appears to confirm, albeit with some limitations and possible confounding factors, a trend of an increase in such lesions over time. As a consequence, endoscopists and clinicians in daily clinical practice, as well as future strategies for screening campaigns, should take into account the possible increase in proximal colonic proliferative disorders. In this regard, the whole colon should be considered as a potential target for neoplastic changes, and partial colon examinations should be avoided or limited to particular conditions. Novel endoscopic instruments with higher resolution power could result in an improvement *per se* in the detection of colonic lesions. However, besides the technical devices, better bowel preparation (cecum cleaning), the constant improvement of endoscopists’ skills, and a standardized technical endoscopic approach^[28] are all fundamental basic tools that can improve the endoscopic examination quality in order to obtain a more accurate observation of the whole colon.

COMMENTS

Background

Proximalization of colorectal cancer during the last decades has been variously reported. Data for the right shift in polyps are scant and controversial.

Research frontiers

Data from large cohorts of patients followed for decades and relatively short studies suggest a change in colonic proliferative disease during recent years, with an increase in right-sided lesions.

Innovations and breakthroughs

The data show a proximalization of proliferative colonic lesions (cancers and polyps), in a single referral center, in two different periods of time with a ten-year interval between these periods.

Applications

These results should be interpreted with caution, due to many possible unavoidable biases that may interfere when undertaking this type of study, either for different endoscopic/surgical approaches or for biological factors. However, the authors suggest that this phenomenon that may have important implications either for improvement of endoscopic accuracy or for screening programs.

Peer review

The study involves a single institution but covers 2 separate 5 year periods 10 years apart. The authors compared data regarding the anatomic location of both colorectal cancers and colonic polyps found by colonoscopy during each period. The data suggest that proximal proliferative lesions have increased in prevalence, particularly in males. That proximal colonic premalignant and malignant conditions have increased in prevalence over the last 2 decades has also been shown in other studies. In order to be sure that comparisons between 2 separate periods are valid, even within a single institution, the procedures in question should be as identical as possible, allowing for changes in technology, operator experience, etc.

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Value of adipokines in predicting the severity of acute pancreatitis: Comprehensive review

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Abstract

AIM: To analyze the prognostic value of adipokines in predicting the course, complications and fatal outcome of acute pancreatitis (AP).

METHODS: We performed the search of PubMed database and the systemic analysis of the literature for both experimental and human studies on prognostic value of adipokines in AP for period 2002-2012. Only the papers that described the use of adipokines for

prediction of severity and/or complications of AP were selected for further analysis. Each article had to contain information about the levels of measured adipokines, diagnosis and verification of AP, to specify presence of pancreatic necrosis, organ dysfunction and/or mortality rates. From the very beginning, study was carried out adhering to the PRISMA checklist and flowchart for systemic reviews. To assess quality of all included human studies, the Quality Assessment of Diagnostic Accuracy Studies tool was used. Because of the high heterogeneity between the studies, it was decided to refrain from the statistical processing or meta-analysis of the available data.

RESULTS: Nine human and three experimental studies were included into review. In experimental studies significant differences between leptin concentrations at 24 and 48 h in control, acute edematous and acute necrotizing pancreatitis groups were found ($P = 0.027$ and $P < 0.001$). In human studies significant differences between leptin and resistin concentrations in control and acute pancreatitis groups were found. 1-3 d serum adiponectin threshold of 4.5 $\mu\text{g/mL}$ correctly classified the severity of 81% of patients with AP. This threshold yielded a sensitivity of 70%, specificity 85%, positive predictive value 64%, negative predictive value 88% (area under curve 0.75). Resistin and visfatin concentrations differ significantly between mild and severe acute pancreatitis groups, they correlate with severity of disease, need for interventions and outcome. Both adipokines are good markers for parapancreatic necrosis and the cut-off values of 11.9 ng/mL and 1.8 ng/mL respectively predict the high ranges of radiological scores. However, the review revealed that all nine human studies with adipokines are very different in terms of methodology and objectives, so it is difficult to generalize their results. It seems that concentrations of the leptin and resistin increases significantly in patients with acute pancreatitis compared with controls. Serum levels of adiponectin, visfatin and especially resistin (positive correlation with Acute Physiology and Chronic

Health Evaluation II, Ranson and C-reactive protein) are significantly different in mild acute pancreatitis and severe acute pancreatitis patients, so, they can serve as a markers for the disease severity prediction. Resistin and visfatin can also be used for pancreatic and parapancreatic necrosis prediction, interventions needs and possible, outcome.

CONCLUSION: High levels of adipokines could allow for prediction of a severe disease course and outcome even in small pancreatic lesions on computed tomography scans.

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Key words: Adipokines; Acute; Pancreatitis; Severity; Prediction

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Karpavicius A, Dambrauskas Z, Sileikis A, Vitkus D, Strupas K. Value of adipokines in predicting the severity of acute pancreatitis: Comprehensive review. *World J Gastroenterol* 2012; 18(45): 6620-6627 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i45/6620.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i45.6620>

INTRODUCTION

Acute pancreatitis (AP) is a common disease with a wide spectrum of severity. Its incidence is about 30-113 cases per 100 000 individuals, with the overall mortality rate of 10%-15%^[1-4]. Most episodes of AP are mild and self-limiting, but up to 10%-20% of patients develop severe AP with mortality ranging from 29% to 43%^[5,6]. Over past two decades mortality rate in early phase of AP associated with the systemic inflammatory response syndrome decreased significantly, but mortality in the late phase remains high. It is believed that the cause of death in late phase is predominantly linked to the development of infected necrosis and septic complications^[7], thus resulting in multiple organ failure and severe sepsis^[8,9]. Because of the systemic complications and high mortality, a considerable interest in the early prediction of the disease course and severity remains.

Pancreatic enzyme levels poorly correlate with the severity of AP, thus prognosis is commonly based on clinical scores. The first disease specific prognostic score was proposed by Ranson in 1974, which later was complemented by a number of pancreatitis specific and organ failure scores, including Glasgow/Imrie (1984), Acute Physiology and Chronic Health Evaluation (APACHE) II (1985), Multiple Organ Dysfunction Score (1995), Sequential Organ Failure Assessment (1998), Pancreatitis Outcome Prediction (2007), Bedside Index of Severity in Acute

Pancreatitis (2009), and many others. Although, the accuracy of such scores is high enough (Table 1)^[10-14], all of them are multifactorial and rather uncomfortable for everyday use, so a great attention is still given for seeking a single prognostic marker. The most widely explored and described single predictor is C-reactive protein (CRP), which remains very useful, because it is accurate, cheap, and widely available. However, its concentration reaches a peak on third day of the disease, so it has a greatest prognostic value approximately 48 h after the onset of the symptoms. Optimal cut-off value recommended by almost all societies for disease course prediction is 150 mg/L. CRP has sensitivity of 80%, specificity of 84% with area under curve (AUC) 0.84 in predicting severity of the disease 48 h after admission with a cut-off of 150 mg/L^[15]. There is little data on the value of CRP on prediction of development of pancreatic necrosis. Some studies demonstrated that a cut-off of as low as 71 mg/L is sufficient to predict development of clinically significant (volume > 30%) necrosis with a sensitivity of 78.79%, specificity of 71.43% and AUC 0.766^[12].

The main problem remains, that neither prognostic scores nor single predictors can't accurately predict the disease course and severity, development of pancreatic or peripancreatic necrosis, and outcomes during the first hours or even days of hospitalization. Therefore, there is a great stimulus for seeking new accurate and easy to use predictors. Perhaps, the least studied group of predictors in AP is adipokines, including adiponectin, leptin, resistin and visfatin.

Adiponectin is being produced exclusively in adipocytes and plays an important role in the inhibition of the inflammatory response^[16,17]. Adiponectin depresses nuclear factor kappa B signaling in endothelial cells and adipocytes, induces the anti-inflammatory cytokine interleukin (IL)-10 and IL-1 receptor antagonist in leukocytes^[18-20].

Leptin is an adipocyte-derived hormone that acts centrally in the hypothalamus to regulate body waste and peripheral energy expenditure^[21]. The presence of leptin and expression of its receptors have been detected in other tissues, also in pancreas^[22]. This suggests, that leptin may modulate pancreatic function and inflammatory response in pancreatitis.

Resistin and visfatin are the adipohormones, produced by neutrophils, macrophages, bone marrow and WAT^[23,24]. They can induce the synthesis of pro-inflammatory cytokines, such as IL-6, IL-1 β , tumor necrosis factor alpha, that is why their role in inflammatory response has been suggested^[24-27].

It is now widely accepted, that white adipose tissue is an active endocrine organ, which is also involved in pathogenesis of AP. Peripancreatic fat cells necrosis might cause a massive release of cytokines (IL-1, IL-6, tumor necrosis factor) and adipokines, that possibly cause multi-organ dysfunction and whole body metabolic changes. It is hypothesized that the extent of peripancreatic fat-cell necrosis determines the severity of pancreatitis, and an early increase of adipocyte-specific marker proteins might serve as predictor of the clinical course^[28].

Table 1 Pancreatitis prediction scores

Score	Sensitivity (%)			Specificity (%)			AUC		
	Course	Necrosis	Mortality	Course	Necrosis	Mortality	Course	Necrosis	Mortality
Ranson, 1974	84	77	100	90	88	77	0.94	0.85	0.95
Glasgow/Imrie, 1984	70	82	89	83	73	70	0.84	0.82	0.80
APACHE II, 1985	70	63	100	72	69	66	0.78	0.72	0.90
MODS, 1995	73	69	89	81	74	90	0.84	0.78	0.93
SOFA, 1996/1998	76		87	69		90	0.81		0.93
POP, 2007	83	51	78	71	95	86	0.86	0.71	0.89
BISAP, 2009	38	33	57	92	91	88	0.81	0.78	0.82

APACHE: Acute Physiology and Chronic Health Evaluation; MODS: Multiple Organ Dysfunction Score; SOFA: Sequential Organ Failure Assessment; POP: Pancreatitis Outcome Prediction; BISAP: Bedside Index of Severity in Acute Pancreatitis; AUC: Area under curve.

Table 2 Reviewer judgments of methodological quality of included human studies according to the Quality Assessment of Diagnostic Accuracy Studies tool

	Konturek <i>et al.</i> ^[301]	Leśniowski <i>et al.</i> ^[331]	Duarte-Rojo <i>et al.</i> ^[341]	Tukiainen <i>et al.</i> ^[351]	Sharma <i>et al.</i> ^[361]	Schäffler <i>et al.</i> ^[371]	Schäffler <i>et al.</i> ^[381]	Schäffler <i>et al.</i> ^[391]	Daniel <i>et al.</i> ^[401]
Patients spectrum	No	No	Yes	Yes	No	Yes	Yes	Yes	Yes
Selection criteria	Unclear	No	No	No	Yes	No	No	No	Yes
Reference standart	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes
Period between IT and RS	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Verification	Unclear	Yes	No	Yes	Yes	No	No	No	Yes
Same RS	No	Yes	No	No	Yes	No	No	No	Yes
RS independence on IT	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
IT replication	Yes	No	No	No	No	No	Yes	Yes	Yes
RS replication	No	No	No	No	No	No	No	No	No
IT interpretation	No	No	No	No	No	No	No	No	No
RS interpretation	Yes	No	No	No	No	No	No	No	No
Data in practice	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Report	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Withdrawals	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Total	8 yes	7 yes	6 yes	8 yes	9 yes	7 yes	8 yes	8 yes	11 yes

IT: Index test; RS: Reference standart.

The aim of this study is to analyze and review the available information about the prognostic value of adipokines in predicting the course of AP, development of pancreatic and peripancreatic necrosis, infectious complications, need for interventional treatment, and fatal outcome. The main objective of the study is to compare the prognostic value of adipokines with already well established single predictors and multifactorial scores in the clinical context.

MATERIALS AND METHODS

We performed the search of PubMed database (service of the United States National Library of Medicine that includes citations from MEDLINE and other life science journals for biomedical articles) and the systemic analysis of the literature for both experimental and human studies on prognostic value of adipokines in AP for period 2002-2012. Keywords (keywords and textwords) for the search were adipokines, adipocitokines, visfatin, resistin, adiponectin, leptin, acute pancreatitis, pancreatic necrosis, peripancreatic necrosis. Further we searched the references of identified articles to find additional sources of information. Only articles in English language were

included in the analysis. Dual publications were excluded. All identified papers (title, abstract and subsequently full text) were independently evaluated by two investigators. Only the papers that described the use of adipokines for prediction of severity and/or complications of AP were selected for further analysis. To be included in the systematic review, each article had to contain information about the levels of measured adipokines, diagnosis and verification of AP, to specify presence of pancreatic necrosis, organ dysfunction and/or mortality rates. All disagreements were resolved by discussion with other two investigators. From the very beginning study was carried out adhering to the PRISMA checklist and flowchart for systemic reviews.

To assess quality of all included human studies the Quality Assessment of Diagnostic Accuracy Studies tool was used^[29]. Quality assessment was performed independently by three researches and all disagreements were resolved by review and discussion with the fourth investigator. The result of the human studies quality assessment is shown in Table 2. Based on the judges' evaluation 8 of 9 studies got seven or more "yes", so the overall quality of included studies was good. However, all studies were very different. Four of them analyzed only one adipo-

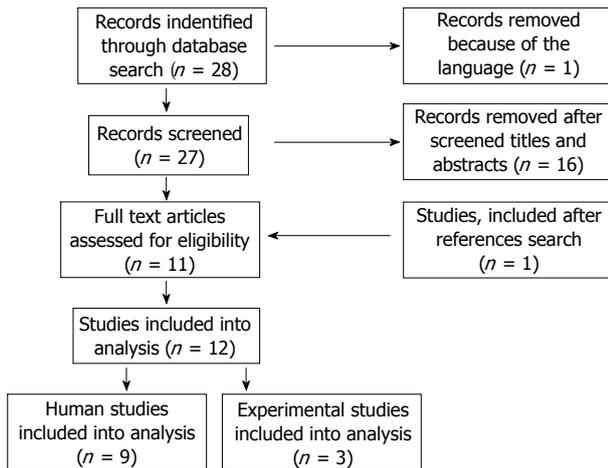


Figure 1 Selection of the studies for systematic review (PRISMA flow-chart). Records identified through database search, $n = 28$.

kine, two adipokines were analyzed in three studies, and the remaining two studies analyzed three adipokines. In two studies adipokines concentration was measured only in control and AP groups, without distinction of mild and severe acute pancreatitis.

Statistical analysis

Because of the high heterogeneity between the studies, lack of the uniform diagnostic criteria and high variation of the assessed adipokines profile it was decided to refrain from the statistical processing or meta-analysis of the available data.

RESULTS

Through database search 28 records were identified. After screening the titles and abstracts, 16 records were removed, because adipokines were not used for prediction of the disease course. One record was removed because of the language. In reference search one additional study was found. So, nine human and three experimental studies were further analyzed (Figure 1).

All three experimental studies were performed on rats. The only one adipokine leptin was analyzed. In all studies significant differences between leptin concentrations in control and acute pancreatitis groups was found^[30-32], one study analyzed leptin concentrations in control, acute edematous pancreatitis (AEP) and acute necrotizing pancreatitis (ANP). Significant difference at 12 h was found between controls and ANP group. At 24 and 48 h significant difference was found between controls and both AEP and ANP groups^[32] (Table 3).

All nine human studies (Table 4) with adipokines are very different in terms of methodology and objectives, so it is difficult to generalize their results. It seems that concentrations of the leptin and resistin increases significantly in patients with AP compared with controls. Serum levels of adiponectin, visfatin and especially resistin (positive correlation with APACHE II, Ranson and CRP) are significantly different in severe acute pancreatitis (SAP),

Table 3 Summary of the experimental studies on the prognostic value of adipokines in rats

Study	Groups	n	Leptin, ng/mL	P value
Konturek <i>et al.</i> ^[30]	AP	6-8	7.5 (4.3-18.4)	$P < 0.01^1$
	Controls	6-8	2.1 (1.0-11.8)	
Yavuz <i>et al.</i> ^[31]	AP	10	1.92 ± 0.1	$P < 0.001$
	CP	10	1.86 ± 0.13	
Kerem <i>et al.</i> ^[32]	Controls	10	0.78 ± 0.12	$P = 0.027$ and $P < 0.001^2$
	AEP	30		
	ANP	30		
	Controls	30		

AP: Acute pancreatitis; CP: Chronic pancreatitis; AEP: Acute edematous pancreatitis; ANP: Acute necrotizing pancreatitis; CIP: Caerulein-induced pancreatitis. ¹The induction of CIP resulted in a significant increase of plasma levels of leptin; ²At 12 h leptin levels in ANP was higher than in controls, at 24 and 48 h leptin levels in AEP and ANP were higher than in controls.

mild acute pancreatitis (MAP) patients, so, they can serve as a markers for the disease severity prediction. Resistin and visfatin can also be used for pancreatic and parapancreatic necrosis prediction, interventions needs and possible, outcome.

DISCUSSION

Human studies (Table 4) began in 2002, when Konturek *et al.*^[30] found, that median plasma leptin levels in AP were significantly increased as compared with controls. In 2007, Leśniowski *et al.*^[33] found a significant differences between resistin concentrations in AP and control groups.

In study of Duarte-Rojo *et al.*^[34] there was no significant independent association between leptin serum levels and severity of AP or fatal outcome. The similar results are published from Tukiainen *et al.*^[35]: on admission plasma leptin levels do not correlate with AP severity. This study also did not confirm correlation between adiponectin levels and severity of AP. Despite of this, in 2009, Sharma *et al.*^[36] has shown, that 1-3 d serum adiponectin threshold of 4.5 $\mu\text{g}/\text{mL}$ correctly classified the severity of 81% of patients with AP. This threshold yielded a sensitivity of 70%, specificity 85%, positive predictive value 64%, negative predictive value 88% (AUC 75%).

Promising results are published from Schäffler *et al.*^[37-39] group. They began their trial in 2006 and finished in 2011 with 41 SAP and 9 MAP patients. This study has shown, that resistin and visfatin concentrations has significant differences between MAP and SAP groups, they correlate with severity of disease, need for interventions and outcome. Both adipokines are good markers for parapancreatic necrosis and the cut-off values of 11.9 ng/mL and 1.8 ng/mL respectively allow to predict the high ranges of radiological scores. These results are consistent with Daniel *et al.*^[40] study in 2010, which demonstrates, that resistin and visfatin may be possibly used for AP prognosis and disease monitoring.

Although Schäffler group provides some cut-off values of adipokines, which are associated with high radiological

Table 4 Summary of the human studies on the prognostic value of adipokines

Study	Patients and methods	Results	Conclusions
Konturek <i>et al</i> ^[30]	Prospective observational study (<i>n</i> = 45) Diagnosis of AP based on Atlanta criteria Adipokines studied: leptin Adipokines evaluated between 48-72 h of illness onetime AP (<i>n</i> = 15) <i>vs</i> controls (<i>n</i> = 30)	Leptin: AP/controls- 7.5 (4.3-18.4) ng/mL/2.1 (1.0-11.8) ng/mL	Median plasma leptin levels in AP were significantly increased as compared with controls
Duarte-Rojo <i>et al</i> ^[34]	Prospective observational study (<i>n</i> = 52) Diagnosis of AP based on typical clinical manifestations with at least a 3-fold increase of serum amylase and/or lipase Whenever uncertainty about diagnosis existed, CT-scan was performed to confirm/rule out AP Severe AP was considered when patients developed one or more local or systemic complications according to the Atlanta classification of AP Adipokines studied: leptin Adipokines evaluated onetime during the 1 d of hospital stay MAP (<i>n</i> = 38) <i>vs</i> SAP (<i>n</i> = 14)	There was no statistically significant association between leptin serum levels and severity of AP There was no difference in leptin measurements between patients favorable and fatal outcomes (<i>P</i> = 0.34) Time of evolution from onset of pain did not alter leptin values There was a positive correlation of BMI and leptin (<i>r</i> = 0.476, <i>P</i> < 0.001) in the whole group Predicted severity by modified Ranson's criteria correlated with Atlanta criteria (<i>r</i> = 0.414, <i>P</i> = 0.002); however, it did not correlate with leptin levels	Results do not support human leptin as a major pro-inflammatory signal involved in AP, nor as a protective and anti-inflammatory mediator It seems neither to be the link between obesity and a higher rate of complications in AP; nor a prognostic marker
Tukiainen <i>et al</i> ^[35]	Prospective observational study (<i>n</i> = 24) AP and SAP defined by Atlanta criteria Adipokines studied: leptin, adiponectin Adipokines evaluated on admission, on days 2-4, and on days 5-7 MAP (<i>n</i> = 12) <i>vs</i> SAP (<i>n</i> = 12)	In patients with SAP highest value of CRP was 349 mg/L (284-476 mg/L), with MAP 119 mg/L (11-367 mg/L) Leptin on admission SAP/MAP [6.1 (1.6-72.9) ng/L]/[9.0(2.5-36.5) ng/L], (<i>P</i> > 0.05); on days 2-4, 7.7 (1.6-13.9) ng/L/3.8(1.6-12.9) ng/L, (<i>P</i> > 0.05) Adiponectin on admission SAP/MAP, [5642 (1201-19 400) ng/L]/[6314 (1980-24 340) ng/L], (<i>P</i> > 0.05)	Plasma levels of adiponectin and leptin do not correlate with AP severity on admission and during the first week of the disease
Schäffler <i>et al</i> ^[37]	Pilot prospective observational study (<i>n</i> = 23) Diagnosis of AP was based on clinical, laboratory and radiological findings during CT and/or ultrasound examination Adipokines studied: leptin, adiponectin, resistin Adipokines evaluated daily for 10 d after admission SAP (<i>n</i> = 20) <i>vs</i> MAP (<i>n</i> = 3) and patients with high points <i>vs</i> low points on radiological scores	Balthazar score: 4 (1-5), Schroeder score: 5 (1-7), Necrosis score: 2(1-4) Ranson: 3 (0-7), Apache II: 12 (4-37) Resistin has a significant positive correlation with Ranson score (<i>r</i> = 0.6, <i>P</i> = 0.002) and with Apache II score (<i>r</i> = 0.5, <i>P</i> = 0.019) Resistin: intervention group/no intervention, 32.4 ± 10.7 ng/L/15.8 ± 5.1 ng/L, <i>P</i> = 0.026 Leptin and relative changes in leptin values were positively and significantly correlated with CRP levels (<i>r</i> = 0.6, <i>P</i> = 0.007 and <i>P</i> = 0.003, respectively) Resistin cut-off value of > 9.2 ng/mL (10 d mean value) can provide a PPV of 91.9% in predicting Schroder score of > 3 (specificity 85%, sensitivity 75%, AUC 0.9, <i>P</i> < 0.0001) Leptin cut-off value of 15.0 ng/mL can provide a PPV of 88% in predicting Schroder score of > 3 (specificity 85%, sensitivity 50%, AUC 0.72, <i>P</i> < 0.0001) Day 1 resistin proved to predict a Schroder score > 3 with a PPV of 93.3%, cut-off 6.95 ng/mL, specificity 87.5%, sensitivity 93.3%; AUC 0.9, <i>P</i> = 0.002	Serum adipokines might be the new useful early markers of disease severity in AP
Leśniowski <i>et al</i> ^[33]	Prospective observational study (<i>n</i> = 79) All AP was classified as grade B according to Balthazar CT score Adipokines studied: adiponectin, resistin Adipokines evaluated onetime during the first day of hospitalization AP (<i>n</i> = 39) <i>vs</i> controls (<i>n</i> = 40)	Resistin: AP/controls, 8.38 ± 4.87 ng/mL/3.58 ± 1.51 ng/mL, <i>P</i> < 0.05 Adiponectin: AP/controls, 119.38 ± 61.75 ng/mL/133.77 ± 55.38 ng/mL, <i>P</i> > 0.05 CRP: AP/controls, 23.21 ± 8.75 ng/mL/3.95 ± 1.06 mg/L, <i>P</i> < 0.01 Weak positive correlation between serum resistin and CRP was observed (<i>r</i> = 0.57, <i>P</i> < 0.05) No correlation between selected adipocytokines and BMI was noticed	Serum concentrations of resistin may possibly represent the useful early marker of inflammatory response in AP
Sharma <i>et al</i> ^[36]	Prospective observational study (<i>n</i> = 60) Diagnosis of AP based on Atlanta criteria SAP was defined as the presence of cardiovascular, pulmonary, and/or renal system dysfunction during the initial hospital admission during for at least 48 h Adipokines studied: adiponectin Adipokines evaluated on admission and subsequently up to 30th hospital day MAP (<i>n</i> = 27) <i>vs</i> SAP (<i>n</i> = 33)	Serum adiponectin levels from days 1 to 3 were significantly lower for patients with SAP [median 3.74 (0.83-8.92) µg/L] than those with MAP [6.58 (1.31-15.37) µg/L], <i>P</i> = 0.02 Serum adiponectin levels from days 4 to 7 were lower for patients with SAP [median 4.53 (0.94-18.2) µg/L] than those with MAP [8.06 (2.11-17.72) µg/L], <i>P</i> = 0.01 1-3 d serum adiponectin threshold of 4.5 µg/mL correctly classified the severity of 81% of patients with AP This threshold yielded a sensitivity of 70%, specificity 85%, PPV 64%, NPV 88%, AUC 0.75	Serum adiponectin levels are significantly lower in patients with SAP than those with MAP and could serve as inverse marker of systemic inflammatory response to pancreatic injury

Daniel <i>et al</i> ^[40]	Prospective observational study ($n = 62$) Diagnosis of AP was based on at least threefold elevated serum amylase level, as well as ultrasonography and CT In all cases AP was classified as C according to Balthazar's CT score and as severe according to Ranson's criteria (3 points) Adipokines studied: resistin Adipokines evaluated on 1, 2, 3 and 5 d of hospitalization SAP ($n = 32$) vs controls ($n = 30$)	On first day of observation, the median serum CRP level was 51.9 ± 46.1 mg/L, significantly higher than in control group (3.44 ± 3.04 mg/L, $P = 0.01$), and further increased at third day of hospitalization (102.6 ± 55.1 mg/L, $P < 0.05$), slightly decreasing on fifth day of hospitalization (78 ± 47.7 mg/L) The values observed at third and fifth day of hospitalization were significantly higher than in the control group ($P < 0.001$) One day of admission and third day of the hospitalization the mean serum resistin concentration was 12.9 ± 6.38 ng/mL and 17.4 ± 4.23 ng/mL, respectively Both values were significantly higher than in the control group (4.06 ± 2.63 ng/mL, $P < 0.05$) At fifth day of hospitalization serum resistin concentration increase further to 25.8 ± 8.14 ng/mL, which was significantly higher than at first and third day ($P < 0.05$) of hospital stay Significant correlation between CRP and resistin ($r = 0.43$, $P < 0.05$) during the hospital stay was found	Resistin may be useful early marker in edematous form of AP
Schäffler <i>et al</i> ^[38,39]	Prospective observational study ($n = 50$) Diagnosis of AP was based on clinical, laboratory and radiological findings during CT and/or ultrasound examination All patients were divided into three groups: first - with higher radiological score's points, second - with lower radiological score's points and third - no CT scan (mild pancreatitis) Adipokines studied: leptin, adiponectin, resistin, visfatin Adipokines were measured daily from admission till 10 d of hospital stay SAP ($n = 41$) vs MAP ($n = 9$) and patients with high points vs low points on radiological scores	Balthazar score: 4.0 (1-5), Schroeder score: 4.5 (1-7), Necrosis score: 1.5 (1-4), Ranson: 3 (0-8), Apache II: 12 (0-45) Admission resistin levels has positive an significant correlation with Apache II score ($r = 6$, $P < 0.001$) and with Ranson score ($r = 0.4$, $P = 0.013$) Admission resistin cut-off value of > 11.9 ng/mL can provide a PPV of 89% in predicting Schroeder score of > 3 (specificity 80%, sensitivity 70%, AUC 0.8, $P < 0.002$) Admission resistin cut-off value of > 11.9 ng/mL can serve as a positive predictor of a Balthazar score > 3 and Necrosis score > 2 Admission visfatin cut-off value of > 1.8 ng/mL can provide a PPV of 93.3% in predicting Schroeder score of > 3 (specificity 81.8%, sensitivity 93.3%, AUC 0.89, $P < 0.001$, likelihood ratio 5.1, post-test probability 93.0%) Admission visfatin concentration can also predict Necrosis score > 2 (PPV 48.3, specificity 40.0%, sensitivity 93.8%, AUC 0.77, $P < 0.004$, likelihood ratio 1.5, post-test probability 70.0%) and Balthazar score > 3 (PPV 79.3, specificity 57.1%, sensitivity 88.9%, AUC 0.74, $P < 0.011$, likelihood ratio 2.1, post-test probability 55.0%)	Resistin and visfatin levels are highly elevated in patients with SAP when compared to patients with MAP Both adipokines levels are positively correlated with clinical severity, clinical end points and needs for interventions A single measurement of serum resistin or visfatin on the day of admission is a highly significant and positive predictive marker in predicting peripancreatic necrosis

AP: Acute pancreatitis; SAP: Severe acute pancreatitis; MAP: Mild acute pancreatitis; BMI: Body mass index; CRP: C-reactive protein; AUC: Area under curve; PPV: Positive predictive value; NPV: Negative predictive value; CT: Computed tomography.

scores ranges, there is no precise cut-off values in order to predict disease severity on admission, development of pancreatic and parapancreatic necrosis, infectious complications, need for interventional treatment and fatal outcome. Therefore it is difficult to compare the prognostic value of adipokines with other prognostic systems.

It is clear, that obesity complicates the course of acute pancreatitis and it is associated with higher incidence of local complications, organ failure and increased mortality risk^[41,42]. Adipose tissue doesn't accumulate contrast, making it difficult to evaluate it's necrosis on computed tomography (CT) scans. Thus, adipokines could be a useful markers for adipose tissue necrosis. High levels of adipokines could allow for prediction of a severe disease course and outcome even in small pancreatic lesions on CT scans, but further research is needed. Therefore, it is appropriate to initiate a multicenter study, with a sufficient number of AP patients and controls. All patients must be evaluated with the same clinical score, adipokines should be investigated all at once and CT scans should be standardized in time. We believe, that such a study could provide a more definitive answer about the value of the

adipokines in predicting the course and the outcomes of AP in a clinical setting.

COMMENTS

Background

Acute pancreatitis (AP) is a common disease with a wide spectrum of severity. The main problem remains, that neither prognostic scores nor single predictors can't accurately predict the disease course and severity, development of pancreatic or peripancreatic necrosis, and outcomes during the first hours or even days of hospitalization. Therefore, there is a great stimulus for seeking new accurate and easy to use predictors. Perhaps, the least studied group of predictors in AP is adipokines. The aim of this study was to analyze the prognostic value of adipokines in predicting the course, complications and fatal outcome of AP.

Research frontiers

It is now widely accepted, that white adipose tissue is an active endocrine organ, which is also involved in pathogenesis of AP. Peripancreatic fat cells necrosis might cause a massive release of and adipokines, that possibly cause multi-organ dysfunction and whole body metabolic changes. It is hypothesized that the extent of peripancreatic fat-cell necrosis determines the severity of pancreatitis, and an early increase of adipocyte-specific marker proteins might serve as predictor of the clinical course.

Innovations and breakthroughs

It seems that concentrations of the leptin and resistin increases significantly in patients with AP compared with controls. Serum levels of adiponectin, visfatin

and especially resistin (positive correlation with Acute Physiology and Chronic Health Evaluation II, Ranson and C-reactive protein) are significantly different in mild and severe AP patients, so, they can serve as a markers for the disease severity prediction. Resistin and visfatin can also be used for pancreatic and parapancreatic necrosis prediction, interventions needs and possible, outcome.

Applications

Adipokines could be a useful markers for adipose tissue necrosis. High levels of adipokines could allow for prediction of a severe disease course and outcome even in small pancreatic lesions on computed tomography scans, but further research is needed.

Terminology

AP: Acute inflammatory process of the pancreas with variable involvement of other regional tissues or remote organ systems; Mild AP: Associated with minimal organ dysfunction and an uneventful recovery; Severe AP: Associated with organ failure and/or local complications such as necrosis, abscess or pseudocyst; Adipokines: Cytokines secreted by adipose tissue.

Peer review

In the current review, the authors have presented the current knowledge on the role of adipocytokines in predicting severity of AP. The search criteria were scientific and the data has been presented in an easily comprehensible manner.

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Trends in the eradication rates of *Helicobacter pylori* infection for eleven years

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Abstract

AIM: To evaluate the trends in the eradication rate of *Helicobacter pylori* (*H. pylori*) over the past 11 years in a single center.

METHODS: This retrospective study covered the period from January 2000 to December 2010. We evalu-

ated 5746 patients diagnosed with gastric ulcers (GU), duodenal ulcers (DU), GU + DU, or nonpeptic ulcers associated with an *H. pylori* infection. We treated them annually with the 2 wk standard first-line triple regimen, proton pump inhibitor (PPI) + amoxicillin + clarithromycin (PAC; PPI, clarithromycin 500 mg, and amoxicillin 1 g, all twice a day). The follow-up test was performed at least 4 wk after the completion of the 2 wk standard *H. pylori* eradication using the PAC regimen. We also assessed the eradication rates of 1 wk second-line therapy with a quadruple standard regimen (PPI *b.i.d.*, tripotassium dicitrate bismuthate 300 mg *q.i.d.*, metronidazole 500 mg *t.i.d.*, and tetracycline 500 mg *q.i.d.*) after the failure of the first-line therapy. Statistical analysis was performed with 95%CI for the differences in the annual eradication rates.

RESULTS: A total of 5746 patients [2333 males (58.8%), 1636 females (41.2%); mean age of males *vs* females 51.31 ± 13.1 years *vs* 52.76 ± 13.6 years, *P* < 0.05, total mean age 51.9 ± 13.3 years (mean ± SD)] were investigated. Among these patients, 1674 patients were excluded: 35 patients refused treatment; 18 patients ceased *H. pylori* eradication due to side effects; 1211 patients had inappropriate indications for *H. pylori* eradication, having undergone stomach cancer operation or chemotherapy; and 410 patients did not undergo the follow-up. We also excluded 103 patients who wanted to stop eradication treatment after only 1 wk due to poor compliance or the side effects mentioned above. Finally, we evaluated the annual eradication success rates in a total of 3969 patients who received 2 wk first-line PAC therapy. The endoscopic and clinical findings in patients who received the 2 wk PAC were as follows: gastric ulcer in 855 (21.5%); duodenal ulcer in 878 (22.1%); gastric and duodenal ulcer in 124 (3.1%), erosive, atrophic gastritis and functional dyspepsia in 2055 (51.8%); and other findings (e.g., MALToma, patients who wanted to receive the therapy even though they had no abnormal endoscopic finding) in 57 (0.5%).

The overall eradication rate of the 2 wk standard first-line triple regimen was 86.5%. The annual eradication rates from 2000 to 2010 were 86.7%, 85.4%, 86.5%, 83.3%, 89.9%, 90.5%, 88.4%, 84.5%, 89.1%, 85.8%, and 88.3%, sequentially ($P = 0.06$). No definite evidence of a significant change in the eradication rate was seen during the past eleven years. The eradication rates of second-line therapy were 88.9%, 82.4%, 85%, 83.9%, 77.3%, 85.7%, 84.4%, 87.3%, 83.3%, 88.9%, and 84% ($P = 0.77$). The overall eradication rate of 1 wk quadruple second-line therapy was 84.7%. There was no significant difference in the eradication rate according to the *H. pylori* associated diseases.

CONCLUSION: This study showed that there was no trend change in the *H. pylori* eradication rate over the most recent 11 years in our institution.

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Key words: *Helicobacter pylori*; Eradication; Proton pump inhibitor; Therapy; Clarithromycin

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INTRODUCTION

Helicobacter pylori (*H. pylori*) has been one of the most common human infections worldwide and is associated with a number of gastrointestinal diseases, including chronic gastritis, peptic ulcer disease, and gastric malignancy^[1]. In multicenter studies, it has been shown that triple therapy with a proton pump inhibitor (PPI), clarithromycin 500 mg and either amoxicillin 1000 mg or metronidazole 500 mg, all taken twice daily, is one of the most effective treatments for *H. pylori* eradication^[2]. However, there is no consensus on the length of treatment among the various management guidelines, including the Asia-Pacific consensus guideline^[3] and the consensus statements from North America^[4] and Europe^[5]. European guidelines recommend 1 wk of treatment, whereas in the United States, it is recommended that the triple standard regimen be given for 10-14 d. The second Asia-Pacific consensus guideline recommends a 7-14 d standard regimen; however, it mentions that 14 d triple therapy confers a limited advantage

over 7-d triple therapy in *H. pylori* eradication rates. In recent years, a decrease in the eradication success rate of 1 wk of triple therapy has been reported due to antibiotic resistance, especially to clarithromycin^[6]. Although clarithromycin resistance is increasing year by year, the current recommended first-line therapy for *H. pylori* infection is PPI, amoxicillin, and clarithromycin for 7-14 d in Korea^[7]. The aim of this retrospective observational study was to investigate the trend in the 2 wk PPI-based standard regimen, which included amoxicillin and clarithromycin, under the unfavorable conditions of increasing antibiotic resistance. In addition, we also studied the trend in the eradication success rate of 1 wk second-line therapy that consisted of bismuth-containing quadruple therapy including PPI, metronidazole, and tetracycline.

MATERIALS AND METHODS

Patients

We retrospectively investigated the annual *H. pylori* eradication success rate of patients who visited our hospital from January 2000 to December 2010 and who had been diagnosed as *H. pylori*-infected by at least one positive result from an *H. pylori* culture test, microscopy of a biopsy specimen, or ¹³C-urea breath test. Patients were excluded due to the following reasons: patient refusal of treatment, abandonment of *H. pylori* eradication treatment, inappropriate indications for *H. pylori* eradication because of a stomach cancer operation or chemotherapy, or follow-up loss after *H. pylori* eradication treatment. We also excluded patients receiving 1 wk PPI + amoxicillin + clarithromycin treatment (PAC; PPI: omeprazole, lansoprazole, pantoprazole, rabeprazole, or esomeprazole, clarithromycin 500 mg, and amoxicillin 1 g, all twice a day) that exhibited poor compliance or adverse effects. We evaluated the success rate of *H. pylori* eradication for all patients who received the 2 wk, first-line standard *H. pylori* eradication PAC regimen. We also evaluated the success rate of eradication of 1 wk bismuth-containing quadruple therapy (PPI *b.i.d.*, tripotassium dicitrate bismuthate 300 mg *q.i.d.*, metronidazole 500 mg *t.i.d.*, and tetracycline 500 mg *q.i.d.*) of patients who failed *H. pylori* eradication treatment by the standard PAC regimen. Among these, patients who did not want treatment, patients who ceased *H. pylori* eradication treatment, and patients lost to follow-up after *H. pylori* eradication treatment were excluded. In the end, we evaluated the success rate of *H. pylori* eradication for a total of 399 patients who received a 1 wk, second-line bismuth-containing quadruple therapy *H. pylori* eradication regimen. This study was approved by the institutional review board of Hallym University Chuncheon Hospital.

Diagnosis of *H. pylori* infection and assessment of *H. pylori* eradication

H. pylori infection was defined according to at least one of the following three tests: (1) a positive rapid urease test (CLO test, Delta West, Bentley, Australia) by gastric mucosal biopsy from the lesser curvature of the mid-antrum or

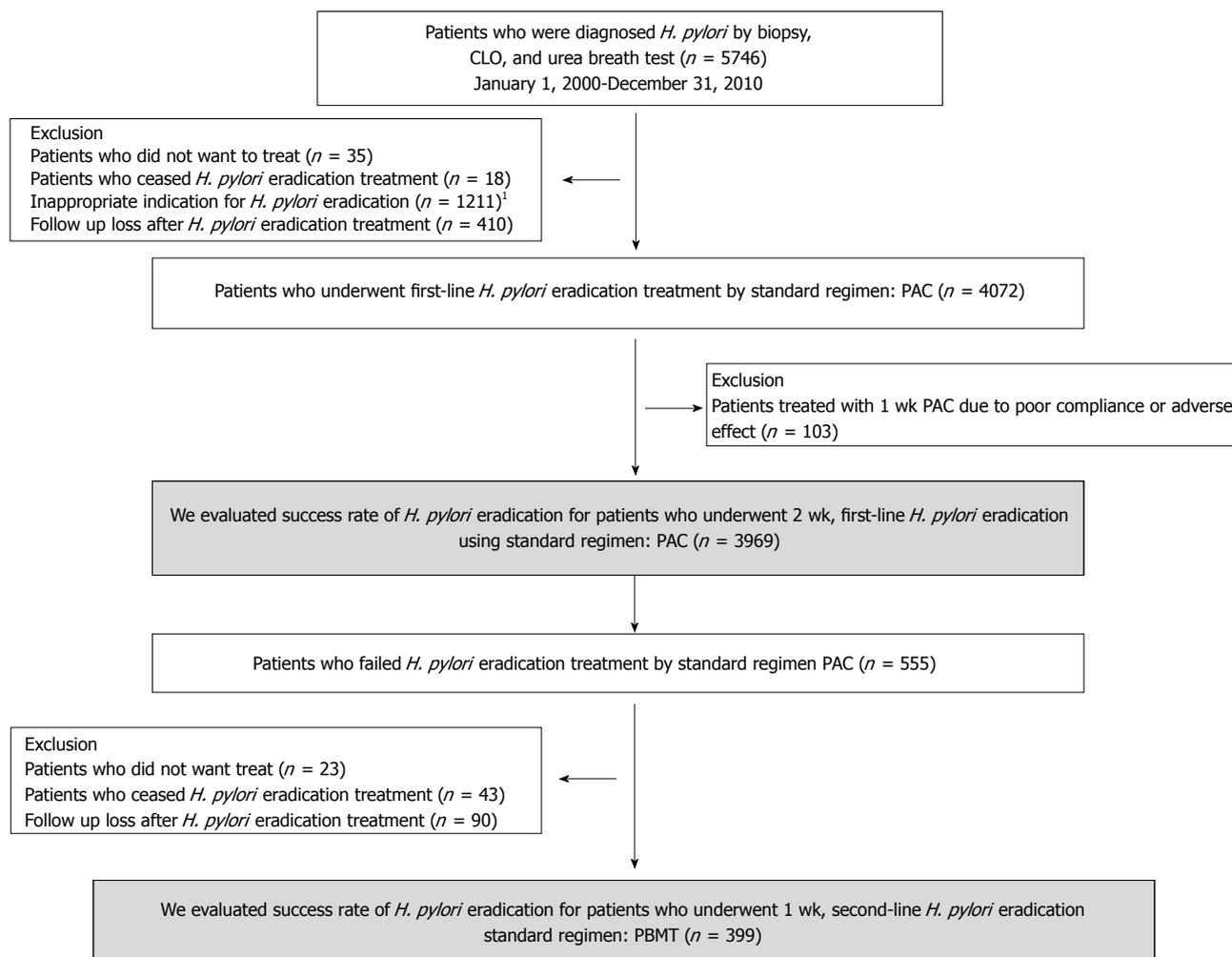


Figure 1 Flow of the study. ¹Inappropriate indications for *Helicobacter pylori* (*H. pylori*) eradication: Stomach cancer operation or chemotherapy. PAC: Proton pump inhibitor *b.i.d.* + amoxicillin 1 g *b.i.d.* + clarithromycin 500 mg; PBMT: Proton pump inhibitor *b.i.d.* + tripotassium dicitrate bismuthate 300 mg *q.i.d.* + metronidazole 500 mg *t.i.d.* + tetracycline 500 mg *q.i.d.*

mid-body; (2) histological evidence of *H. pylori* by modified Giemsa staining in the lesser and greater curvature of the mid-antrum or mid-body, respectively; or (3) a positive C-urea breath test. *H. pylori* eradication was defined as a negative ¹³C-urea breath test or a combination of the rapid urease test, Giemsa staining, and culture when follow-up endoscopy was necessary. The follow-up test was performed at least 4 wk after the completion of the 2 wk standard *H. pylori* eradication using the PAC regimen.

Statistical analysis

Statistical analysis was performed with 95%CI for the differences in the annual eradication rates from January 2000 to December 2010. Continuous variables were analyzed by Student's *t* test, and categorical variables, by the χ^2 test or Fisher's exact test. A *P* value of < 0.05 was considered to be statistically significant.

RESULTS

A total of 5746 patients [2333 males (58.8%), 1636 fe-

males (41.2%); mean age of males *vs* females 51.31 ± 13.1 years *vs* 52.76 ± 13.6 years, *P* < 0.05, total mean age 51.9 ± 13.3 years (mean ± SD)] were retrospectively investigated for the period of January 2000 to December 2010 in this study. The retrospective assessment flow is summarized in Figure 1. Among these patients, 1674 patients were excluded: 35 patients refused treatment; 18 patients ceased *H. pylori* eradication treatment due to side effects, such as abdominal discomfort, diarrhea, taste disturbance, or nausea; 1211 patients had inappropriate indications for *H. pylori* eradication therapy, having undergone a stomach cancer operation or chemotherapy; and 410 patients did not undergo the follow-up assessment for *H. pylori* eradication after the 2 wk PAC treatment. We also excluded 103 patients who wanted to stop the eradication treatment after only 1 wk due to poor compliance or the side effects mentioned above. Finally, we evaluated the annual eradication success rates in a total of 3969 patients who received 2 wk PAC therapy. Demographic characteristics are summarized in Table 1. Of the patients included in the study, 735 (18.5%) were current smokers, and 28.7%

Table 1 Baseline characteristics of the *Helicobacter pylori* eradication population

Baseline characteristics	Data n (%)
Sex	
Male	2333 (58.8)
Female	1636 (41.2)
Age (yr, mean \pm SD)	51.9 \pm 13.3
Current smoker	735 (18.5)
Alcohol intake	1140 (28.7)
Endoscopic and clinical diagnosis	
GU	855 (21.5)
DU	878 (22.1)
GU + DU	124 (3.1)
Non-ulcer dyspepsia ¹	2055 (51.8)
Other ²	57 (1.4)

¹Functional dyspepsia, erosive gastritis, atrophic gastritis; ²Gastric cancer, MALToma. GU: Gastric ulcers; DU: Duodenal ulcers.

were alcohol drinkers. Endoscopic and clinical findings in patients who received the 2 wk PAC were as follows: gastric ulcer in 855 (21.5%); duodenal ulcer in 878 (22.1%); gastric and duodenal ulcer in 124 (3.1%), erosive, atrophic gastritis and functional dyspepsia in 2055 (51.8%), and other findings (e.g., MALToma, patients who wanted to receive the therapy even though they had no abnormal endoscopic finding) in 57 (1.4%). When endoscopy is not indicated, C¹³ urea breath tests (3030, 76.3%), biopsies (22, 0.6%), and CLO test (503, 12.7%) are accepted to determine the outcome of *H. pylori* eradication therapy. In some cases, biopsy + CLO test (329, 8.2%) or CLO test + C¹³ urea breath test was used for the diagnosis of *H. pylori* eradication therapy. Successful eradication rates for each year were follows: 2000, 86.7%; 2001, 85.4%; 2002, 86.5%; 2003, 83.3%; 2004, 89.9%; 2005, 90.5%; 2006, 88.4%; 2007, 84.5%; 2008, 89.1%; 2009, 85.8%; and 2010, 88.3% ($P = 0.06$). Figure 2 summarizes the annual eradication rates year by year from 2000 to 2010. The overall eradication rate was 86.5%, that is, 3435 of 3969 patients who received the 2 wk PAC (95%CI: 85.4% to 87.6%). The P value was 0.09. The annual eradication rates of the 2 wk PAC regimen revealed a relatively constant rate over the years. According to endoscopic and clinical findings, the eradication rates were not significantly different by year. We also investigated the eradication rates of 1 wk bismuth-containing quadruple therapy for 555 patients who failed the *H. pylori* eradication treatment using the 2 wk PAC therapy. Among the 555 patients, 156 patients were excluded for the following reasons: 23 patients declined treatment, 43 patients ceased *H. pylori* eradication treatment due to poor compliance or side effects such as diarrhea, nausea, vomiting, or stool color change, and 90 patients were lost to follow-up after *H. pylori* eradication treatment. Finally, we found the rates of successful eradication in 399 patients who received 1 wk bismuth-containing quadruple second-line therapy for each year to be as follows: 2000, 88.9%; 2001, 82.4%; 2002, 85.0%; 2003, 83.9%; 2004, 77.3%; 2005, 85.7%; 2006, 84.4%; 2007, 87.3%; 2008, 83.3%; 2009, 88.9%; and 2010, 84.0% ($P =$

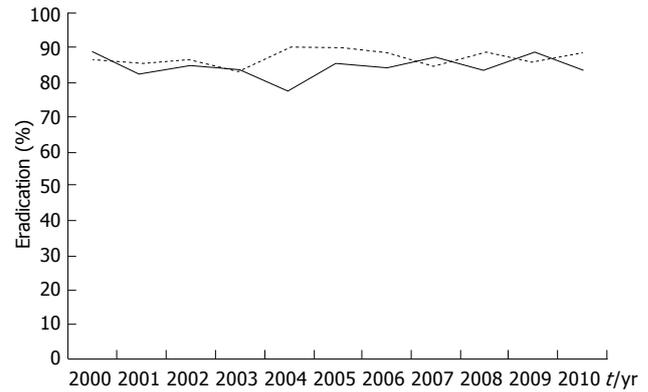


Figure 2 Efficacy of 2 wk first-line (a dotted line) proton pump inhibitor *b.i.d.* + amoxicillin 1 g *b.i.d.* + clarithromycin 500 mg and 1 wk second-line (a solid line) bismuth-containing quadruple therapy for the eradication of *Helicobacter pylori* by year.

0.77). Figure 2 summarizes the annual eradication rates year by year from 2000 to 2010. The overall eradication rate was 84.7%, and the rate among those who received 1 wk second-line therapy was 338 of 399 patients (95%CI: 80.9% to 87.9%). The P value was 0.07. The annual eradication rates of the 1 wk second-line therapy revealed a relatively constant rate over the years.

DISCUSSION

The PAC regimen is one of the most widely used therapies for the first-line treatment of *H. pylori* infection. A recent study reported the eradication rate of *H. pylori* with one wk standard triple therapy to be 75% in Korea^[8]. There is still no general consensus regarding the optimal duration of triple therapy for *H. pylori* eradication, as mentioned above. A recent large, multicenter, double-blind, randomized study concluded that the 1 wk and 2 wk standard triple regimens for *H. pylori* eradication are similar in terms of efficacy, safety, and patient compliance^[9]. Two meta-analyses have reported that 2 wk triple therapy achieves considerably better results than 1 wk therapy^[10,11]. Recently, many studies have reported that the efficacy of the standard triple regimen has decreased^[12-14] because of the increased antibiotic resistance rate; that is, the standard triple regimen of PPI, amoxicillin, and clarithromycin administered to patients infected with the clarithromycin-resistant strain was not successful^[15]. Although the general consensus is that a 1 wk PAC is preferable, we have treated patients infected by *H. pylori* with the 2 wk PAC to overcome increasing antibiotic resistance. Our data on the 2 wk PAC regimen shows a relatively constant rate over the year from 2000 to 2010. A recent chronological analysis of the results of meta-analyses performed between 1998 and 2010 showed that first-line standard triple regimens achieved eradication rates of around only 80% (intention-to-treat)^[16]. Given that the overall eradication rate in this study was 86.5% (3435 of 3969 patients who received the 2 wk PAC), we concluded that the 2 wk PAC regimen had an accept-

able efficacy for *H. pylori* eradication. Many investigators have commented that the decreasing eradication rate using standard triple therapy is due to increasing antibiotic resistance. However, a number of factors, including resistance, which varies widely, influence the success of antibiotic regimens. The resistance rates of antibiotics that are widely used in eradication therapy, including amoxicillin and tetracyclines, are relatively lower than clarithromycin and metronidazole^[15]. Therefore, the true challenge for clinical practice lies mainly in resistance to metronidazole or clarithromycin. In Korea, the rates of resistance to clarithromycin and metronidazole were reported from 1.6% up to 29.7% and from 35.7% up to 49.6% from 1996 to 2006, respectively^[17]. This is a major factor in the reduced effectiveness of triple regimens containing clarithromycin^[18]. Clarithromycin must bind to ribosomes to kill *H. pylori*. Resistance is associated with failure to bind to ribosomes, such that resistance cannot be overcome by increasing the dose or duration^[15]. However, patients do not always have perfect compliance. Because *H. pylori* treatment failures may also occur independently of resistance, that is, treatment may fail but the organism remains susceptible to the antibiotic^[19], we concluded that the 2 wk standard PAC regimen may be fit for use in eradicating *H. pylori*. Bacteria oscillate between a phenotypically resistant and a phenotypically susceptible state, during which they can be eradicated. To extend the duration of treatment such that the antibiotic will be present during at least one period of susceptibility may be an alternative option to overcome antibiotic resistance^[20]. Consistent with the results from Korea in this study, several authors have reported an increasing trend in clarithromycin resistance rates in other areas, such as the United States^[21], Turkey^[22], *etc.* Nevertheless, exceptional cases where clarithromycin resistance rates have remained stable have also been reported^[23]. These results strongly suggest that there is an institutional and geographical difference in the antibiotic resistance of *H. pylori*. This fact and our results in this study suggest that the 2 wk PAC regimen may be effective in eradicating *H. pylori* in some countries or limited geographical areas. In a recent review, the eradication rates of quadruple therapy were 75%-95%^[24]. In Korea, where the resistance to metronidazole is high, the eradication rates of second line quadruple therapy have been reported to be 54.5%-76.7% and 70.4%-83.9% in intention to treat and per protocol analysis^[25,26], respectively, based on studies in which the therapeutic durations varied from 7 to 14 d. Metronidazole is a prodrug that is activated by *H. pylori* enzymes to become active within the cell. There are a number of different enzyme pathways that can accomplish this task, and clinically, by increasing the dose and duration, it is possible to overcome, at least partially, metronidazole resistance^[27]. Thus, there is the possibility that quadruple therapy might be more effective with after a treatment duration of longer than 1 wk. However, some studies have shown the benefit of eradication with prolonged treatment durations^[28], while

others have not^[29,30]. We previously reported that 1 wk bismuth-containing quadruple therapy can be as an effective as 2 wk therapy after the failure of the first-line eradication therapy. In this study, the eradication rates of 1 wk bismuth-containing quadruple therapy have no significant differences from the consecutive, annual eradication rates, in spite of increasing metronidazole resistance in Korea. Therefore, although 1 wk bismuth-containing quadruple therapy is not effective up to more than 90% yet, we concluded that 1 wk bismuth-containing quadruple therapy can be used to treat patients who failed the first *H. pylori* eradication. To raise the eradication rate of *H. pylori*, when the clarithromycin resistance rate is higher than 20%, it is recommended that drug sensitivity tests be carried out prior to eradication^[5]. However, there are several limitations to performing a culture before the first-line treatment for *H. pylori* infection. Cultures are expensive, owing to the costs of the endoscopic procedures, and they are time-consuming. Therefore, cultures are not always available on a routine basis. Until now, cultures for *H. pylori* have mainly been used to perform epidemiological and pharmacologic research. Because extending the duration of the first line PAC regimen from 1 wk to 2 wk may improve the efficacy of the *H. pylori* eradication rate, the 2 wk PAC regimen is preferable for treating *H. pylori*. However, this study does have limitations. One is that it is a retrospective, observational study. Therefore, there was some bias, such as an uneven diagnostic method for the determination of the outcome of *H. pylori* eradication therapy in each year. The other endoscopic findings were not even taken in each year. Additionally, we could not obtain the data on antibiotic resistance including amoxicillin, clarithromycin, metronidazole, and tetracyclin in accordance with the eradication rate.

In conclusion, we show the efficacy of a 2 wk PAC regimen and 1 wk bismuth-containing quadruple therapy has not changed across the 2000 to 2010 period in South Korea. The efficacy of 2 wk PAC and 1 wk quadruple second line therapy is by no means acceptable and satisfactory.

COMMENTS

Background

In recent years, a decrease in the eradication success rate of 1 wk of triple therapy has been reported due to antibiotic resistance, especially to clarithromycin. Although clarithromycin resistance is increasing year by year, the currently recommended first-line therapy for *Helicobacter pylori* (*H. pylori*) infection is proton pump inhibitor (PPI), amoxicillin, and clarithromycin for 7-14 d in South Korea. This retrospective, observational study intended to investigate the trend in the 2 wk PPI-based standard regimen including amoxicillin and clarithromycin under the unfavorable conditions of increasing antibiotic resistance.

Research frontiers

The overall eradication rate of the 2 wk standard first-line triple regimen from 2000 to 2010 was 86.5%. No definite evidence of a significant change in the eradication rate was seen during the past eleven years. The overall eradication rate of 1 wk bismuth-containing quadruple, second-line therapy from 2000 to 2010 was 84.7%. The annual eradication rates of the 1 wk second-line therapy revealed a relatively constant rate over the year.

Innovations and breakthroughs

Authors show that the efficacy of 2 wk PPI + amoxicillin + clarithromycin (PAC)

regimen and 1 wk bismuth-containing quadruple therapy did not change over the period from 2000 to 2010 in South Korea. The efficacy of the 2 wk PAC and 1 wk quadruple second line therapy is by no means acceptable and satisfactory.

Applications

Authors conclude that the 2 wk standard PAC regimen may be fit for use in eradicating *H. pylori*. Bacteria oscillate between a phenotypically resistant and a phenotypically susceptible state, during which they can be eradicated. To extend the duration of treatment such that the antibiotic will be present during at least one period of susceptibility may be an alternative option to overcome antibiotic resistance.

Peer review

In this study, the authors retrospectively evaluated the eradication rate of *H. pylori* in a single center in the Republic of Korea for the period of January 2000 to December 2010. In 3969 patients who received a two weeks standard first-line triple therapy, an overall eradication rate of 86.5% has been found, with no significant ($P = 0.09$) difference in the annual eradication rate during the eleven years. Furthermore, in the 399 patients who failed *H. pylori* eradication and received a 1-wk second-line therapy, an overall eradication rate of 84.7% has been found, with a relatively constant rate over the years. The authors conclude that there was no trend of change in the *H. pylori* eradication rate over the last 11 years in their institution and that a 2-wk first-line and a 1-wk second-line therapy can still be used in Korea.

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UGT1A1 predicts outcome in colorectal cancer treated with irinotecan and fluorouracil

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Abstract

AIM: To evaluate effects of UDP-glucuronosyltransferase 1A1 (*UGT1A1*) and thymidylate synthetase (*TS*) gene polymorphisms on irinotecan in metastatic colorectal cancer (mCRC).

METHODS: Two irinotecan- and fluorouracil-based regimens, FOLFIRI and IFL, were selected as second-

line therapy for 138 Chinese mCRC patients. Genomic DNA was extracted from peripheral blood samples before treatment. *UGT1A1* and *TS* gene polymorphisms were determined by direct sequencing and restriction fragment length polymorphism, respectively. Gene polymorphisms of *UGT1A1**28, *UGT1A1**6 and promoter enhancer region of *TS* were analyzed. The relationship between genetic polymorphisms and clinical outcome, that is, response, toxicity and survival were assessed. Pharmacokinetic analyses were performed in a subgroup patients based on different *UGT1A1* genotypes. Plasma concentration of irinotecan and its active metabolite SN-38 and inactive metabolite SN-38G were determined by high performance liquid chromatography. Differences in irinotecan and its metabolites between *UGT1A1* gene variants were compared.

RESULTS: One hundred and eight patients received the FOLFIRI regimen, 29 the IFL regimen, and one irinotecan monotherapy. One hundred and thirty patients were eligible for toxicity and 111 for efficacy evaluation. One hundred and thirty-six patients were tested for *UGT1A1**28 and *6 genotypes and 125 for promoter enhancer region of *TS*. Patients showed a higher frequency of wild-type *UGT1A1**28 (TA6/6) compared with a Caucasian population (69.9% vs 45.2%). No significant difference was found between response rates and *UGT1A1* genotype, although wild-type showed lower response rates compared with other variants (17.9% vs 24.2% for *UGT1A1**28, 15.7% vs 26.8% for *UGT1A1**6). When *TS* was considered, the subgroup with homozygous *UGT1A1**28 (TA7/7) and non-3RG genotypes showed the highest response rate (33.3%), while wild-type *UGT1A1**28 (TA6/6) with non-3RG only had a 13.6% response rate, but no significant difference was found. Logistic regression showed treatment duration was closely linked to clinical response. In toxicity comparison, *UGT1A1**28 TA6/6 was associated with lower incidence of grade 2-4 diarrhea (27.8% vs 100%), and significantly reduced the risk of grade 4 neutropenia compared with TA7/7 (7.8% vs 37.5%).

Wild-type *UGT1A1**6 (G/G) tended to have a lower incidence of grade 3/4 diarrhea *vs* homozygous mutant (A/A) genotype (13.0% *vs* 40.0%). Taking *UGT1A1* and *TS* genotypes together, lower incidence of grade 2-4 diarrhea was found in patients with non-3RG *TS* genotypes, when TA6/6 was compared with TA7/7 (35.3% *vs* 100.0%). No significant association with time to progression (TTP) and overall survival (OS) was observed with either *UGT1A1* or *TS* gene polymorphisms, although slightly longer TTP and OS were found with *UGT1A1**28 (TA6/6). Irinotecan PK was investigated in 34 patients, which showed high area under concentration curve (AUC) of irinotecan and SN-38, but low AUC ratio (SN-38G / SN-38) in those patients with *UGT1A1**28 TA7/7.

CONCLUSION: A distinct distribution pattern of *UGT1A1* genotypes in Chinese patients might contribute to relatively low toxicity associated with irinotecan and 5-fluorouracil in mCRC patients.

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Key words: Irinotecan; Fluorouracil; UDP-glucuronosyltransferase1A1; Thymidylate synthetase; Polymorphisms; Pharmacokinetics; Treatment outcome; Toxicity; Metastatic colorectal cancer

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INTRODUCTION

The current management of metastatic colorectal cancer (mCRC) uses fluorouracil-based regimens in combination with either oxaliplatin or irinotecan. These regimens mainly differ in their toxicity profiles with neutropenia and late diarrhea being associated with irinotecan-based therapy versus neurotoxicity with oxaliplatin-based treatment.

In our previous studies on the relationship of UDP-glucuronosyltransferase1A1 (*UGT1A1*) polymorphisms and irinotecan-related diarrhea, we found that *UGT1A1**28 genotype was significantly associated with the occurrence of diarrhea, while the polymorphisms of *UGT1A7* and *UGT1A9* variants were found to be unrelated. Mutated *UGT1A1**28 genotype was seen infrequently in the Chinese population, therefore, this might explain the lower

incidence of late diarrhea in our patients treated with irinotecan compared to that in the Caucasian population^[1,2]. However, the role of *UGT1A* in clinical response to irinotecan- and 5-fluorouracil (5-FU)-based treatment, non-diarrhea toxicities and prognosis remains unclear. In addition, thymidylate synthetase (TS), the enzyme targeted by 5-FU, deserves more attention because most patients that receive irinotecan are treated with irinotecan and 5-FU in combination.

UGT1A polymorphisms have become the focus of irinotecan pharmacokinetics and toxicity research because they are involved in metabolism of cytotoxic SN-38 (an active metabolite of irinotecan) to inactive SN-38 glucuronide (SN-38G) (Figure 1). Di Paolo *et al*^[3] have demonstrated that *UGT1A1* polymorphisms are closely correlated with glucuronidation rates, and patients with higher concentration of SN-38G are less susceptible to irinotecan-induced toxicity. Although, several clinical trials have confirmed that patients carrying different genotypes of *UGT1A1* had varied degrees of tolerance to irinotecan, it is still unclear whether *UGT1A1* has any influence on treatment efficacy. Three studies tested if *UGT1A1* isoforms had any impact on treatment outcome, however, their conclusions were inconsistent^[4-6].

Irinotecan is often combined with 5-FU in mCRC treatment, therefore, 5-FU should be taken into consideration for response and toxicity evaluation as well. *In vivo*, the active metabolite of 5-FU inhibits TS activity by forming complexes with TS and 5, 10-methylene-tetrahydrofolate (Figure 1)^[7]. Evidence supports the determinant role of TS promoter region polymorphism in 5-FU treatment efficacy and tolerance^[8,9]. Given higher accuracy and consistency in testing polymorphisms, tremendous efforts have been put into using TS polymorphism as a genetic marker for predicting clinical response, toxicity and prognosis in mCRC patients treated with 5-FU^[10-13].

This study aimed to evaluate the effects of *UGT1A1*/*TS* polymorphisms in Chinese mCRC patients treated with irinotecan and fluorouracil including toxicity and clinical outcome.

MATERIALS AND METHODS

Drug administration

Two regimens were selected for this study: (1) FOLFIRI: irinotecan (Camptosar; Pfizer, United States) 180 mg/m² 90-min *i.v.* infusion on day 1; leucovorin 200 mg/m² *i.v.* infusion on days 1 and 2; followed on days 1 and 2 by 5-FU 400 mg/m² *i.v.* bolus, then 600 mg/m² *i.v.* over 22 h continuous infusion; repeated every 2 wk; (2) IFL: irinotecan 125 mg/m² as a 90-min *i.v.* infusion on days 1, 8, 15 and 22; leucovorin 20 mg/m² *i.v.* infusion on days 1, 8, 15 and 22; 5-FU 500 mg/m² *i.v.* bolus on days 1, 8, 15 and 22; every 6 wk.

Patient eligibility

The criteria for inclusion were: at least 18 years old; histologically confirmed mCRC; failed or intolerant to oxalip-

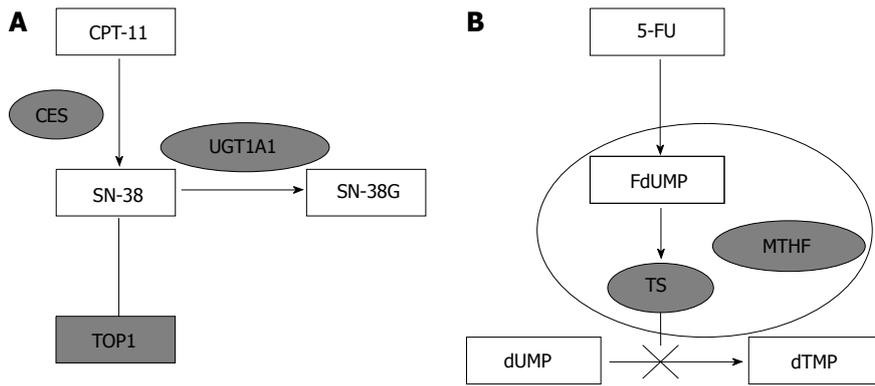


Figure 1 Schematic diagram of UDP-glucuronosyltransferase1A1 and thymidylate synthetase. A: UDP-glucuronosyltransferase1A1 (UGT1A1) is the main enzyme involved in the glucuronidation of SN-38 (SN-38G). Single-nucleotide polymorphisms (SNPs) of UGT1A1 are the key factor in irinotecan metabolism; B: Thymidylate synthetase (TS) is the main target of 5-fluorouracil (5-FU). The ternary complex of TS, active metabolite of 5-FU (FdUMP) and methyl-tetra-hydrofolic acid (MTHF) inhibits DNA synthesis. SNP of TS affects the expression of enzyme and 5-FU efficacy. CES: Carboxylesterases; TOP1: Topoisomerase-1.

atin-based regimens; the Eastern Cooperative Oncology Group (ECOG) performance status 0-2; no chemotherapy at least 4 wk before study enrollment; life expectancy > 3 mo; neutrophil count $\geq 1.5 \times 10^9/L$, platelet count $\geq 8 \times 10^{10}/L$, serum creatinine ≤ 1.25 upper limit normal (ULN), total bilirubin ≤ 1.25 ULN, alanine aminotransferase and aspartate aminotransferase ≤ 2.5 ULN (≤ 5 ULN with liver metastasis); normal electrocardiogram. Written informed consent was required and the study was performed in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. All patients' information and samples were collected from trial participating centers.

Evaluation of response and toxicity

Tumor response was assessed by RECIST 1.0^[14] based on the results of computed tomography. Time to progression (TTP) was defined as the time from the start of treatment to the date of progression. Overall survival (OS) was defined as the time from the start of the treatment to the date of death. Toxicity was assessed according to the NCI-CTC 3.0^[15].

Genomic DNA extraction, polymerase chain reaction and genotyping assay

Two-milliliter peripheral blood samples were collected before starting treatment and frozen at -20°C . Genomic DNA was extracted from these samples using the Peripheral Blood Extraction Kit (Tiangen, China). The fragments of UGT1A1*28 and UGT1A1*6 were amplified by polymerase chain reaction (PCR). The Primer 5.0 software was used to design the sense primer (5'-GC-CAGTTCAACTGTTGTTGC-3') and antisense primer (5'-GTCCGTCAGCATGACATCAA-3'). Each 25- μL PCR reaction mixture included 5 ng DNA template, 1 mmol/L dNTPs, 0.4 mmol/L MgSO_4 , 0.25 $\mu\text{mol/L}$ primers, 0.5 U KOD-plus polymerase (TOYOBO, Japan) and $10 \times$ KOD plus buffer. The PCR profile included 2.5 min denaturation at 94°C , 35 cycles of 30 s at 94°C , 60 s at 57°C , 60 s at 68°C , with a final 7-min extension at 68°C . The PCR products were sequenced by ABI-3730 DNA analyzer and all single-nucleotide polymorphisms (SNPs) were analyzed by Polyphred 5.04 with additional manual proofreading.

Restriction fragment length polymorphism testing for promoter enhancer region of TS

Promoter enhancer region of 2R or 3R and G>C SNP in 3R were selected for testing. PCR-based restriction fragment length polymorphism was applied to detect these variants. The PCR conditions were same as described above with sense primer of 5'-GTGGCTCCTGCGTTTCCCC-3' and antisense primer of 5'-GCTCCGAGCCGCCACAGGCATGGCGCGG-3'. The PCR profile included initial 15 cycles with annealing temperature at 63°C , and another 30 cycles with annealing temperature at 62°C . The PCR products were electrophoresized in 3% agarose gel to differentiate 215-bp 2R genotype and 243-bp 3R genotype. 3R gene polymorphism was detected *via* electrophoresis after enzyme digestion. After *Hae*III treatment, 3RG genotype was separated to fragments of 66, 47, 45, 44, 28 and 13 bp, whereas 3RC genotype was separated to fragments of 94, 47, 45, 44 and 13 bp. The 20- μL reaction system contained 1 μL *Hae* III Takara, 2 μL $10 \times$ M buffer, 17 μL purified PCR products for 37°C overnight incubation.

Pharmacokinetics study

Five-milliliter heparinized blood samples were collected before administration of irinotecan, 1 and 1.5 h during infusion, and 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 36, 48, 60 and 72 h after termination of infusion. Plasma samples were obtained by 3000 r/min centrifugation for 10 min and kept at -40°C . The concentrations of the drug and its metabolites were determined by high performance liquid chromatography with postcolumn fluorescence derivatization (HPLC-FLD). Two hundred microliters plasma was added to 50 μL 1 ng/mL camptothecin, 150 μL methanol, and 200 μL acetonitrile, and vortexed for 2 min. The mixture was then centrifuged at 15 000 r/min for 10 min. Supernatant (200 μL) was placed with 100 μL 1 mol/L HCl before HPLC analysis. Agilent 1100 chromatographic system consisted of quaternary pumps, an automatic sample injection system, fluorescence detector, and Znerstisil ODS-C18 Column. The mobile phases were eluted through the column and contained acetonitrile-sterile water (75:75 by volume) and phosphate buffer at pH 4. Irinotecan was kindly donated by Pfizer and camptothecin was provided by National Institute for the Control of Pharmaceutical and Biological Products. SN-38 was synthesized by Shanghai Zhangjiang Biochemical

Table 1 Association of UDP-glucuronosyltransferase1A1 and thymidylate synthetase genotypes with tumor response

Genotypes	Tumor response	
	n (%)	P value ¹
UGT1A1*28		
TA 6/6	14 (17.9)	0.446
TA 6/7, TA 7/7	8 (24.2)	
UGT1A1*6		
G/G	11 (15.7)	0.217
G/A, A/A	11 (26.8)	
TS promotor		
3RG/3RG	3 (17.6)	0.880
3RG/3RC, 3RG/2RG	5 (23.8)	
3RC/3RC, 3RC/2RG, 2RG/2RG	12 (19.4)	

¹Fisher's exact test for all genotypes. Tumor response including complete and partial response, 111 patients were assessable for tumor response. UGT1A1: UDP-glucuronosyltransferase1A1; TS: Thymidylate synthetase.

Company. Blank human plasma was obtained from Blood bank of 307 Hospital. The lowest limit of quantification of irinotecan, SN-38 and SN-38G were 50 ng/mL, 1.25 ng/mL and 5 ng/mL, respectively, with within-day and between-day imprecision < 15%. C_{max} and T_{max} were observed values. AUC_{0-t} was acquired by linear trapezoidal approximation. $AUC_{0-\infty} = AUC_{0-t} + Ct/k_e$.

Statistical analysis

The χ^2 test was used to determine if the allele frequencies were matched to Hardy-Weinberg equilibrium. Associations between genotypes, clinical response and toxicity in Chinese and Caucasian populations were assessed by using Fisher's exact test and Cochran-Armitage trend test. Kaplan-Meier estimates and the log-rank test were used in TTP and OS analysis. Cox regression and logistic regression models were examined in multivariate analysis with and without time variables, respectively. The linkage between genotypes and pharmacokinetic parameters was analyzed by Mann-Whitney *U* and Kruskal-Wallis tests. Two-sided tests were used to determine statistically significant *P* values ($P < 0.05$) using SPSS 13.0 software.

RESULTS

Between September 2005 and April 2009, 138 mCRC patients were enrolled. Of these, 130 patients were eligible for toxicity evaluation and 111 qualified for treatment efficacy evaluation. Of 138 patients, 108 received the FOL-FIRI regimen, 29 the IFL regimen, and one irinotecan monotherapy. The median age of patients was 52 years (range: 26-81 years) with 63% being male. One hundred and eleven patients were followed until cancer progression or death. The overall response rate (complete + partial response) was 19.5%. The median TTP was 5.6 mo (95%CI: 3.9-7.3) and the median OS was 17 mo (95%CI: 11.9-22.1). The incidence of grade 3/4 neutropenia was 29.5%, and 17.4% participants developed grade 3/4 late diarrhea. However, the incidences in the FOLFIRI regimen were decreased, with 21.8% grade 3/4 neutropenia

Table 2 Association of UDP-glucuronosyltransferase1A1 in combination with thymidylate synthetase genotypes with tumor response

UGT1A1*28	3RG/3RG	TS promoter	
		3RG/3RC, 3RG/2RG	3RC/3RC, 3RC/2RG, 2RG/2RG
TA6/6	2/11 (18.1)	4/13 (30.8)	6/44 (13.6)
TA6/7	1/4 (25)	1/6 (16.7)	5/15 (33.3)
TA7/7	0/2 (0)	0/2 (0)	1/3 (33.3)

Tumor response including complete and partial response, 111 patients were assessable for tumor response, no significant difference was found (Fisher's exact test). UGT1A1: UDP-glucuronosyltransferase1A1; TS: Thymidylate synthetase.

(95%CI: 13.7%-29.9%) and 10.9% grade 3/4 late diarrhea (95%CI: 4.8%-16.9%).

UGT1A1 and TS polymorphisms correlation with clinical response

We tested UGT1A1 genotypes in 136 patients and TS promoter enhancer region in 125 (Figure 2). We observed that the response rates of UGT1A1*28 alleles TA6/6 and TA6/7 + TA7/7 were 17.9% and 24.2%, respectively, and those of UGT1A1*6 alleles G/G and G/A + A/A were 15.7% and 26.8%, respectively, without statistical significance observed.

Based on mRNA transcriptional activity, TS was categorized into 3RG/3RG homozygous group with highest activity, 3RG heterozygous group (3RG/3RC and 3RG/2RG) with median activity, and non-3RG group (3RC/3RC, 3RC/2RG and 2RG/2RG) with lowest activity. Although not statistically significant, 3RG/3RG homozygous group was found with lowest response rate (17.6%) (Table 1). Taking *UGT1A1* and *TS* genotypes together, the subgroup with TA7/7 and non-3RG genotypes showed the highest response rate of 33.3%. Furthermore, under the same non-3RG background, no significant difference in response rate was found between TA6/6 and TA7/7 (13.6% vs 33.3%, $P = 0.154$) (Table 2). A multivariate logistic regression model was fitted to our data. The covariates included: chemotherapy regimen, treatment duration, ECOG performance status, sex, age, *UGT1A1* genotypes, and TS promoter enhancer region, but only treatment duration (odds ratio 1.268, 95%CI: 1.027-1.565, $P = 0.027$) was closely linked to clinical response.

UGT1A1 polymorphisms correlation with toxicity

The enzyme activities of UGT1A1*28 alleles from highest to lowest were TA6/6, TA6/7 and TA7/7. TA7/7 showed the highest incidence of grade 2-4 late diarrhea ($P < 0.0005$). Compared with TA6/6, the risk ratio of TA6/7 and TA7/7 was 2.6 (95%CI: 1.20-5.63). Although not statistically significant, a trend of higher incidence of grade 3/4 diarrhea was observed in patients with lower activity genotypes. A similar finding was obtained in the UGT1A1*6 study. G/G with the highest activity was associated with lowest incidence of grade 3/4 diarrhea (trend

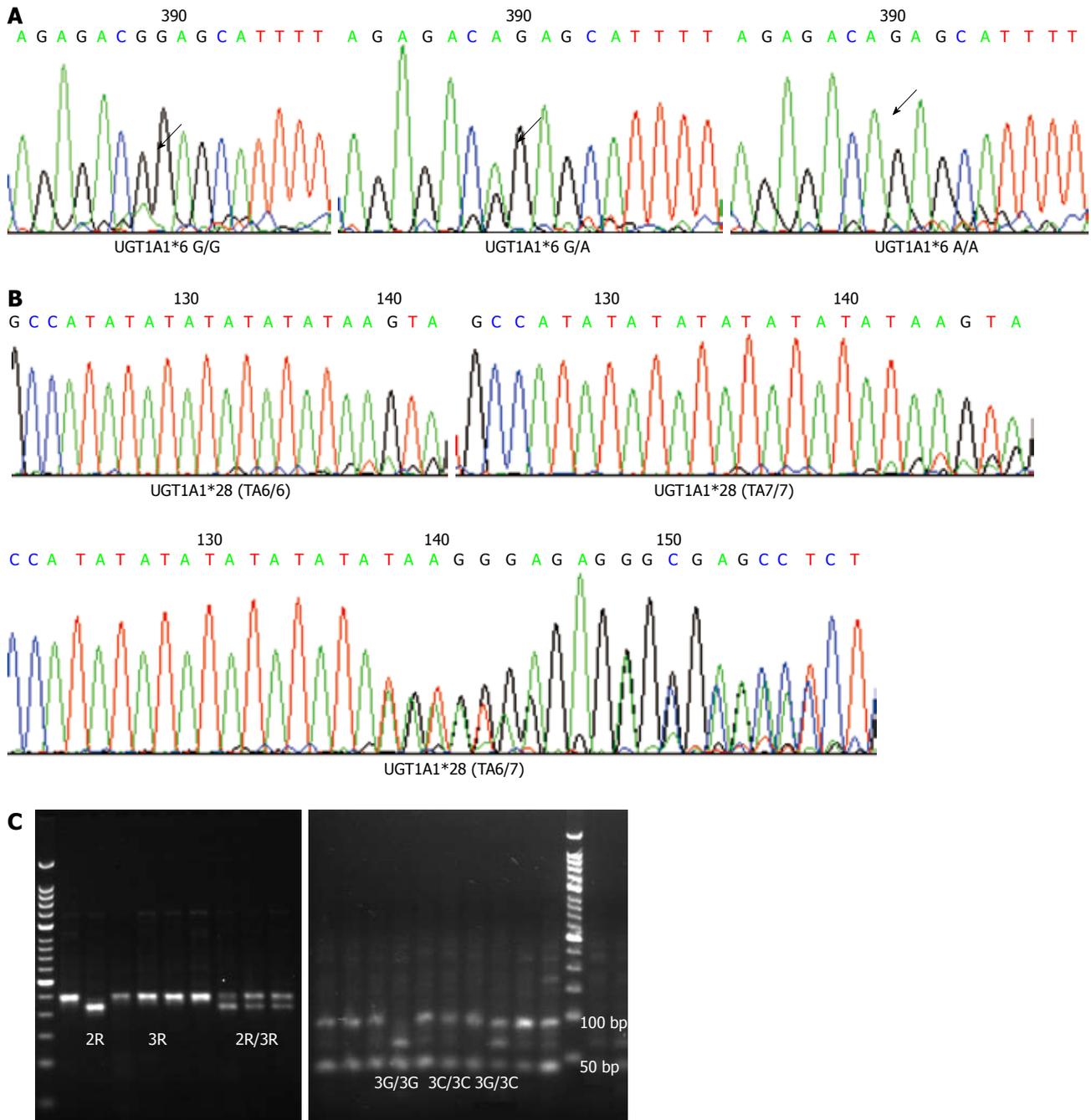


Figure 2 Analysis of UDP-glucuronosyltransferase1A1*28 and *6 by DNA sequencing and thymidylate synthetase by restriction fragment length polymorphism. A: 211G>A of UDP-glucuronosyltransferase1A1 (UGT1A1)*6; B: (TA) nTAA variant of UGT1A1*28; C: Polymerase chain reaction-based restriction fragment length polymorphism for 2R or 3R and G>C single-nucleotide polymorphism in 3R located in the 5'-UTR region of the thymidylate synthetase gene.

test $P = 0.057$). Compared with G/G, the risk ratio of grade 3/4 diarrhea in patients with G/A and A/A was 2.18 (95%CI: 0.87-5.42) (Table 3). In addition, UGT1A1*28 showed a similar correlation pattern with neutropenia. Grade 4 neutropenia was dominantly observed in TA7/7 patients ($P = 0.043$, trend test, $P = 0.017$). Compared with TA6/6, the risk ratio of grade 3/4 neutropenia in patients carrying TA6/7 and TA7/7 was 1.75 (95%CI: 0.79-3.88). By contrast, no association between UGT1A1*6 and grade 3/4 neutropenia was found (Table 3). Among three

TS groups, homozygous 3RG/3RG showed least incidence of grade 3/4 late diarrhea (15.8%) and neutropenia (26.3%), although not significantly different from the other two groups ($P > 0.05$). Further subgroup analysis of non-3RG TS genotypes found that patients with TA6/6 were less prone to grade 2-4 late diarrhea than TA7/7 (35.3% *vs* 100.0%, $P = 0.019$). Meanwhile, a trend of lower incidence of grade 3/4 neutropenia was observed in patients with TA6/6, although the association was not statistically significant (29.4% *vs* 50.0%, $P = 0.582$).

Table 3 Association of UDP-glucuronosyltransferase 1A1 genotypes with toxicity

Genotypes	Delayed diarrhea						Neutropenia					
	Grades 2-4			Grades 3/4			Grades 3/4			Grade 4		
	n (%)	P value ¹	P value ²	n (%)	P value ¹	P value ²	n (%)	P value ¹	P value ²	n (%)	P value ¹	P value ²
UGT1A1*28												
TA 6/6	25 (27.8)	< 0.0005	< 0.0005	14 (15.6)	0.228	0.178	23 (25.6)	0.088	0.055	7 (7.8)	0.043	0.017
TA 6/7	2 (37.5)			6 (18.8)			10 (31.3)			2 (6.3)		
TA 7/7	8 (100)			3 (37.5)			5 (62.5)			3 (37.5)		
UGT1A1*6												
G/G	24 (31.2)	0.564	0.346	10 (13)	0.12	0.057	23 (29.9)	1	0.748	8 (10.4)	0.857	0.473
A/G	19 (39.6)			11 (22.9)			14 (29.2)			4 (8.3)		
A/A	2 (40)			2 (40)			1 (20)			0 (0)		

¹Fisher's exact test for all genotype; ²Exact test of Cochran-Armitage trend test across genotypes. Toxicity grade by National Cancer Institute Common Toxicity Criteria version 3.0. A total of 130 patients were assessable for toxicity. Significant differences were found on grades 2-4 delayed diarrhea and grade 4 neutropenia for UGT1A1*28 genotypes. UGT1A1: UDP-glucuronosyltransferase1A1.

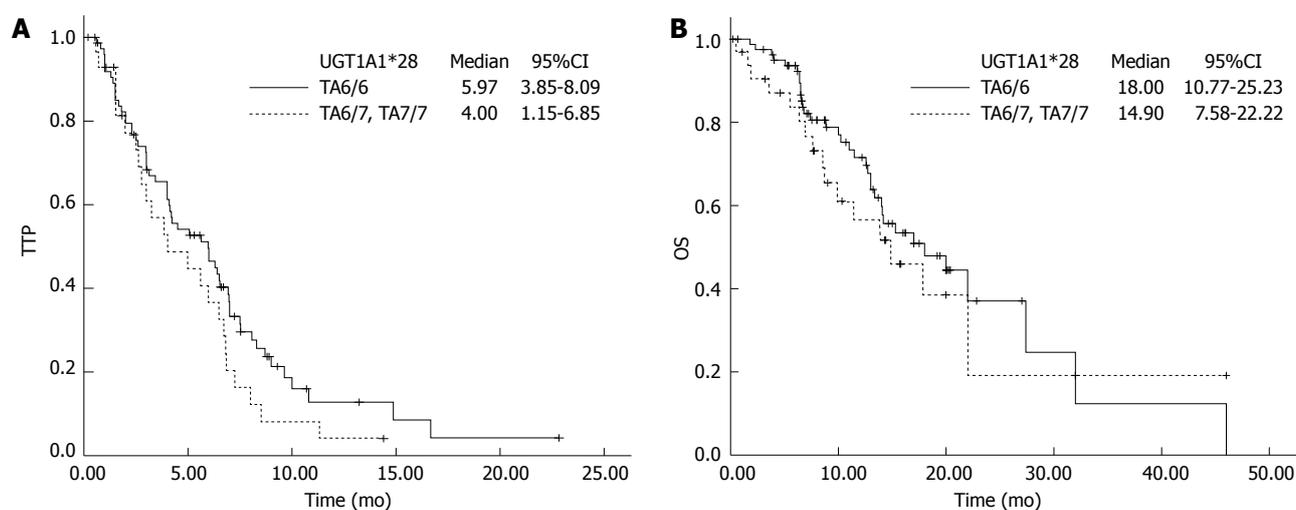


Figure 3 Time to progression and overall survival according to UDP-glucuronosyltransferase1A1*28 genotypes. A: Time to progression (TTP): Patients with TA6/6 were 5.97 (95%CI: 3.85-8.09), patients with TA6/7 and TA7/7 were 4.00 (95%CI: 1.15-6.85), $P = 0.154$; B: Overall survival (OS): Patients with TA6/6 were 18.00 (95%CI: 10.77-25.23), patients with TA6/7 and TA7/7 were 14.90 (95%CI: 7.58-22.22), $P = 0.444$. No significant difference was found between TA6/6 (wild type) and mutated genotypes. UGT1A1: UDP-glucuronosyltransferase1A1.

UGT1A1 polymorphisms correlation with TTP and OS

In 111 patients available for survival outcome analysis, no significant association with TTP and OS was observed with either UGT1A1*28 or UGT1A1*6, although slightly longer TTP and OS were found with UGT1A1*28 (TA6/6) (Figure 3). An additional study of 108 patients on TS revealed that there was no significant association between TS genotype and TTP/OS.

We further examined if the length of treatment duration was related to genotype. Patients with UGT1A1*28 (TA6/6) had a relatively longer treatment time compared with TA6/7 + TA7/7 genotype; near to statistical significance [9.57 ± 6.82 wk (range: 2-32 wk) *vs* 6.76 ± 5.05 wk (range: 1-19 wk), $P = 0.078$]. Although the 3RG homozygous group had longer treatment duration, it failed to display a significant correlation [3RG *vs* non-3RG, 9.03 ± 6.78 wk (range: 1-23 wk) *vs* 8.55 ± 6.16 wk (range: 2-32 wk), $P = 0.895$]. The longest TTP (6.9 mo) and OS (20 mo) were

found in the subgroup with TA6/6 and 3RG genotypes, but the differences were still insignificant compared with other subgroups ($P = 0.46$ and 0.37 , respectively). Independent variables that might affect OS were tested *via* Cox regression model analysis. These variables were ECOG performance status, age, sex, chemotherapy regimen, UGT1A1 and TS polymorphisms. Only chemotherapy regimen and ECOG were independent prognostic factors affecting OS. Higher ECOG score increased the death risk > 3 times [hazard ratio (HR): 3.325, 95% CI: 1.913-5.777, $P < 0.0005$]. Compared with IFL, FOLFIRI showed a 69% decline in death risk (HR: 0.312, 95%CI: 0.132-0.738, $P = 0.008$). For TTP, the Cox model suggested that chemotherapy regimen and sex were independent prognostic factors. Treatment with FOLFIRI lowered the risk of disease progression by 78% (HR: 0.217, 95%CI: 0.104-0.451, $P < 0.0005$). Female sex reduced the progression risk to 60% (HR: 0.608, 95%CI: 0.387-0.968, $P = 0.036$).

Table 4 Association between UDP-glucuronosyltransferase 1A1 genotypes and pharmacokinetics parameters

Genotype	AUC CPT-11		AUC SN-38		AUC SN-38G		AUC SN-38G/AUC SN-38 ratio	
	Median	P value	Median	P value	Median	P value	Median	P value
UGT1A1*28								
TA6/6	5554.57	0.163 ¹	176.40	0.149 ¹	582.14	0.988 ¹	3.178	0.158 ¹
TA6/7	6919.11		213.25		591.86		3.330	
TA7/7	9049.03		390.00		584.63		1.488	
UGT1A1*6								
G/G	5811.73	0.953 ²	189.62	0.320 ²	529.96	0.234 ²	2.924	0.591 ²
G/A	6582.17		231.50		679.17		3.759	
UGT1A1*28+*6								
TA6/6 + G/G	5297.41	0.282 ¹	169.62	0.084 ¹	529.96	0.386 ¹	2.924	0.188 ¹
TA6/7 + G/G, TA6/6 + G/A	6649.50		217.87		679.17		3.680	
TA7/7 + G/G, TA6/7 + G/A	6213.46		297.89		584.63		1.488	
UGT1A1*28								
TA6/6, TA6/7	6030.13	0.884 ²	191.92	0.067 ²	582.14	1.000 ²	3.177	0.057 ²
TA7/7	9049.03		390.00		584.63		1.488	
UGT1A1*28+*6								
TA6/6 + G/G, TA6/7 + G/G, TA6/6 + G/A	6030.13	0.748 ²	183.37	0.061 ²	582.14	0.915 ²	3.177	0.078 ²
TA7/7 + G/G, TA6/7 + G/A	6213.46		297.89		584.63		1.488	

¹Kruskal-Wallis test; ²Mann-Whitney *U* test. 34 patients were assessable for pharmacokinetics parameters. CPT-11: Irinotecan; UGT1A1: UDP-glucuronosyltransferase1A1; AUC: Area under concentration curve.

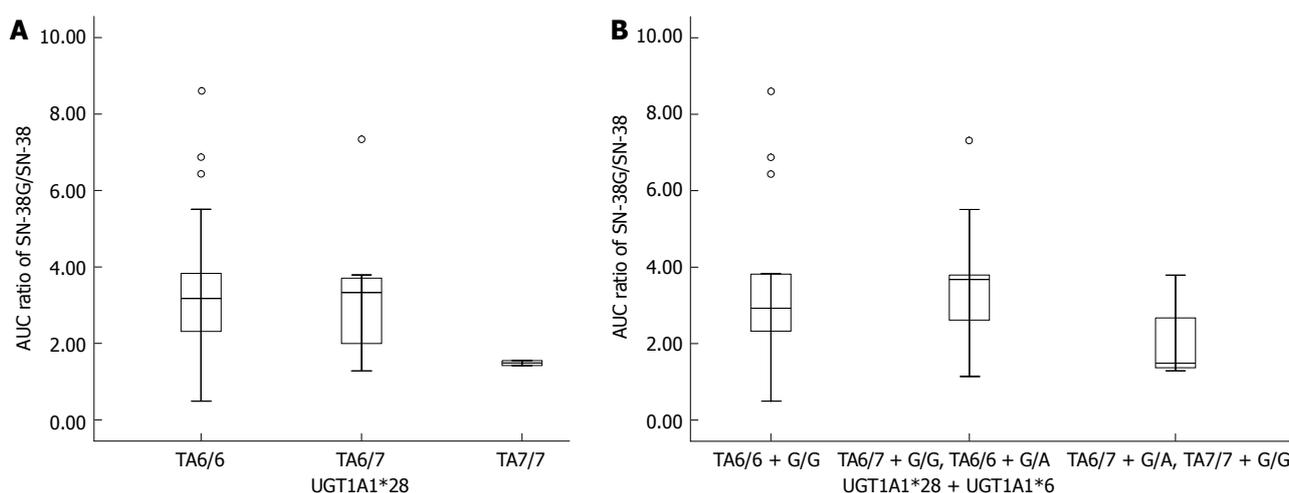


Figure 4 Area under concentration curve ratios of SN-38G/SN-38 comparison according to UDP-glucuronosyltransferase1A1*28 genotypes and UDP-glucuronosyltransferase1A1*28 + *6 group. A: Area under concentration curve (AUC) ratios comparison by UDP-glucuronosyltransferase1A1 (UGT1A1)*28: The AUC ratio of TA7/7 was less than half of the TA6/6 ratio (1.488 vs 3.178, $P = 0.158$); B: AUC ratios comparison by UGT1A1*28 + *6 group: Lower AUC ratio was found in the double mutation group (TA6/7 + G/A and TA7/7 + G/G), compared with the other two groups [wild type or single mutation group (1.488 vs 3.177, $P = 0.078$)].

UGT1A1*28 and UGT1A1*6 correlation with pharmacokinetics

Thirty-four patients were enrolled in a pharmacokinetics study. Among UGT1A1*28 alleles, there were 22 with TA6/6, 10 with TA6/7, and two with TA7/7. Among UGT1A1*6 alleles, there were 25 with G/G and nine with G/A. Among them, two participants were found with a double heterozygous genetic profile; TA6/7 + G/A. The pharmacokinetic parameters showed that the area under concentration curve (AUC) values of irinotecan and SN-38 had an increasing trend among UGT1A1*28 alleles, and TA7/7 appeared to have the highest AUC values, although the AUC values of SN-38G in these three alleles showed no significant difference. Another indicator of SN-38 metabolic rate, the AUC ratio of SN-38G

to SN-38 demonstrated that, compared with the other two alleles, the AUC ratio of TA7/7 was lower and close to statistical significance ($P = 0.057$). Due to no A/A allele being identified in UGT1A1*6, we were unable to study the effect of A/A on AUC. The combinations of UGT1A1*28 and UGT1A1*6 were divided into three groups: group 1 with wild type (TA6/6 + G/G); group 2 with one- mutated site variants (TA6/6 + G/A, TA6/7 + G/G); and group 3 with two-mutated site variants (TA6/7 + G/A, TA7/7 + G/A, TA7/7 + G/G). The higher AUC values of irinotecan and SN-38 and lower AUC ratio were found in group 3, and compared with the other two groups, the difference was near to statistical significance ($P = 0.061$ and 0.078 , respectively) (Table 4). The plot of AUC ratios (Figure 4) clearly illustrated that

group 3 had lower AUC ratio than the other two groups. Regarding UGT1A1*28, the AUC ratio of TA7/7 was less than half of the TA6/6 ratio (1.488 *vs* 3.178, $P = 0.158$).

DISCUSSION

Many western studies on the relationship of UGT1A polymorphisms and irinotecan toxicity and response have suggested that UGT1A1*28 is significantly associated with irinotecan-induced toxicity^[16-18]. In particular, patients bearing UGT1A1*28 (TA7/7) have a high possibility to experience severe neutropenia and diarrhea. Based on this, a warning is labeled on irinotecan that patients with UGT1A1*28 (TA7/7) should start with a reduced dose of irinotecan, although the details of how to adjust the dose have not been specified^[19]. By contrast, research in Asian countries has shown a lower incidence of UGT1A1*28 (TA7/7), while UGT1A1*6 (A/A) is more often found and may replace UGT1A1*28 as a key regulator in UGT1A1 expression^[20-22].

Our early studies have found that few carrying UGT1A1*28 (TA7/7) might be the reason that less than expected diarrhea was observed in Chinese patients treated with irinotecan-based chemotherapy. Nevertheless, other UGT1A members, such as UGT1A1*6, UGT1A7*3 and UGT1A9*1 have a trend towards a high incidence of toxicity, and this association is racially different^[1]. Although, recent data have implicated that UGT1A7 and UGT1A9 might be useful in predicting irinotecan-related toxicity^[7], it might not be applicable for Asian patients because UGT1A1*6 is highly linked with UGT1A7 and UGT1A9 in the Asian population. In addition, Fujita *et al*^[4] and Toffoli *et al*^[5] have proposed that the homozygous UGT1A1*28 (TA7/7) is a predictive marker for better treatment outcome and longer survival time. The results of our study confirmed that only 6.6% of Chinese patients had UGT1A1*28 (TA7/7), whereas a higher frequency of TA6/6 was identified compared with that in Caucasians (69.9% *vs* 45.2%, $P = 0.002$). The allele distribution difference might account for lower incidence of severe late diarrhea and grade 3/4 neutropenia in Chinese patients treated with irinotecan. At the same time, we discovered that the association between UGT1A1*6 and grade 3/4 late diarrhea was close to statistically significance. Furthermore, we did not find that treatment efficacy was discounted by either UGT1A1*28 or UGT1A1*6 variants. Besides that, multivariate logistic regression analysis failed to show that UGT1A1/TS polymorphisms and chemotherapy regimen had any effect on clinical response, but indicated that longer treatment time increased response rate by 26%. No OS benefit was obtained in patients with high incidence of toxicity. This could be caused by shorter treatment duration due to drug intolerance.

Several studies have indicated that TS polymorphism is associated with 5-FU toxicity and prognosis. Although the results are inconclusive, it is well accepted that the allele 3RG/3RG is correlated with long OS and low

toxicity^[23,24]. We introduced TS polymorphism into treatment outcome and toxicity evaluation of irinotecan/5-FU chemotherapy and found that patients carrying UGT1A1*28 (TA6/6) and 3RG/3RG seemed to experience mild toxicity, but relatively low tumor response. Similar to UGT1A1*28, the OS of patients with 3RG/3RG was extended because therapy was well tolerated and its duration was prolonged. However, these observed differences did not reach statistical significance. We conjectured that the differences of treatment response and toxicity between TS genotypes could be narrowed by the synergistic effect of 5-FU combined with irinotecan regimen.

Unfortunately, the combination of two genetic markers was unable to increase the predictive accuracy. In our study, blood samples were used for polymorphism testing. Possibly, examining TS polymorphism and expression level in tumor tissues might yield more convincing data.

The *in vivo* metabolism of irinotecan is complicated. Our data showed that the plasma concentrations of irinotecan and its metabolites varied significantly in each patient. These variations are strongly related to the polymorphisms of many metabolic enzymes and transport proteins. We found that the AUC values of irinotecan and SN-38 were gradually elevated from TA6/6 to TA7/7, whereas the AUC ratios of SN-38G/SN-38 were decreased from TA6/6 to T7/7 ($P = 0.057$). A similar trend was found for UGT1A1*6. If UGT1A1*28 and UGT1A1*6 were taken together, the AUC ratios of group 1 (wild type), group 2 (one-mutated site) and group 3 (two mutated sites) were shifted from the highest to the lowest. Clearly, both alleles had effects on irinotecan metabolism.

Toffoli *et al*^[5] concluded that for Caucasians, UGT1A1*28 not only affected irinotecan-related toxicity, but also changed the drug metabolic rate. On the contrary, reports from Asia suggested that UGT1A1*6 was involved in irinotecan pharmacokinetics and toxicity. Han *et al*^[21] discovered that few UGT1A1*28 variants were found in the Korean population, while more UGT1A1*6 variants were observed. They claimed that the role of UGT1A1*28 in predicating irinotecan pharmacokinetics and toxicity could be replaced by UGT1A1*6 for Koreans. The research from Jada *et al*^[24] also downplayed the role of UGT1A1*28 in the irinotecan metabolic pathway. However, Minami *et al*^[20] contradicted this hypothesis by showing that both UGT1A1*28 and UGT1A1*6 were involved in irinotecan pharmacokinetics. Thus, for the Japanese population, the homozygous (UGT1A1*28/*28 and UGT1A1*6/*6) and the heterozygous (UGT1A1*6/*28) reduced the AUC ratios of SN-38G/SN-38. Our data favored Minami's conclusion that both UGT1A1*28 and UGT1A1*6 participated in glucuronidation.

Derived from the AUC ratios of UGT1A1*28 and UGT1A1*6 variants, we estimated that genetic alterations could inactive the metabolic efficiency of SN-38 by 50%. Therefore we recommend a 50% dose reduction of irinotecan to treat group 3 patients with double-site mutations. Minami *et al*^[20] described that the ratio of SN-38 AUC to irinotecan dose was 2.4 in group 3, while the ratio was 1.4

in group 1. Based on their findings, it would be rational to lower the initial dose of irinotecan by 50% for group 3 patients. Another study by Innocenti *et al.*²⁵ investigated the association between the pharmacokinetics of UGT1A1*28 variants and neutropenia. They recommended that a 20% decrease in drug dose should be considered in patients with UGT1A1*28 (TA7/7).

To date, no clinical trial has used genotypes for determining the proper dose for initial irinotecan-based therapy. We recommend a 50% dose reduction of irinotecan for patients with double-site mutations to avoid severe toxicity, and ensure better efficacy with sufficient treatment given. However, accurate dose modification for patients with different *UGT1A1* genotype is difficult. The limitations of our pharmacokinetics study were lack of UGT1A1*6 A/A information and small sample size. A large-scale prospective trial focused on dose modification of irinotecan will be our next work.

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COMMENTS

Background

Irinotecan is a prodrug that is hydrolyzed by carboxylesterase *in vivo* to form an active metabolite SN-38. SN-38 is further conjugated and detoxified by UDP-glucuronosyltransferase (UGT) to yield its β -glucuronide. Remarkable inter-individual variations in the pharmacokinetics and clinical outcomes have been reported. Genetic polymorphisms of UDP-glucuronosyltransferase 1A1 (UGT1A1), especially UGT1A1*28 and UGT1A1*6 are important determinants of individual variations in Asian patients. 5-Fluorouracil (5-FU) is a fundamental component of all chemotherapeutic combinations for treatment of colorectal cancer (CRC). Thymidylate synthetase (TS) is the main intracellular target of fluoropyrimidines. The TS gene polymorphisms are known to influence the activity of TS, which are related to 5-FU clinical response. The allele containing the triple repeat (3R) in 5'-UTR of the TS is associated with 3-4-fold translational efficiency compared with the double repeat allele (2R), and a G>C base change in 3R alleles makes the transcriptional activity of the 3R allele as low as that of the 2R allele.

Research frontiers

Current studies have revealed the relationship of UGT1A polymorphisms and irinotecan related toxicity and pharmacokinetics. The polymorphisms of UGT1A1*28 are considered to be important in the Caucasian population, while UGT1A1*6 seems to be more important than UGT1A1*28 in Asian studies. It is still unclear whether UGT1A1 has any influence on treatment efficacy. Three studies tested if UGT1A1 isoforms had any impact on treatment outcome, however, their conclusions were inconsistent. TS polymorphisms are of the most broadly studied genetic variants in CRC. It is accepted that the 3R allele is associated with increased expression levels of TS and poorer outcome in patients treated with 5-FU-based regimens. However, the role of gene polymorphisms of UGT1A1 and TS on clinical response to irinotecan and 5-FU-based treatment, non-diarrhea toxicities and prognosis remains unclear.

Innovations and breakthroughs

Only 6.6% of Chinese patients had UGT1A1*28 (TA7/7), whereas higher frequency of TA6/6 was identified compared with that in Caucasians (69.9% vs 45.2%, $P = 0.002$). Mutant variants of UGT1A1*28 and UGT1A1*6 were associated with increased toxicity and decreased SN-38 disposition, but the response rate did not increase accordingly, although this group of patients surely had a trend towards a better response. This probably related to shorter treatment time in patients carrying double-site mutations. Derived from the area under concentration curve ratios of UGT1A1*28 and UGT1A1*6 variants, authors estimated that genetic alterations could inactivate the metabolic efficiency of SN-38 by 50%.

Therefore, the authors recommend a 50% dose reduction of irinotecan to treat patients with double-site mutations.

Applications

To date, no clinical trial has used genotypes for determining the proper dose for initial irinotecan-based therapy. The authors recommend a 50% dose reduction of irinotecan for patients with double-site mutations to avoid severe toxicity, and ensure better efficacy with sufficient treatment given. However, accurate dose modification for patients with different *UGT1A1* genotypes is difficult. A large-scale prospective trial focusing on dose modification of irinotecan will be next work.

Peer review

Polymorphisms predict response and toxicity in patients with metastatic CRC treated with irinotecan and 5-FU. This study is interesting, and the manuscript is worthy of publication.

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Safety of lamivudine treatment for chronic hepatitis B in early pregnancy

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Abstract

AIM: To evaluate the safety of lamivudine (LAM) treatment for chronic hepatitis B in early pregnancy.

METHODS: A total of 92 pregnant women who received LAM treatment either before pregnancy or in early pregnancy were enrolled in this study. All of the pregnant women volunteered to take lamivudine during pregnancy and were not co-infected with hepatitis C virus, human immunodeficiency virus, cytomegalovirus, or other viruses. All infants received passive-active immunoprophylaxis with 200 IU hepatitis B immunoglobulin and three doses of 10 µg hepatitis B vaccines (0-1-6 mo) according to the guidelines for the prevention and treatment of chronic hepatitis B. Adverse events were observed throughout the entire pregnancy and perinatal period, and the effectiveness of lamivudine treatment for blocking mother-to-infant transmission of hepatitis B virus (HBV) was evaluated. All adverse events in mothers and infants during pregnancy and the perinatal period and the HBV mother-to-infant transmission blocking rate were compared with the literature.

RESULTS: Among the 92 pregnant women, spontaneous abortions occurred in 11 cases, while 3 mothers had a second pregnancy after the initial abortion; 72 mothers delivered 73 live infants, of whom 68 infants were followed up for no less than 6 mo, and 12 mothers were still pregnant. During pregnancy, the main maternal adverse events were vaginitis (12/72, 16.7%), spontaneous abortion (11/95, 11.6%), and gestational diabetes (6/72, 8.3%); only one case had 1-2 degree elevation of the creatine kinase level (195 U/L). During the perinatal period, the main maternal adverse events were premature rupture of the membranes (8/72, 11.1%), preterm delivery (5/72, 6.9%), and meconium staining of the amniotic fluid (4/72, 5.6%). In addition, 2 infants were found to have congenital abnormalities; 1 had a scalp hemangioma that did not change in size until 7 mo, and the other had early cerebral palsy, but with rehabilitation training, the infant's motor functions became totally normal at 2 years of age. The incidence of adverse events among the mothers or abnormalities in the infants was not higher than that of normal mothers or HBV-infected mothers who did not receive lamivudine treatment. In only 2 cases, mother-to-infant transmission blocking failed; the blocking rate was 97.1% (66/68), which was higher than has been previously reported.

CONCLUSION: Lamivudine treatment is safe for chronic HBV-infected pregnant mothers and their fetuses with a gestational age of less than 12 wk or throughout the entire pregnancy.

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Key words: Pregnancy; Chronic hepatitis B; Lamivudine; Safety; Hepatitis B virus

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a global health problem; about two billion people have a past or present HBV infection, of whom 350 million are chronic HBV carriers. Each year, about one million people die of liver failure, cirrhosis, or primary hepatocellular carcinoma, all of which are associated with HBV infection. Lamivudine (LAM) is the first approved oral nucleoside analog (NA) used to treat HBV infection (100 mg/d), and the appearance of LAM marked a new era in the treatment of chronic HBV infection. However, treatment duration is very long, and some patients may become pregnant during that time. Although some studies have previously reported on the safety of LAM treatment for chronic HBV infection in late pregnancy^[1-3], there are very few reports about the safety of LAM treatment in early pregnancy^[4-6]. The objective of this study was to evaluate the safety of LAM treatment for chronic hepatitis B in early pregnancy or throughout the entire pregnancy, to provide information about how to block mother-to-infant transmission of HBV in chronic HBV-infected fertile women.

MATERIALS AND METHODS

Patients

All patients were chronic HBV-infected fertile women from our outpatient department who either intended to become pregnant or were already in early pregnancy (with a gestational age of less than 12 wk). Three groups of patients were considered: (1) those who were on NA treatment and could not stop treatment; (2) those who had never taken any NA treatment, with alanine aminotransferase (ALT) > 2 times the upper limit of normal (ULN) for the reference range, HBV DNA > 1×10^5 copies/mL, but failed the traditional liver protection and enzyme reducing treatment; and (3) those who had received NA treatment, but later had virological breakthrough and liver function rebound without LAM drug-resistance. If patients from the above groups planned to become pregnant or had an accidental pregnancy and agreed to LAM treatment after thorough communication and consideration, they were included in this study after providing their written, informed consent.

All participants took screening tests before or in early pregnancy to rule out infection with hepatitis C virus, hepatitis delta virus, human immunodeficiency virus (HIV), syphilis, toxoplasmosis, rubella, cytomegalovirus, and herpes simplex. In this way, these tests helped to exclude any potentially hereditary diseases and any other diseases that needed to be treated. Furthermore, all participants routinely took folic acid before or during early

pregnancy to prevent embryonic neural tube defects.

Methods

All participants undertook routine screening tests, and additionally liver function and HBV serology (HBV markers and HBV DNA) were measured every 12 wk. All adverse events that occurred during pregnancy and all neonatal abnormalities were recorded. The rate of blocking vertical transmission of HBV was also determined.

All infants received passive-active immunoprophylaxis with 200 IU hepatitis B immunoglobulin (HBIG) and three doses of 10 µg hepatitis B vaccines (0-1-6 mo) according to the guidelines for the prevention and treatment of chronic hepatitis B^[7]. One month later, after all vaccinations (7-8 mo), liver function and HBV serology were measured again to evaluate the blocking rate. All infants underwent routine physical examination, hearing screening, and tests for congenital phenylketonuria and hypothyroidism. All neonatal abnormalities were observed for up to 2 years.

Laboratory tests

Liver function and HBV serology were tested in the hospital's clinical laboratory. HBV DNA was detected with an HBV real-time PCR amplification kit from Kehua Biological Company (Shanghai, China), which can detect HBV DNA at levels as low as 500 copies/mL. HBV markers were detected by enzyme-linked immunosorbent assay kits (Abbot Labs, North Chicago, IL, United States) on an ARCHITECT i2000 automatic immunoassay analyzer (Abbott) according to the manufacturer's instructions. Hearing screening was performed with the ECHO-SCREEN from Madsen Company (Germering, Germany). Heel blood was taken on filter paper from the infants after 72 h of breastfeeding, and specimens were sent to the Beijing Neonatal Diseases Screening Center to rule out congenital phenylketonuria and hypothyroidism.

RESULTS

Mothers' basic information

From January 1, 2007 to December 31, 2011, 92 HBV-infected women took LAM treatment before or during early pregnancy. Of these, one mother underwent *in vitro* fertilization with the transfer technique and later delivered twin infants. By the end of 20 wk' gestational age, 11 fetuses aborted, and 3 mothers had a second pregnancy after initial abortion. Ultimately, 72 mothers delivered 73 live infants, of whom 68 infants were followed-up for more than 6 mo, 47 were followed-up for more than 1 year, and 16 were followed-up for more than 2 years. At the end of follow-up, 12 mothers were still pregnant (Figure 1).

Table 1 summarizes the clinical information for the mothers who chose lamivudine treatment before or in early pregnancy, including their demographic characteristics, HBV infection status, and treatment history. All participants were chronic hepatitis B patients, except

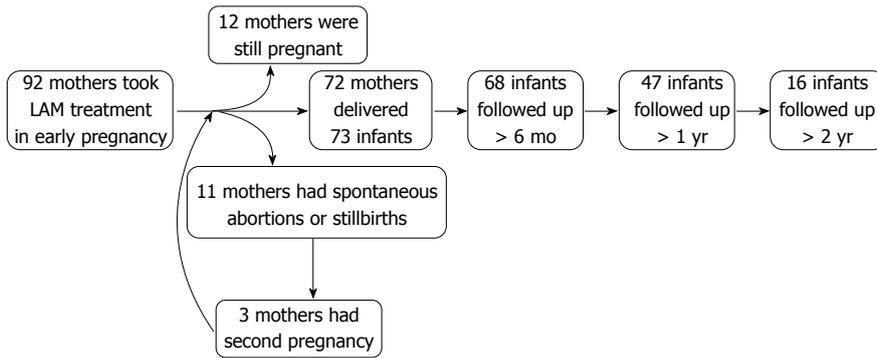


Figure 1 Basic information of mothers and follow-up times for mothers and infants. LAM: Lamivudine.

Table 1 Clinical information of mothers who took lamivudine treatment before or during early pregnancy *n* (%)

	Treated population (<i>n</i> = 92)
Average age (yr)	30.5 ± 3.1
Primipara	88 (95.7)
Cirrhosis	2 (2.2)
HBeAg positivity rate	74 (80.4)
HBV DNA > 10 ⁵ copies/mL in early pregnancy	16 (17.4)
Treatment before pregnancy	
Naive	15 (16.3)
LAM	41 (44.6)
ADV	28 (30.4)
LAM→ADV	3 (3.3)
ETV	2 (2.2)
ETV→ADV	1 (1.1)
IFN + LdT	2 (2.2)

LAM: Lamivudine; ADV: Adefovir dipivoxil; ETV: Entecavir; LdT: Telbivudine; IFN: Interferon; HBeAg: Hepatitis B e antigen.

for 2 cases with compensated cirrhosis, and none of the participants were HBV carriers with normal liver function. Most cases had a history of NA treatment before pregnancy; therefore, only 16 cases (17.4%) had HBV DNA > 10⁵ copies/mL in early pregnancy. Of these 2 cirrhosis patients, one had refused to take any antiviral treatment despite a long history of abnormal liver function due to her concerns about the safety of NA drugs during pregnancy. However, when she later developed cirrhosis, she started to take LAM treatment and became pregnant; the other patient had concomitant autoimmune hepatitis and was taking ursodesoxycholic acid in addition to adefovir dipivoxil, and was later switched to LAM treatment before pregnancy. In early pregnancy, she ceased ursodesoxycholic acid treatment. In addition, 2 cases had previous peripheral neuropathy or myopathy when they took interferon and telbivudine (LdT) combination treatment; both switched to LAM treatment and later became pregnant.

Adverse events in mothers and infants during pregnancy and the perinatal period

Fetal monitoring during pregnancy: Among the 92 women who took LAM treatment in early pregnancy, there were 95 pregnancies. Before 20 wk' gestational age, 4 cases had a threatened abortion, but symptoms of miscar-

riage disappeared following aggressive treatment; 11 had developmental arrest or natural abortion, with an abortion rate of 11.6%. Ultimately, 72 mothers delivered 73 live infants; 3 had developmental retardation monitored with ultrasound but development later normalized after improving the mothers' nutritional status and intravenous hydration. There were no other fetal developmental abnormalities reported and no stillbirths.

Monitoring mothers during pregnancy and the perinatal period:

All maternal adverse events and laboratory abnormalities during pregnancy and the perinatal period are summarized in Table 2. The top 3 adverse events for mothers during pregnancy were vaginitis (16.7%), gestational diabetes (8.3%), and arrhythmia or abnormal electrocardiogram (5.6%). Only one case had 1-2 degree elevation of the creatine kinase (CK) level (195 U/L), and none of the other adverse events could be associated with LAM treatment. Abnormal ALT levels ($\geq 2 \times$ ULN) occurred in 16 cases (22.2%). Of these, 15 cases were in naïve patients without previous antiviral treatment who took LAM treatment only due to abnormal liver function in early pregnancy. The other case stopped LAM treatment before pregnancy and later developed severe hepatitis in early pregnancy, after which LAM was restarted, and her ALT level soon became normal, with her HBV DNA below 500 copies/mL.

However, another patient who took LAM treatment in early pregnancy decreased her HBV DNA by only 1.32 log in 12 wk, after which she was switched to LdT treatment from week 26. In addition, 6 cases had HBV DNA breakthrough with a LAM resistance rate of 8.3%; of these, 3 cases had HBV DNA rebound to above 10⁶ copies/mL, and ADV was added from week 28. For the other 3 cases, ADV was added postpartum. The top 3 adverse events for mothers during the perinatal period were premature rupture of the membranes (11.1%), preterm delivery (6.9%), and meconium staining of the amniotic fluid (5.6%). There were no cases of postpartum hemorrhage or perinatal mortality. In addition, HBV DNA was monitored in mothers antepartum, and 63 cases (87.5%) had HBV DNA below 500 copies/mL.

Postpartum infant monitoring

Of the 92 mothers who took LAM treatment before or

Table 2 Adverse events and laboratory abnormalities during pregnancy and the perinatal period (*n* = 72) (%)

	Adverse events	Cases
Pregnancy	Vaginitis	12 (16.7)
	Gestational diabetes	6 (8.3)
	Arrhythmia/abnormal ECG	4 (5.6)
	Nausea and vomiting	3 (4.2)
	Common cold	3 (4.2)
	Oligohydramnios	3 (4.2)
	Polyhydramnios	2 (2.8)
	Greater than moderate anemia (Hb < 9 g/L)	2 (2.8)
	Placenta previa	2 (2.8)
	Inguinal hernia	1 (1.4)
	Hypothyroidism	1 (1.4)
	ALT ≥ 2 × ULN	16 (22.2)
	HBV DNA breakthrough	6 (8.3)
	Elevated total bilirubin	2 (2.8)
	Thrombocytopenia	2 (2.8)
	Elevated CK (1-2 degrees)	1 (1.4)
	Perinatal period	Premature rupture of the membranes
Preterm		5 (6.9)
Meconium staining of the amniotic fluid 2-3 degrees		4 (5.6)
Antepartum hemorrhage		3 (4.2)
Placenta accreta		1 (1.4)

ECG: Electrocardiogram; Hb: Hemoglobin; ALT: Alanine aminotransferase; ULN: Upper limit of normal; HBV: Hepatitis B virus; CK: Creatine kinase.

in early pregnancy, 72 delivered 73 live infants. Only 3 infants (4.1%) were low birth weight (birth weight < 2500 g); all the other infants had normal weights. None of the infants had abnormal hearing, congenital phenylketonuria, or hypothyroidism on testing. Of the 73 infants, 68 were followed-up for no less than 6 mo, while 47 were followed-up for more than 1 year, and 16 were followed-up for more than 2 years. Two infants were found to have abnormalities postpartum: 1 had 2 scalp hemangiomas (1.5 cm × 1.5 cm and 1.5 cm × 2.0 cm, respectively), which did not change in size until 7 mo, when the parents planned to arrange surgery for the infant; the other infant had early cerebral palsy at 8 mo, but with rehabilitation training, the infant's motor functions became totally normal at 2 years of age. Sixteen babies were followed up for 2 to 4 years, and showed no signs of abnormal intelligence or growth.

Monitoring the mother-to-infant transmission blocking rate

Of the 73 live infants, 68 completed all of the examinations required for evaluating if there was mother-to-infant transmission. Vertical transmission was successfully blocked in 66 infants, for a blocking rate of 97.1%. One mother took LAM every other day by herself because she was concerned about LAM's influence on the infant. She later developed virological breakthrough with HBV DNA increasing to 6.38×10^7 copies/mL. The other mother, who had HBV DNA of 3.6×10^3 copies/mL antepartum, experienced HBV-S mutation during treatment, resulting in blocking failure (the infant had HBsAg(-), HBV DNA(+) and abnormal ALT levels at both 7 mo and 1 year).

DISCUSSION

HBV infection is a serious public health problem worldwide. According to WHO statistics, about 5% of mothers are chronic HBV carriers^[8], and the hepatitis B surface antigen positivity rate among fertile women in some high epidemic areas, such as Africa and South Asia, can be as high as 9.2%-15.5%^[9-11]. About one-third of HBV-infected women enter into the immune clearance phase before or during pregnancy, with a high HBV DNA load and abnormal ALT levels. They are not only faced with a high risk of mother-to-infant transmission, but they also have an increased chance of hepatic disease exacerbation during pregnancy, threatening the safety of both mother and infant^[12-14]. It is currently not recommended for chronic HBV-infected fertile women who are not pregnant to take interferon or NA treatment, in line with the guidelines for the prevention and treatment of chronic hepatitis B^[7]. The antiviral efficacy of interferon is limited, and several side effects hinder its use, resulting in treatment failure. NA treatment alone results in only 20% of hepatitis B e antigen (HBeAg)-positive patients achieving HBeAg seroconversion, with only 12% able to stop treatment with a sustained virological response^[15]. Therefore, many chronic HBV-infected fertile women become pregnant during treatment.

It is very dangerous for pregnant women to stop taking antiviral treatment during pregnancy when they have not met the withdrawal standard, as it may exacerbate liver disease, threatening the safety of both mother and infant. However, the safety of infants exposed to antiviral drugs in utero throughout the entire pregnancy is of particular concern, especially in early pregnancy, which is vital for fetal development. Although LAM is already approved as an optional antiviral drug for use in pregnancy, and several studies have reported its safety in late pregnancy^[1-3], all of the studies regarding its safety before or during early pregnancy come from HIV-infected pregnant women^[16]. Furthermore, most of these patients received combination therapy. So far, there have been very few reports about the safety of LAM treatment in chronic HBV-infected women before or during early pregnancy, and systematic observations have been scarce^[4-6,17].

The present study showed that the abortion rate for HBV-infected women who took LAM treatment in early pregnancy was 11.6%, which was not higher than that of non HBV-infected mothers or HBV-infected mothers according to previous reports (11%-16% and 16.7%-21.9%, respectively)^[6,18,19]. Overall, 72 mothers delivered 73 live infants; none were stillborn. Three fetuses had developmental retardation during pregnancy monitoring, although their development later normalized after treatment; no other fetal developmental abnormalities were reported. Only 4.1% of infants had low birth weight, which was similar to that of women without or with HBV infection according to previous reports (2.7%-7.8% and 5.0%-10.4%, respectively)^[20]. None of the infants had abnormal hearing, congenital phenylke-

tonuria, or hypothyroidism on testing. Two infants were found to have abnormalities (scalp hemangiomas and early cerebral palsy), with a congenital abnormality rate of 2.7%, which is similar to the data from HIV-infected women (3.1%) who took antiretroviral treatment (including LAM) in early pregnancy from 1989 to 2011^[16]. According to the literature, the congenital abnormality rates for infants born to mothers without or with HBV infection were 5.1%-6.3% and 7.2%-10%, respectively^[6,20,21]. The present report suggests that it is safe for fetuses to be exposed to LAM in utero for the entire pregnancy or in early pregnancy, and it does not affect fertilization or fetal development or result in congenital abnormalities. Neither does it affect postnatal development.

The most common adverse event for mothers during pregnancy and the perinatal period was vaginitis (16.7%), among which 7 were vulvovaginal candidiasis (9.7%) and 5 were bacterial vaginosis. However, vaginitis is a common genital infectious disease in pregnant and non-pregnant women and the incidence of vaginitis in our study group was similar to that reported in the literature; it has been reported that the detection rate of candidal vaginitis in pregnant women is about 10%^[22,23], and the incidence of bacterial vaginosis among Asian women is 6.1%^[24]. Other adverse events included gestational diabetes, gestational hypertension, nausea and vomiting of pregnancy, oligohydramnios, polyhydramnios, placenta previa, anemia, pre-eclampsia, premature rupture of the membranes, and preterm delivery. The incidence of the above adverse events was not higher than that of mothers without or with HBV infection according to previous reports^[25-29]. Only one patient had 1-2 degree elevation of serum CK levels, and none of the other adverse events could be associated with LAM treatment. These results suggest that it is safe for HBV-infected pregnant women to take LAM treatment in early pregnancy or throughout the entire pregnancy.

Pregnancy can not only increase the burden on the liver, but it can also increase adrenal cortical hormone levels, boosting HBV replication and activation of hepatitis B. Therefore, for HBV-infected women, the average increase in the HBV DNA level was 0.4 log in late pregnancy or postpartum, and 25% of HBeAg(-) pregnant women had an increase of HBV DNA > 1 log, accompanied by elevation of ALT levels in late pregnancy or postpartum^[13,29]. The incidence of severe hepatitis during the perinatal period (in late pregnancy and one month postpartum) was much higher than in nonpregnant women^[12-14,30]. In the present study, all mothers without previous antiviral treatment maintained normal ALT levels throughout the entire pregnancy, and none of them had severe hepatitis. Fifteen mothers who took NA treatment previously had abnormal ALT levels in early pregnancy, but ALT normalized after LAM treatment, and the pregnancy continued. Interestingly, one mother stopped LAM treatment before pregnancy and later developed severe hepatitis in early pregnancy. However, all symptoms later disappeared, liver function normalized

and the HBV DNA load became undetectable when she restarted LAM. In addition, 87.5% of patients had HBV DNA below 500 copies/mL antepartum, and the LAM resistance rate was only 8.3% during pregnancy. This suggests that LAM can effectively suppress HBV DNA replication in HBV-infected pregnant women, maintain normal liver function, and lower the incidence of hepatic disease during pregnancy, improving the prognosis of HBV-infected pregnant women.

According to previous reports, for infants born to HBeAg-positive mothers, even after they received passive-active immunoprophylaxis with HBIG and three doses of hepatitis B vaccines, vertical transmission was not blocked in 7%-16.3%^[31]. For mothers with an increased HBV DNA load, vertical transmission was not blocked in as many as 23.4%-32% of infants^[32]. In the present study, 80.4% of mothers was HBeAg-positive, and all of them had a high HBV DNA load before LAM treatment. However, 63 (87.5%) mothers had HBV DNA below 500 copies/mL antepartum, and the blocking rate was 97.1%; blocking failed in only 2 cases. The first mother had poor compliance, resulting in HBV DNA breakthrough, while the other had an HBV-S mutation during treatment, resulting in failure of vaccine immunization. These results suggest that LAM treatment could increase the vertical transmission blocking rate in HBeAg-positive mothers.

This study suggests that it is safe and effective for chronic HBV-infected pregnant women to take LAM treatment in early pregnancy. Treatment does not increase complications or adverse events for mothers during pregnancy or the perinatal period, has no effect on fertilization or embryonic development, and does not increase the incidence of congenital abnormalities in infants. Furthermore, it increases the blocking rate of mother-to-infant transmission. In conclusion, the benefits of taking LAM treatment in early pregnancy outweigh the risks. However, the sample size of this study was small, and the follow-up time was limited; both need to be optimized in later studies to provide greater insight.

COMMENTS

Background

Chronic hepatitis B virus (HBV) infection is prevalent throughout the world; 2 billion people have been or are infected with HBV worldwide, with 350 million people having chronic infections. Each year, more than 1 million people die of HBV-related liver failure, cirrhosis, or primary hepatic carcinoma. Interferon and nucleos(t)ide analogs (NAs) are available for the antiviral treatment of hepatitis B. However, the efficacy of interferon is suboptimal, and interferon is associated with scores of adverse effects, so that many patients fail or cannot tolerate treatment. Treatment with NAs requires long-term persistence, as in the case of human immunodeficiency virus; stopping the medicine frequently leads to relapse of the disease. As women undergoing treatment cannot halt the medicine at will, pregnancy often occurs during the on-treatment period. However, the safety profile of NAs has not been verified. The goal of this study was to observe the safety profile of lamivudine for the mother and fetus throughout the entire pregnancy.

Research frontiers

In recent years, with many studies evaluating the efficacy and safety profile, the administration of lamivudine and other NAs during the third trimester to block HBV transmission has been a hot topic. However, studies regarding lamivudine

treatment during the first trimester or the entire period of pregnancy are scarce.

Innovations and breakthroughs

This study observed adverse events throughout the entire period of pregnancy in women taking lamivudine, including abortion, ectopic pregnancy, and complications related to pregnancy. The study also observed intrauterine deformations of the fetus and abnormalities after birth in detail and maintained long-term follow-up of some neonates.

Applications

The study provides preliminary evidence of the safety profile for pregnant women taking lamivudine. It also sets an example for further studies exploring the safety profile of NAs in pregnant women.

Peer review

The manuscript describes safety of lamivudine treatment in early pregnancy. They compared maternal and infant abnormality, HBV DNA level and mutations, and blocking rates of mother-to infant transmission between cases with and without lamivudine treatment. They concluded that lamivudine treatment in early treatment is safe. The study is important, because patients with HBV infection often show elevation of HBV DNA levels and may have alanine aminotransferase flare.

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Overexpression of lysine specific demethylase 1 predicts worse prognosis in primary hepatocellular carcinoma patients

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Abstract

AIM: To investigate the clinicopathological features and prognostic value of lysine specific demethylase 1 (LSD1) in hepatocellular carcinoma (HCC).

METHODS: We examined LSD1 expression in 60 paired liver cancer tissues and adjacent noncancerous tissues by quantitative real time polymerase chain reaction (qRT-PCR) and Western blotting. In addition, we analyzed LSD1 expression in 198 HCC samples by immunohistochemistry. The relationship between LSD1 expression, clinicopathological features and patient survival was investigated.

RESULTS: Immunohistochemistry, Western blotting,

and qRT-PCR consistently confirmed LSD1 overexpression in HCC tissues compared to adjacent non-neoplastic tissues ($P < 0.01$). Additionally, immunostaining showed more LSD1-positive cells in the higher tumor stage (T3-4) and tumor grade (G3) than in the lower tumor stage (T1-2, $P < 0.001$) and tumor grade (G1-2, $P < 0.001$), respectively. Moreover, HCC patients with high LSD1 expression had significantly lower 5-year overall survival rates ($P < 0.001$) and lower 5-year disease-free survival rates ($P < 0.001$), respectively. A Cox proportional hazards model further demonstrated that LSD1 over-expression was an independent predictor of poor prognosis for both 5-year disease-free survival [hazards ratio (HR) = 1.426, 95%CI: 0.672-2.146, $P < 0.001$] and 5-year overall survival (HR = 2.456, 95%CI: 1.234-3.932, $P < 0.001$) in HCC.

CONCLUSION: Our data suggest for the first time that the overexpression of LSD1 protein in HCC tissues indicates tumor progression and predicts poor prognosis.

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Key words: Hepatocellular carcinoma; Lysine specific demethylase 1; Tumor progression; Prognosis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, especially in Asia^[1]. In China, HCC ranks behind gastric and esophageal cancer with the third highest mortality rate among all malignant carcinomas, leads to approximately 110 000 deaths every year, and accounts for 45% of total HCC deaths worldwide^[2]. Multiple risk factors have been associated with the initiation and development of HCC; these include chronic infection with hepatitis B, C or D virus, aflatoxin, alcohol abuse, hereditary metabolic liver diseases, and diabetes mellitus^[1,3]. Like most other cancers, hepatocarcinogenesis is a multistep process that involves multiple genetic alterations that may activate oncogenes and/or inactivate tumor suppressor genes, ultimately leading to the malignant transformation of hepatocytes.

Histone demethylase lysine specific demethylase 1 (LSD1), the first histone demethylase that was discovered as a nuclear homolog of amine oxidases, removes the methyl groups from mono- and dimethylated lysine (Lys) 4 of histone H3 (H3K4me1/2) and Lys9 of histone H3 (H3K9me1/2). LSD1 is essential for mammalian development and is involved in many biological processes, including cell-type differentiation, gene activation and gene repression^[4]. A recent study indicated that LSD1 might promote cell phase transition (deficiency in LSD1 led to partial cell cycle arrest in G2/M) and cell proliferation, suggesting that its over-expression might promote tumorigenesis^[5]. The expression of LSD1 has been associated with tumor recurrence during therapy in various cancers, further implicating LSD-1 as a tumor promoter^[6-8]. A tissue cDNA microarray analysis also demonstrated the presence of LSD1 transactivation in lung and colorectal carcinomas^[7]. LSD1 knockdown with small interfering (si) RNAs resulted in the suppression of proliferation of various bladder and lung cancer cell lines^[7]. To the best of our knowledge, there is little available data regarding the involvement of *LSD1* genes in hepatic tumorigenesis. In this study, we investigated LSD1 expression in HCC and its correlation with the clinicopathological features of patients with HCC, including patient survival.

MATERIALS AND METHODS

Patients and tissue samples

The study was approved by the Research Ethics Committee of Xinhua Hospital, which is affiliated to Shanghai Jiaotong University School of Medicine, Shanghai, China. Informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to accepted ethical and legal standards.

A total of 198 patients who presented with primary HCC and later underwent curative liver resection at Xinhua Hospital affiliated to Shanghai Jiaotong University School of Medicine, Shanghai, China, were included in this retrospective study. The tissue samples used in this study were retrieved from the tissue bank of the Depart-

Table 1 Clinicopathological features and the expression of lysine specific demethylase 1 in 198 hepatocellular carcinoma patients

Characteristics	n	LSD1 (%)		P value
		High expression	Low expression	
Gender				
Male	101	58	43	
Female	97	54	43	NS
Age (yr)				
≥ 50	112	61	51	
< 50	86	51	35	NS
Tumor stage				
T1	39	5	34	
T2	42	21	21	
T3	76	47	29	
T4	41	39	2	< 0.001
Tumor grade				
G1	45	9	34	
G2	114	63	51	
G3	39	38	1	< 0.001
Growth pattern				
Trabecular	147	84	63	
Nontrabecular	51	28	23	NS
Cirrhosis				
Yes	151	87	64	
No	47	25	22	NS
Underlying liver disease				
Alcoholic	21	15	6	
Hepatitis B	136	74	62	
Hepatitis C	30	17	13	
Unknown	11	6	5	NS

LSD1: Lysine specific demethylase 1; NS: Not significant.

ment of Pathology in the Xinhua Hospital affiliated with Shanghai Jiaotong University School of Medicine. The patients had been diagnosed with HCC between 2001 and 2006. None of the patients recruited in this study had undergone preoperative chemotherapy or radiotherapy. HCC diagnosis was based on World Health Organization criteria. Tumor differentiation was defined according to the Edmondson grading system. Liver function was assessed using the Child-Pugh scoring system. Tumor staging was determined according to the sixth edition of the tumor-node-metastasis (TNM) classification of the International Union against Cancer. The clinicopathological features of 198 patients are summarized in Table 1. In addition, 60 self-pairs of HCC specimens (10 TNM stage I, 16 TNM stage II, 24 TNM stage III, and 10 TNM stage IV) and adjacent non-neoplastic liver tissues were snap frozen in liquid nitrogen and stored at -80 °C following surgery for quantitative real time polymerase chain reaction (qRT-PCR) assay and western blot analysis. The median follow-up period was 8.6 years. Postoperative surveillance included routine clinical and laboratory examinations every third month, computed tomography scans of the abdomen, and radiographs of the chest every third month. After 5 years, the examination interval was extended to 12 mo.

Immunohistochemistry analysis

Immunohistochemical staining was carried out follow-

ing the protocol of our previous study^[9-11]. The primary antibody against LSD1 was a rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., United States) at a dilution of 1:50. The specificity of the primary antibody has been validated by the previous studies of Müller *et al*^[12] and Lü *et al*^[13]. The secondary antibody for the detection of primary antibody was anti-rabbit immunoglobulin G (Sigma, St. Louis, MO, United States). The negative controls were processed in a similar manner with phosphate-buffered saline instead of primary antibody. Further, positive LSD1 expression, as confirmed by western blotting, was used as a positive control for immunostaining. Following hematoxylin counterstaining, immunostaining was scored by two independent experienced pathologists who were blinded to the clinicopathological parameters and clinical outcomes of the patients. The scores of the two pathologists were compared, and any discrepant scores were re-examined for staining by both pathologists until a consensus score was obtained. The number of cells that stained positive for nuclear LSD1 in ten representative microscopic fields was counted, and the percentage of positive cells was calculated. The percentages of cells that were immunoreactive were converted to scores as follows: 0 (0%), 1 (1%-10%), 2 (11%-50%) and 3 (> 50%). Staining intensity was visually scored and stratified as follows: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). A final score was obtained for each case by multiplying the percentage score and the intensity score. Multiplied scores exceeding 5 (median of total scores for LSD1) were considered to indicate low levels of LSD1 expression, while all other scores were considered to indicate high levels of LSD1 expression.

Western blotting

The Western blotting protocol and semiquantitative analysis were carried out following the protocol of Xu *et al*^[14]. LSD1 antibody (rabbit polyclonal antibody, dilution 1:50, Santa Cruz Biotechnology, Inc., United States) was used, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (CW0266, dilution 1:1000, CoWin Biotech) was used as an internal control.

Quantitative RT-PCR

To measure the mRNA expression levels of LSD1, total RNA was extracted from frozen liver tissues using TriZol reagent (Invitrogen) according to the manufacturer's instructions. The extraction was followed by RT-PCR using the TransStart Green qPCR SuperMix (TransGen Biotech). The primer sequences for LSD1 amplification were 5'-CGAACGCACATCAAGACGA-3' for the forward primer and 5'-AGGTGAAGGTGGAGTAGAGGC-3' for the reverse primer. The transcription of GAPDH was used as an internal control for normalization. LSD1 expression levels were calculated relative to GAPDH using the delta-delta computed tomography method^[15].

Statistical analysis

SPSS version 13.0 for Windows (SPSS Inc, IL, United

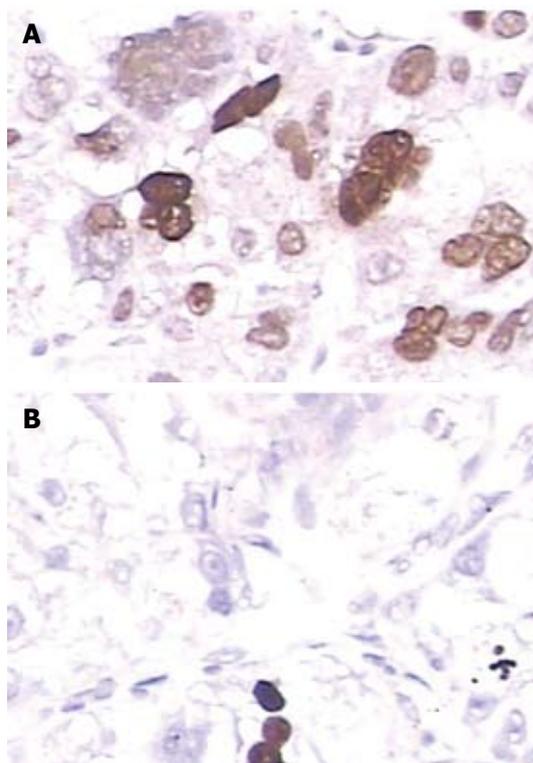


Figure 1 Lysine specific demethylase 1 expression in hepatocellular carcinoma and adjacent non-neoplastic liver tissues. A: High expression of lysine specific demethylase 1 (LSD1) in hepatocellular carcinoma (HCC) samples; B: Low expression of LSD1 in HCC samples (original magnification, 200 \times).

States) and SAS 9.1 (SAS Institute, Cary, NC) were used for statistical analysis. A Fisher's exact test and χ^2 test were performed to assess associations between LSD1 expression and clinicopathological parameters. A Kaplan-Meier method was used for survival analysis, and differences in survival were estimated using the log-rank test. A multivariate survival analysis was performed for all parameters that were significant in the univariate analyses using a Cox regression model. Differences were considered statistically significant when *P* was less than 0.05.

RESULTS

Expression of LSD1 protein and mRNA in HCC

Immunohistochemical analysis revealed that LSD1 staining was mainly localized to the nucleus of noncancerous and malignant epithelial cells (Figure 1). In addition, we found that 112 (56.6%) of 198 HCC tissues had high LSD1 expression, and 86 (43.4%) of 198 HCC tissues had low LSD1 expression. Additionally, 52 (26.3%) of 198 adjacent non-neoplastic liver tissues had high LSD1 expression, and 146 (73.7%) of 198 adjacent nonneoplastic liver tissues had low LSD1 expression. Thus, the LSD1 immunostaining in HCC tissues was significantly higher than the staining in the adjacent non-neoplastic liver tissues (*P* < 0.01). To confirm LSD1 protein expression by an independent method, Western blotting analysis was performed using 60 self-pairs of HCC and adjacent

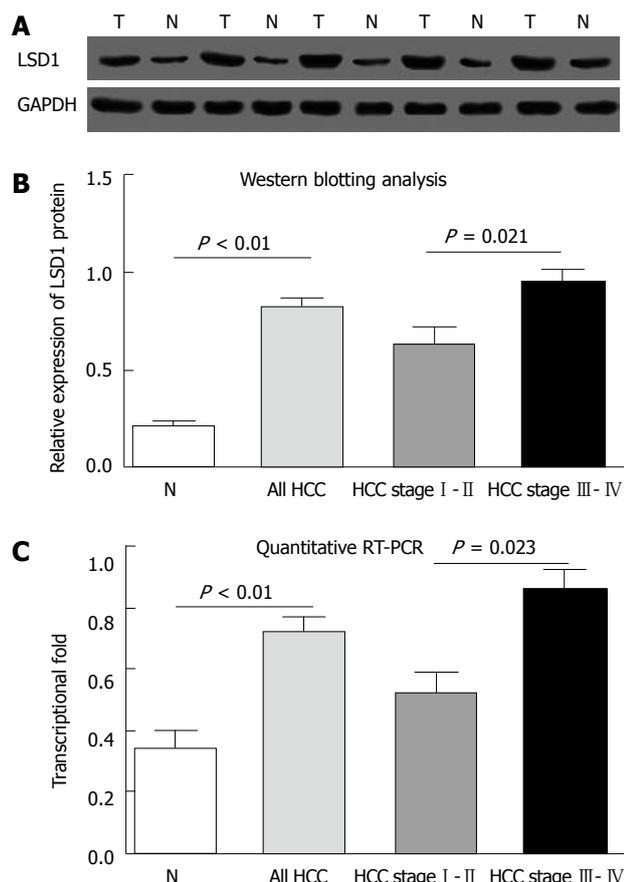


Figure 2 Increased lysine specific demethylase 1 protein and mRNA levels in hepatocellular carcinoma with different tumor node metastasis stages and adjacent non-neoplastic liver tissues. A: Representative Western blotting of lysine specific demethylase 1 (LSD1) protein levels in hepatocellular carcinoma (HCC) tissues (T) and adjacent non-neoplastic liver tissues (N); B: Semiquantitative Western blotting showed that the expression levels of LSD1 protein were significantly higher than those in adjacent non-neoplastic liver tissues ($P < 0.01$). Additionally, the expression levels of LSD1 protein increased with ascending tumor node metastasis (TNM) stages. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. P values, mean and SD were given (t test); C: Quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay showed significantly increased LSD1 mRNA levels in HCC tissues compared with adjacent non-neoplastic liver tissues ($P < 0.01$). Additionally, the expression levels of LSD1 mRNA were increased with ascending tumor TNM stages. GAPDH was used as the internal control. P values, mean and SD were given (Mann-Whitney test).

non-neoplastic liver tissues. Overexpression of LSD1 protein in HCC tissues was compared with adjacent non-neoplastic liver tissues ($P < 0.01$, Figure 2A and B) using this method, and significantly increased LSD1 mRNA levels were detected by qRT-PCR ($P < 0.01$, Figure 2C). The expression levels of LSD1 protein and mRNA in high stage (III-IV) HCC tissues were both significantly higher than the levels of LSD1 protein and mRNA in low stage (I-II) HCC tissues (for protein: $P = 0.021$; for mRNA: $P = 0.023$; Figure 2B and C).

Association of LSD1 expression with the clinicopathological features of HCC

To evaluate whether LSD1 protein expression was associated with clinicopathological features of patients with

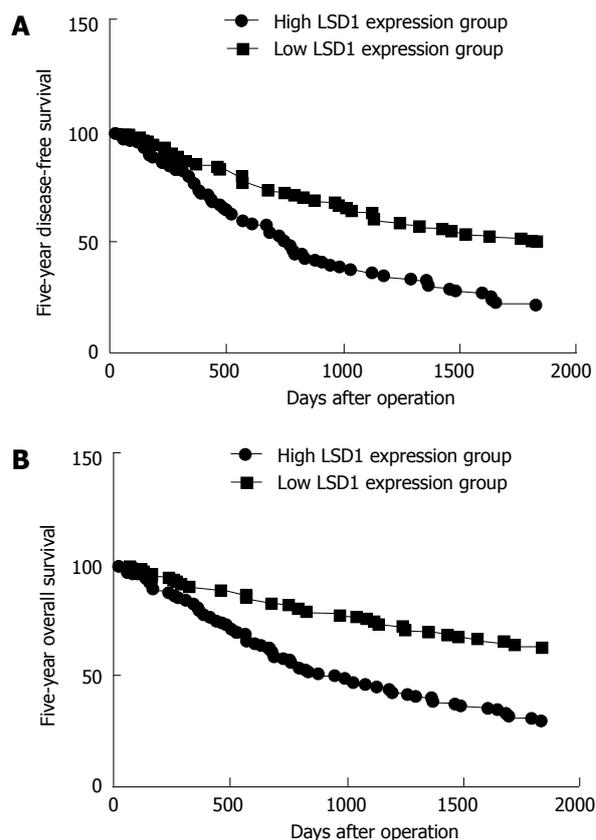


Figure 3 Kaplan-Meier survival curves for lysine specific demethylase 1 expression in hepatocellular carcinoma patients. A: The hepatocellular carcinoma patients with high lysine specific demethylase 1 (LSD1) expression showed significantly shorter disease-free survival ($P < 0.001$); B: Overall survival ($P < 0.001$) rates than those with low LSD1 expression.

HCC, we correlated immunohistochemical LSD1 staining results with T stage, tumor grade, presence of cirrhosis, underlying liver disease, including alcohol abuse, viral hepatitis B and C, sex and age (Table 1). We found more LSD1 positive cells in tissues with higher tumor stages (T3-4) and higher tumor grades (G3) than in the lower tumor stages (T1-2, $P < 0.001$) and tumor grades (G1-2, $P < 0.001$), respectively.

Prognostic values of LSD1 expression in HCC

The 5-year disease-free survival in the group with high LSD1 expression was significantly poorer than the disease-free survival in the group with low LSD1 expression ($P < 0.001$, log-rank test; Figure 3A). A Kaplan-Meier plot of 5-year overall survival curves stratified by LSD1 expression is shown in Figure 3B. There was a significant relationship between LSD1 expression and 5-year overall survival ($P < 0.001$, log-rank test; Figure 3B). In a multivariate Cox model that included tumor size, tumor stage, tumor grading, presence of cirrhosis, gender, age, and LSD1 staining, we found that LSD1 expression independently indicated poor prognosis for both 5-year disease-free survival [hazards ratio (HR) = 1.426, 95%CI: 0.672-2.146, $P < 0.001$; Table 2] and 5-year overall survival (HR = 2.456, 95%CI: 1.234-3.932, $P < 0.001$; Table

Table 2 Multivariate survival analysis of five-year overall and disease-free survival in 198 patients with hepatocellular carcinoma

Parameter	Five-year overall survival			Five-year disease-free survival		
	P value	HR	95%CI	P value	HR	95%CI
Age	0.775	0.867	0.463-1.452	0.714	1.174	0.883-1.853
Gender	0.456	1.121	0.569-1.867	0.634	1.126	0.684-1.846
Tumor stage	< 0.001	1.634	1.142-2.537	< 0.001	1.423	0.784-2.161
Tumor grade	< 0.001	1.154	0.647-1.893	< 0.001	1.023	0.456-1.638
Presence of cirrhosis	0.542	1.143	0.647-1.784	0.427	1.321	0.824-1.917
LSD1 expression	< 0.001	2.456	1.234-3.932	< 0.001	1.426	0.672-2.146

LSD1: Lysine specific demethylase 1; HR: Hazards ratio.

2) in patients with HCC.

DISCUSSION

Genetic alterations are a hallmark of human cancer. In recent years, the field of cancer genomics has made significant advances in the area of cancer-associated genetic lesion identification. Furthermore, the importance of epigenetic changes that occur during HCC development has also been recognized^[16]. Epigenetic changes can take the form of DNA methylation or histone modification^[17]. Histone modifications in the form of selective acetylation, phosphorylation, and methylation serve as switches that alter chromatin structure, allowing posttranscriptional activation or repression of downstream proteins^[18]. Understanding these epigenetic changes will lead to the identification of novel cancer-related genes that may represent attractive targets for cancer treatment and provide new insights into the biology of hepatic cancers. Thus, an integrative approach to hepatic cancer research that combines epidemiological, genetic and epigenetic information has emerged as an important paradigm for cancer therapy^[19]. The methylation status of histone methyltransferases and histone demethylases plays a pivotal role in the regulation of gene expression^[20]. LSD1, the first histone demethylase that was discovered as a nuclear homolog of amine oxidases, removes methyl groups from mono- and dimethylated H3K4me1/2 and H3K9me1/2^[21].

Epigenetic changes in LSD1 have been shown to play a key role in carcinogenesis^[22]. LSD1 can prevent the accumulation of the dimethyl groups of p53, repressing p53-mediated transcriptional up-regulation, preventing apoptosis, and contributing to human carcinogenesis *via* a chromatin modification mechanism. To date, a few studies have indicated that LSD1 may promote cell phase transition (deficiency in LSD1 led to partial cell cycle arrest in G2/M) and cell proliferation, suggesting that LSD1 over-expression might promote tumorigenesis^[5]. The expression of LSD1 has been associated with tumor recurrence during therapy in various cancers, further implicating LSD-1 as a tumor promoter^[6-8]. Tissue cDNA microarray analysis also revealed LSD1 transactivation in lung and colorectal carcinomas^[7]. Knocking down LSD1 with small interfering RNAs resulted in suppression of proliferation of various bladder and lung cancer cell

lines^[7]. However, the association between LSD1 and the survival of HCC patients was not well defined. In this study, we investigated the associations between LSD1 expression levels and clinical features of HCC patients.

In order to demonstrate that the epigenetic changes were associated with genetic changes in lung cancer, we first investigated the expression of LSD1 in HCC clinical samples. Previous studies have demonstrated that LSD1 protein and mRNA levels could act as biomarkers for the identification of patients with more aggressive breast cancer, prostate cancer, lung cancer and neuroblastoma^[23-26]. In our study, we detected LSD1 by immunohistochemistry analysis, Western blotting, and qRT-PCR. Our results showed that LSD1 immunoreactivity was significantly increased in HCC compared with adjacent non-neoplastic liver tissue in a substantial proportion of cases. The over-expression of LSD1 was observed in tumor tissues with higher tumor stage and higher tumor grade. Additionally, our investigation revealed that high LSD1 expression is associated with a significant trend toward both poorer disease-free survival and poorer overall survival. Our study further confirms that high LSD1 expression independently predicts a higher risk of disease relapse or death after multivariate adjustment for other prognostic factors. These observations support the hypothesis that LSD1 may function as an oncogene in HCC and suggest that LSD1 may play an important role in the tumorigenesis of HCC. However, the role of LSD1 in HCC remains to be elucidated. Our data may offer new insight into LSD1 as a potentially important contributor to the progression of HCC and as a new prognostic factor for HCC. As the 198 cases of the present study were all obtained from the Chinese population, the results reported here should be further confirmed in other populations.

In conclusion, our study suggests that LSD1 is over-expressed in HCC tissues compared with their benign counterparts. To the best of our knowledge, this is the first study evaluating the expression levels of LSD1 mRNA and protein in HCC tissues and the association between these expression levels and clinicopathologic parameters. The most important finding of this study is that LSD1 expression may predict a poorer prognosis for HCC patients after surgery. Further studies are needed to investigate the precise function of LSD1 in the progression of HCC.

COMMENTS

Background

Lysine specific demethylase 1 (LSD1) is essential for mammalian development and is involved in many biological processes, such as cell-type differentiation, gene activation and gene repression. Knocking down LSD1 with small interfering RNAs suppressed the proliferation of various bladder and lung cancer cell lines. To be known, little data has been generated with regard to the involvement of LSD1 genes in hepatic tumorigenesis. In this study, the authors investigated LSD1 expression in hepatocellular carcinoma (HCC) and its correlation with clinicopathological features, including the survival of patients with HCC.

Research frontiers

The data suggest for the first time that the overexpression of LSD1 protein in HCC tissues may help predict tumor progression and poor prognosis.

Innovations and breakthroughs

The authors examined LSD1 expression in 60 paired liver cancer tissues and adjacent noncancerous tissues by quantitative real time polymerase chain reaction and Western blotting. In addition, authors analyzed LSD1 expression in 198 HCC samples by immunohistochemistry. The relationships between LSD1 expression and both clinicopathological features and patient survival were investigated.

Applications

These findings provide evidence that the overexpression of LSD1 serves as a biomarker for poor prognosis in HCC. Thus, authors speculate that LSD1 may be a potential target of anti-angiogenic therapy for HCC.

Terminology

LSD1, the first histone demethylase that was discovered as a nuclear homolog of amine oxidases, removes the methyl groups from mono- and dimethylated Lysine (Lys) 4 of histone H3 and Lys9 of histone H3.

Peer review

The authors investigated the expression of LSD1 in HCC and determined its correlation with tumor progression and prognosis. The authors claim that the expression levels of LSD1 protein in HCC tissues correlates with tumor progression and prognosis.

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S-Editor Gou SX L-Editor A E-Editor Li JY

Meta-analysis of laparoscopic vs open liver resection for hepatocellular carcinoma

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Author contributions: Xiong JJ and Altaf K contributed equally to this work; Sutton R, Liu XB and Hu WM designed the research, corrected and approved the manuscript; Xiong JJ and Altaf K developed the literature search and carried out statistical analysis of the studies; Javed MA, Huang W, Mukherjee R and Mai G performed data extraction; Xiong JJ and Altaf K wrote the manuscript; and all authors read and approved the final manuscript.

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Abstract

AIM: To conduct a meta-analysis to determine the safety and efficacy of laparoscopic liver resection (LLR) and open liver resection (OLR) for hepatocellular carcinoma (HCC).

METHODS: PubMed (Medline), EMBASE and Science Citation Index Expanded and Cochrane Central Register of Controlled Trials in the Cochrane Library were searched systematically to identify relevant comparative studies reporting outcomes for both LLR and OLR

for HCC between January 1992 and February 2012. Two authors independently assessed the trials for inclusion and extracted the data. Meta-analysis was performed using Review Manager Version 5.0 software (The Cochrane Collaboration, Oxford, United Kingdom). Pooled odds ratios (OR) or weighted mean differences (WMD) with 95%CI were calculated using either fixed effects (Mantel-Haenszel method) or random effects models (DerSimonian and Laird method). Evaluated endpoints were operative outcomes (operation time, intraoperative blood loss, blood transfusion requirement), postoperative outcomes (liver failure, cirrhotic decompensation/ascites, bile leakage, postoperative bleeding, pulmonary complications, intraabdominal abscess, mortality, hospital stay and oncologic outcomes (positive resection margins and tumor recurrence).

RESULTS: Fifteen eligible non-randomized studies were identified, out of which, 9 high-quality studies involving 550 patients were included, with 234 patients in the LLR group and 316 patients in the OLR group. LLR was associated with significantly lower intraoperative blood loss, based on six studies with 333 patients [WMD: -129.48 mL; 95%CI: -224.76-(-34.21) mL; $P = 0.008$]. Seven studies involving 416 patients were included to assess blood transfusion requirement between the two groups. The LLR group had lower blood transfusion requirement (OR: 0.49; 95%CI: 0.26-0.91; $P = 0.02$). While analyzing hospital stay, six studies with 333 patients were included. Patients in the LLR group were found to have shorter hospital stay [WMD: -3.19 d; 95%CI: -4.09-(-2.28) d; $P < 0.00001$] than their OLR counterpart. Seven studies including 416 patients were pooled together to estimate the odds of developing postoperative ascites in the patient groups. The LLR group appeared to have a lower incidence of postoperative ascites (OR: 0.32; 95%CI: 0.16-0.61; $P = 0.0006$) as compared with OLR patients. Similarly, fewer patients had liver failure in the LLR group than in the OLR group (OR: 0.15; 95%CI: 0.02-0.95; $P =$

0.04). However, no significant differences were found between the two approaches with regards to operation time [WMD: 4.69 min; 95%CI: -22.62-32 min; $P = 0.74$], bile leakage (OR: 0.55; 95%CI: 0.10-3.12; $P = 0.50$), postoperative bleeding (OR: 0.54; 95%CI: 0.20-1.45; $P = 0.22$), pulmonary complications (OR: 0.43; 95%CI: 0.18-1.04; $P = 0.06$), intra-abdominal abscesses (OR: 0.21; 95%CI: 0.01-4.53; $P = 0.32$), mortality (OR: 0.46; 95%CI: 0.14-1.51; $P = 0.20$), presence of positive resection margins (OR: 0.59; 95%CI: 0.21-1.62; $P = 0.31$) and tumor recurrence (OR: 0.95; 95%CI: 0.62-1.46; $P = 0.81$).

CONCLUSION: LLR appears to be a safe and feasible option for resection of HCC in selected patients based on current evidence. However, further appropriately designed randomized controlled trials should be undertaken to ascertain these findings.

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Key words: Hepatocellular carcinoma; Laparoscopy; Open liver resection; Hepatectomy; Meta-analysis

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Xiong JJ, Altaf K, Javed MA, Huang W, Mukherjee R, Mai G, Sutton R, Liu XB, Hu WM. Meta-analysis of laparoscopic vs open liver resection for hepatocellular carcinoma. *World J Gastroenterol* 2012; 18(45): 6657-6668 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i45/6657.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i45.6657>

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common primary cancer worldwide^[1], and the third most common cause of cancer-related deaths with about 600 000 patients dying from the disease annually^[2]. The potential treatment options for HCC include: surgical resection^[3], liver transplantation^[4], chemotherapy and local ablative therapy^[5]. Surgery, either through hepatic resection or liver transplantation, is the best hope for a cure, but is not suitable for those patients who also suffer from significant background cirrhosis^[5]. Liver transplantation should be considered in any patient with cirrhosis and a small (5 cm or less single nodule or up to three lesions of 3 cm or less) HCC. Hepatic resection, on the other hand, should be considered as a primary therapy in every patient with HCC and a non-cirrhotic liver (including fibrolamellar variant). Resection can also be carried out in highly selected patients with hepatic cirrhosis and well preserved hepatic function (Child-Pugh A) who are unsuitable for liver transplantation^[6].

Open liver resection (OLR) has traditionally been accepted as the preferred treatment for resectable HCC in patients with adequate liver reserves^[7]. However, most patients with HCC have significant underlying co-morbidities, including liver diseases such as chronic hepatitis and liver cirrhosis, and hence are at very high risk of developing significant postoperative complications. Laparoscopic surgery is considered to be a safe alternative to open surgical intervention in numerous surgical procedures. Since the first successful report of laparoscopic liver wedge resection in 1992^[8], improvement in surgical instrumentation and experience in laparoscopic treatment for the majority of surgical gastrointestinal conditions, including benign liver diseases, have led to a growing interest in its application for HCC. Recent studies have suggested that the laparoscopic liver resection (LLR) has a number of advantages such as reduction of postoperative pain, operative morbidity, and length of hospitalization, especially for cirrhotic patients with HCC^[9-12]. However, the current literature on LLR for HCC exists in the form of few comparative studies. General application of this approach for treating this disease is still a matter of debate because it is new and data regarding long-term oncologic outcomes (e.g., recurrence) are not robust.

Three published meta-analysis^[13-15] have investigated the advantages and disadvantages of the LLR for HCC. These meta-analyses have reported that LLR was associated with decreased blood loss and requirement for blood transfusion, lower overall postoperative morbidity and shorter hospital stay compared with the OLR. In addition, there was no difference between groups in oncologic outcomes such as positive resection margins and tumor recurrence. Since these meta-analysis included a limited number of studies with fewer cases, data reported were not sufficient to derive conclusions with regards to the overall efficacy and safety of LLR. In the interim, several high-quality studies^[16-20] with more participants have been published. We have therefore undertaken an analysis of 15 studies including 1105 hepatic resections to provide an update on the efficacy of LLR vs OLR for HCC.

MATERIALS AND METHODS

Study selection

PubMed (Medline), EMBASE and Science Citation Index Expanded and Cochrane Central Register of Controlled Trials in the Cochrane Library were searched systematically for all articles published from January 1992 to February 2012 comparing LLR and OLR for HCC. The following medical search headings and keywords were used: "laparoscopy" or "laparoscopic" or "minimally invasive surgery" and "hepatectomy" or "liver resection" or "hepatic resection" and "primary liver carcinoma" or "hepatocellular carcinoma" or "HCC". Only human studies published in English language as full text articles were considered for inclusion. Reference lists of selected articles were also examined to find relevant studies which were not identified during the initial data-

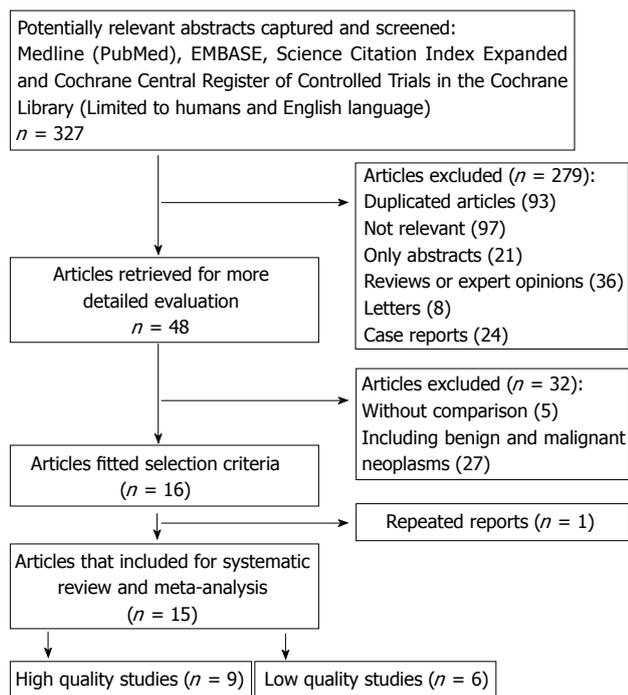


Figure 1 Flow diagram depicting the process of identification and inclusion of selected studies.

base searches. Final inclusion of articles was determined by consensus from two authors; when this failed, a third author adjudicated.

Inclusion and exclusion criteria

Two authors identified and screened the aforementioned databases for potentially eligible studies.

Inclusion criteria: (1) Clear documentation of the operative techniques as “laparoscopic” or “open”; (2) Studies with at least one of the outcomes mentioned; and (3) Where multiple studies came from the same institute and/or authors, either the one of higher quality or the most recent publication was included in the analysis.

Exclusion criteria: (1) Abstracts, letters, editorials, expert opinions, case reports, reviews and studies lacking control groups; (2) Studies with no clearly reported outcomes of interest; (3) Studies dealing with HCC recurrence after hepatectomy; and (4) Studies including patients with benign lesions or other types of malignant liver tumors.

Outcomes of interest

The following outcomes were evaluated in the two approaches.

Operative outcomes: Operative time, intraoperative blood loss and requirement for blood transfusions.

Postoperative outcomes: Hospital stay, liver failure, cirrhotic decompensation/ascites, bile leakage, postoperative bleeding, pulmonary complications (including pleural

effusion and pneumonia), intra-abdominal abscess and mortality.

Oncologic outcomes: Positive resection margins and tumor recurrence.

Data extraction and quality assessment

Data were extracted by two independent observers using standardized forms. The recorded data included patient and study characteristics and surgical details. The quality of studies was assessed using the Newcastle-Ottawa Scale^[21], by examining three factors: patient selection, comparability of the study groups and assessment of outcome. Studies were matched for age, American Society of Anesthesiologists status, presence of cirrhosis, size of tumor and type of hepatic resection undertaken. The maximum numbers of stars in the selection, comparability, and outcome categories were four, two, and three, respectively. Studies achieving six or more stars were considered to be of higher quality^[22]. Only these were included in the final analysis to have the best estimate of the outcome measure.

Statistical analysis

Meta-analysis was performed using Review Manager Version 5.0 software (The Cochrane Collaboration, Oxford, United Kingdom). For continuous variables, treatment effects were expressed as weighted mean difference (WMD) with corresponding 95%CI. For categorical variables, treatment effects were expressed as odds ratio (OR) with corresponding 95%CI. Heterogeneity was evaluated using the χ^2 test, and a P value < 0.1 was considered significant^[23]. The fixed-effects model was initially calculated for all outcomes^[24]. If the test rejected the assumption of homogeneity of studies, random-effects analysis was performed^[25]. Sensitivity analysis were performed by removing individual studies from the data set and analyzing the effect on the overall results to identify sources of significant heterogeneity. Subgroup analysis were also undertaken by including low-quality studies to present cumulative evidence. Funnel plots were constructed to evaluate potential publication bias^[26] based on the operative time, hospital stay and tumor recurrence.

RESULTS

Description of included trials in the meta-analysis

The search strategy initially generated 327 relevant clinical trials. Finally, 16 articles^[10-12,16-20,27-34] were selected for further investigation. Of these, two studies^[12,30] were published by the same institute and had overlapping patient populations; therefore, the higher-quality study^[30] was included. In total, 15 non-randomized comparative studies were identified for final inclusion, out of which 9 were found to be of high quality^[10,11,17,18,20,28,30,32,33]. These were included in the final analyses. Figure 1 shows the process of selecting comparative studies included in our meta-analysis.

Table 1 Characteristics of studies included in the meta-analysis

Study	Year	Country	Group	n	Male/female	Age (yr) (mean ± SD)	Matching ^b	Study quality
Shimada <i>et al</i> ^[27]	2001	Japan	LLR	17	15/2	62 ± 9	1,3,4	*****
Laurent <i>et al</i> ^[28]	2003	France	OLR	38	24/14	63 ± 79		
			LLR	13	10/3	62.6 ± 9.5	1,3,4,5	*****
Kaneko <i>et al</i> ^[29]	2005	Japan	OLR	14	10/4	65.9 ± 5.5		
			LLR	30	18/12	59 ± 8	1,2,3,4,5	*****
Belli <i>et al</i> ^[30]	2007	Italy	OLR	28	18/10	61 ± 10		
			LLR	23	13/10	59.5 ± 6.84	1,2,3,4,5	*****
Endo <i>et al</i> ^[31]	2009	Japan	OLR	23	14/9	62.4 ± 7.7		
			LLR	10	8/2	72 ± 4	3,4	****
Lai <i>et al</i> ^[32]	2009	China	OLR	11	8/3	64 ± 2		
			LLR	25	18/7	59 (35-79) ^a	1,3,4	*****
Sarpel <i>et al</i> ^[11]	2009	United States	OLR	33	21/12	59 (38-77) ^a		
			LLR	20	15/5	63.8 ± 10.3	1,3,4	*****
Aldrighetti <i>et al</i> ^[33]	2010	Italy	OLR	56	45/11	58.3 ± 11.0		
			LLR	16	11/5	65 ± 10	1,2,3,4,5	*****
Tranchart <i>et al</i> ^[10]	2010	France	OLR	16	12/4	71 ± 6		
			LLR	42	15/27	63.7 ± 13.1	1,2,3,4,5	*****
Nguyen <i>et al</i> ^[34]	2011	United States	OLR	42	14/28	65.7 ± 7.1		
			LLR	17	12/5	68	1,3,4,5	*****
Hu <i>et al</i> ^[16]	2011	China	OLR	20	12/8	65		
			LLR	30	20/10	46 ± 12	1,3,4	****
Ker <i>et al</i> ^[19]	2011	China	OLR	30	19/11	48 ± 15		
			LLR	116	92/24	58.31 ± 12.7	1,2	****
Kim <i>et al</i> ^[20]	2011	South Korea	OLR	208	156/52	57.9 ± 11.2		
			LLR	26	18/8	57.84 ± 9.66	1,2,3,4,5	*****
Lee <i>et al</i> ^[17]	2011	China	OLR	29	20/9	57.08 ± 9.78		
			LLR	33	24/9	59 (36-85) ^a	1,2,4	*****
Truant <i>et al</i> ^[18]	2011	France	OLR	50	40/10	58.5 (32-81) ^a		
			LLR	36	31/5	60.6 ± 10.2	1,2,3,4	*****
			OLR	53	47/6	63.3 ± 7.6		

LLR: Laparoscopic liver resection; OLR: Open liver resection. ^aMedian with range; ^b1: Age; 2: American Society of Anesthesiologists physical status score; 3: Presence of cirrhosis; 4: Tumor size; 5: Type of liver resection.

Study and patient characteristics

The characteristics and quality assessments of included studies are shown in Table 1. A total of 550 patients were included: 234 patients in the LLR and 316 patients in the OLR group. The characteristics of patients and surgical details are summarized in Table 2. The sample size of the included studies varied from 21 to 89 patients. The rate of conversion, from laparoscopic to open procedure, ranged from 0% to 19.4%. Patients in most of studies had concurrent hepatitis B infection.

Meta-analysis results

Results of the analyses are shown in Figure 2 and summarized in Table 3.

Operative outcomes: Six high-quality studies^[10,11,18,28,30,33] reported mean operation time, analysis of which showed no statistically significant difference between the two groups (patients 354; WMD: 4.69 min; 95%CI: -22.62-32 min; $P = 0.74$). Similarly, six high-quality studies^[10,18,20,28,30,33] provided detailed data for estimation of blood loss between the two groups. We found that LLR had significantly less intraoperative blood loss compared to OLR [patients 333; WMD: -129.48 mL; 95%CI: -224.76-(-34.21) mL; $P = 0.008$]. Furthermore, the rate of blood transfusions requirement was identified to be significantly lower in the

LLR group as opposed to OLR (trials: 7; patients 416; OR: 0.49; 95%CI: 0.26-0.91; $P = 0.02$). Addition of low-quality trials to these groups did not affect the results.

Postoperative outcomes: Six high-quality studies^[10,18,20,28,30,33] reported on length of hospital stay. Pooled outcome measure favored LLR [patients 333; WMD: -3.19 d; 95%CI: -4.09-(-2.28) d; $P < 0.00001$]. A lower incidence of liver failure was observed in patients undergoing LLR (trials 2, patients 116; OR: 0.15; 95%CI: 0.02-0.95; $P = 0.04$). The incidence of postoperative ascites in seven high-quality trials (patients 416; OR: 0.32; 95%CI: 0.16-0.61; $P = 0.0006$) was found to be significantly lower in LLR group. Six high-quality trials^[10,17,18,20,28,30] revealed no statistically significant difference in the incidence of pulmonary complications between the two groups (patients 384; OR: 0.43; 95%CI: 0.18-1.04; $P = 0.06$). However, when two low-quality trials^[27,31] were also pooled together to get a cumulative result, LLR group seemed to have a lower incidence (patients 460; OR: 0.43; 95%CI: 0.19-0.96; $P = 0.04$).

No significant differences were observed between two operative techniques in terms of other postoperative complications, such as bile leakage (trials 3; patients 205; OR: 0.55; 95%CI: 0.10-3.12; $P = 0.50$), postoperative bleeding (trials 5; OR: 0.54; 95%CI: 0.20-1.45; $P = 0.22$) and mortality (trials 5; patients 474; OR: 0.46; 95%CI:

Table 2 Characteristics of patients and surgical details

Study	Group	Cirrhosis <i>n</i> (%)	Tumor size (cm)	Type of hepatectomy
Shimada <i>et al</i> ^[27]	LLR	13 (76.4)	2.6 ± 0.9	a = 7, b = 10
	OLR	28 (73.6)	2.5 ± 1.0	NA
Laurent <i>et al</i> ^[28]	LLR	NA	3.35 ± 0.89	a = 3, b = 7, c = 3
	OLR	NA	3.43 ± 1.05	a = 4, b = 7, c = 3
Kaneko <i>et al</i> ^[29]	LLR	13 (43.3)	3.0 ± 0.8	a = 10, b = 20
	OLR	NA	3.1 ± 0.9	a = 8, b = 20
Belli <i>et al</i> ^[30]	LLR	23 (100)	3.1 ± 0.7	a = 5, b = 3, c = 15
	OLR	23 (100)	3.24 ± 0.70	a = 6, b = 5, c = 12
Endo <i>et al</i> ^[31]	LLR	6 (60)	3.0 ± 1.5	NA
	OLR	9 (81.8)	4.1 ± 0.8	NA
Lai <i>et al</i> ^[32]	LLR	23 (92)	2.5 (1-7) ¹	a = 6, b = 8, c = 10, d = 1
	OLR	31 (93.9)	2.6 (1-8) ¹	a = 2, b = 18, c = 13
Sarpel <i>et al</i> ^[11]	LLR	9 (45)	4.3 ± 2.1	NA
	OLR	27 (48.2)	4.3 ± 2.2	NA
Aldrighetti <i>et al</i> ^[33]	LLR	9 (56.3)	4 ± 2.2	a = 5, b = 2, c = 9
	OLR	9 (56.3)	4.6 ± 2.5	a = 5, b = 2, c = 9
Tranchart <i>et al</i> ^[10]	LLR	31 (73.8)	3.58 ± 1.75	a = 9, b = 15, c = 10, d = 3, e = 2, f = 3
	OLR	34 (80.9)	3.68 ± 2.09	a = 7, b = 13, c = 10, d = 3, e = 2, f = 7
Nguyen <i>et al</i> ^[34]	LLR	44 (65)	3.0	a = 6, b = 5, e = 6
	OLR	23 (35)	4.5	a = 6, b = 8, e = 6
Hu <i>et al</i> ^[16]	LLR	25 (83.3)	6.7 ± 3.1	NA
	OLR	NA	8.7 ± 2.3	a = 10, b = 20
Ker <i>et al</i> ^[19]	LLR	NA	2.5 ± 1.2	a = 7, c = 97, e = 4, g = 8
	OLR	NA	5.4 ± 3.5	NA
Kim <i>et al</i> ^[20]	LLR	NA	3.15 (1-8) ¹	a = 4, b = 4, c = 13, d = 4, e = 1
	OLR	NA	3.6 (1-19) ¹	a = 3, b = 10, c = 9, d = 5, e = 2
Lee <i>et al</i> ^[17]	LLR	28 (84.8)	2.5 (1.5-9) ¹	a = 18, h = 15
	OLR	32 (64)	2.9 (1.2-9) ¹	a = 10, h = 40
Truant <i>et al</i> ^[18]	LLR	NA	2.9 ± 1.2	a = 22, b or f = 14
	OLR	NA	3.1 ± 1.2	a = 26, b or f = 27

¹Median and (range). LLR: Laparoscopic liver resection; OLR: Open liver resection; NA: Not available; a: Left lateral segmentectomy; b: Segmentectomy; c: Subsegmentectomy; d: Right hepatectomy; e: Left hepatectomy; f: Bisegmentectomy; g: Right anterior sectorectomy; h: Nonanatomical resection.

0.14-1.51; $P = 0.20$).

Two high-quality trials^[20,30] reported intra-abdominal abscess formation in their patient populations. However, one of these did not have any events in both the groups and was subsequently excluded. A subgroup analysis was therefore undertaken including a low-quality study, which also did not show an association of intra-abdominal abscess formation with the type of operative technique (patients 122; OR: 0.72; 95%CI: 0.12-4.54; $P = 0.73$).

Oncologic outcomes: We did not find any significant differences in the rate of positive margins (trials 4; patients 287; OR: 0.59; 95%CI: 0.21-1.62; $P = 0.31$) and tumor recurrence (trials 6; patients 416; OR: 0.95; 95%CI: 0.62-1.46; $P = 0.81$).

Sensitivity and subgroup analysis

Sensitivity analyses were carried out by excluding each in-

dividual study from each outcome measure. These exclusions did not alter the results obtained from cumulative analyses. Additionally, the pooled result of included outcomes was not affected, when either fixed effects or random effects models were used. Subgroup analyses were undertaken for all outcome measures by including low-quality studies as well. These are summarized in Table 3.

Publication bias

The funnel plot was based on the operation time, hospital stay and tumor recurrence, which is shown in Figure 3. As no study lies outside the limits of the 95%CI, there was no evidence of publication bias.

DISCUSSION

LLR is a challenging technique for surgeons as the liver has unique anatomical features which present technical difficulties for parenchymal transections-massive hemorrhage and bile leak from intrahepatic vessels^[11,29]. Presence of cirrhosis in patients undergoing LLR makes parenchymal transection an even more delicate and demanding procedure^[11]. Rare but fatal complications such as a gas embolism caused by the pneumoperitoneum through hepatic venous branches on the hepatic stump during parenchymal division of the liver have also been reported^[27]. On the contrary, increased experience, technical refinement and improvement in surgical equipment have increased the safety of liver resection as a curative treatment for benign or malignant liver lesions^[35-38]. In spite of these advancements, LLR has not been very popular for HCC, partly because of the controversies related to resection margins, tumor seeding, incision related metastasis, and long-term survival^[32]. However, the difference in outcomes between LLR and OLR in HCC has not been evaluated in a randomized controlled trial. Most reported studies are retrospective, single-institution series with a small number of patients, which makes it difficult to interpret outcomes appropriately. In order to overcome these limitations, we have endeavored to pool all the relevant available data and perform a meta-analysis. Although our result is similar to previously reports^[13-15] in some aspects, such as operative blood loss, blood transfusion requirement, length of hospital stay and tumor recurrence, our analysis included more high-quality case-matched studies as well as more patients and therefore, provides an up to date and high-quality evidence regarding the perioperative and long-term outcomes of patients with HCC undergoing LLR *vs* OLR.

We found no significant difference in the 30-d mortality between the two groups. The results from this analysis indicate that LLR was successfully completed in most patients, with a rate of conversion to open surgery ranging from 0% to 19.4%. These results point towards the feasibility of LLR for patients with HCC.

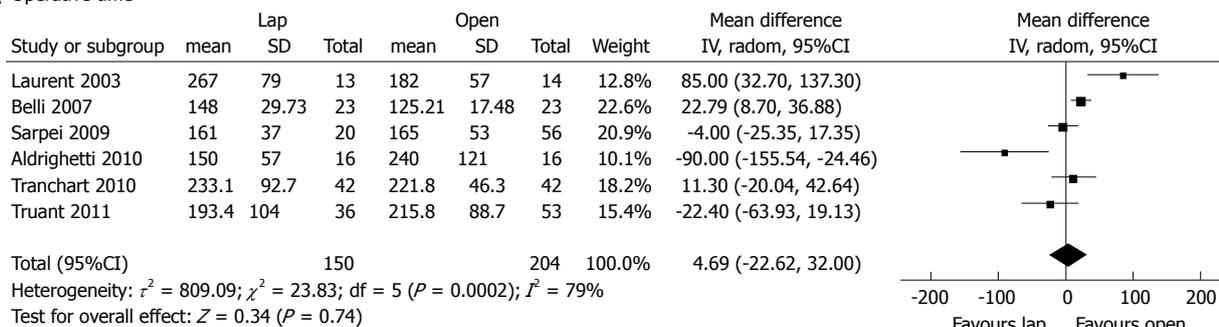
There was no significant difference in operative time between the two techniques, based on our analysis, which can be explained by current advances in surgical instru-

Table 3 Results of meta-analysis comparing laparoscopic vs open hepatectomy (only high-quality studies)

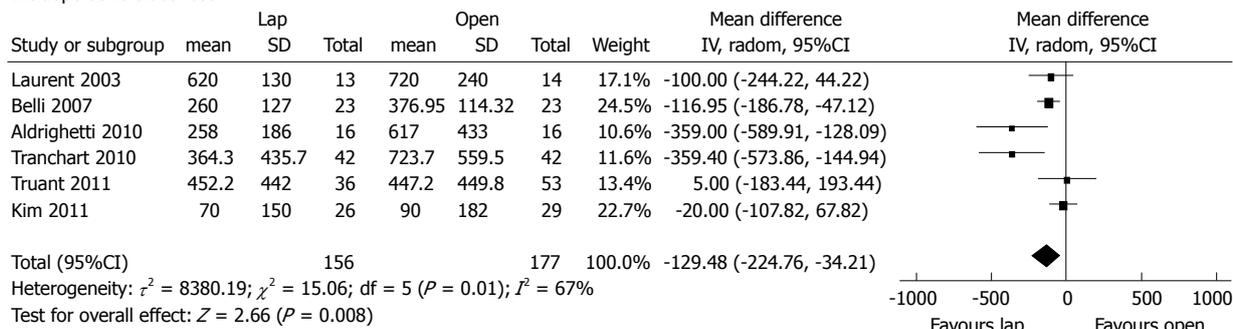
Outcome of interest	No. of studies	No. of patients	OR/WMD	95%CI	P value	Heterogeneity P value	I ² (%)
Operative outcomes							
Operation time (min)	6	354	4.69	-22.62, 32.00	0.74	0.0002	79
Intraoperative blood loss (mL)	6	333	-129.48	-224.76, -34.21	0.008	0.01	67
Blood transfusions requirement	7	416	0.49	0.26, 0.91	0.02	0.89	0
Postoperative outcomes							
Liver failure	2	116	0.15	0.02, 0.95	0.04	1.00	0
Cirrhotic decompensation/ascites	7	416	0.32	0.16, 0.61	0.001	0.95	0
Bile leakage	3	205	0.55	0.10, 3.12	0.50	0.86	0
Postoperative bleeding	5	287	0.54	0.20, 1.45	0.22	0.83	0
Pulmonary complications	6	384	0.43	0.18, 1.04	0.06	0.46	0
Intra-abdominal abscess	2	101	0.21	0.01, 4.53	0.32	-	-
Mortality	8	474	0.46	0.14, 1.51	0.20	0.64	0
Hospital stay	6	333	-3.19	-4.09, -2.28	< 0.00001	0.91	0
Oncologic outcomes							
Surgery margin positive rate	5	287	0.59	0.21, 1.62	0.31	0.65	0
Tumor recurrence	7	416	0.95	0.62, 1.46	0.81	0.93	0

WMD: Weighted mean difference; OR: Odds ratio.

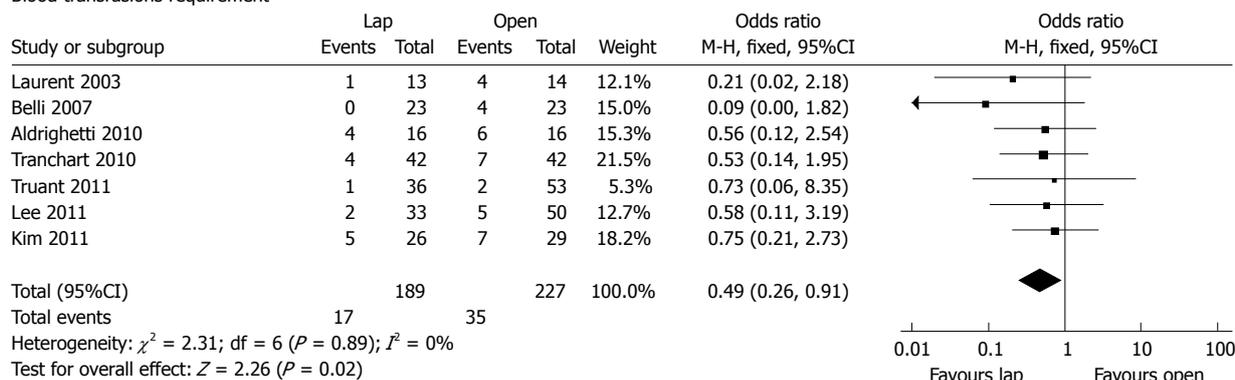
A Operative time



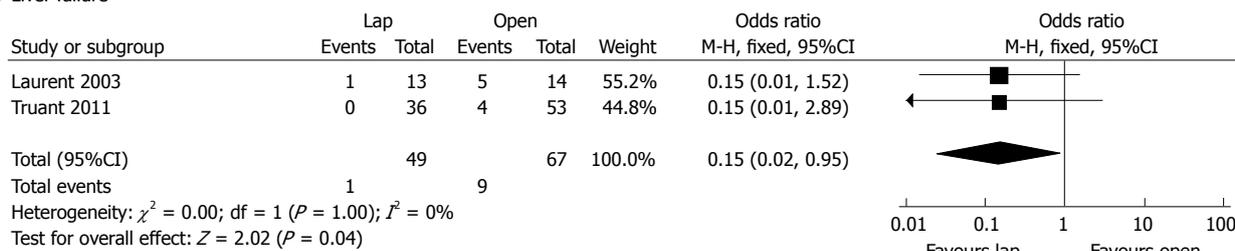
Intraoperative blood loss



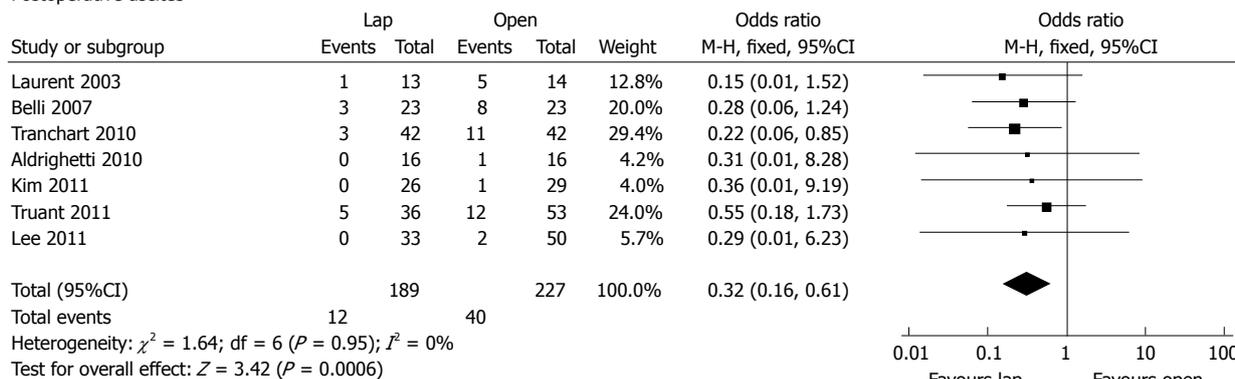
Blood transfusions requirement



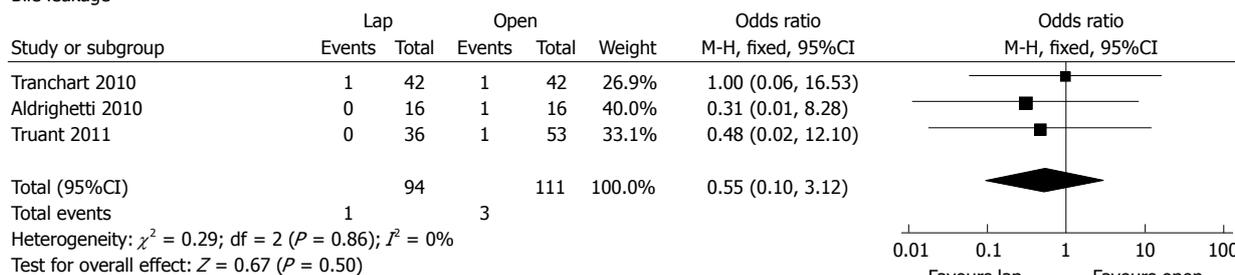
B Liver failure



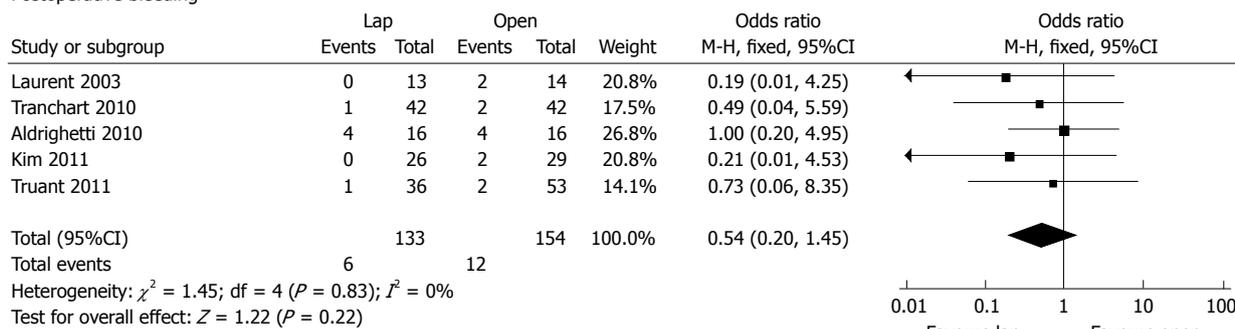
Postoperative ascites



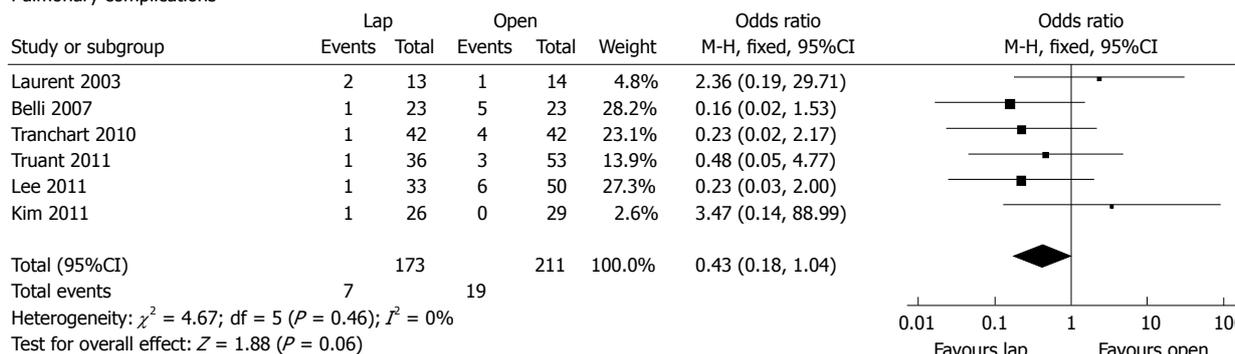
Bile leakage



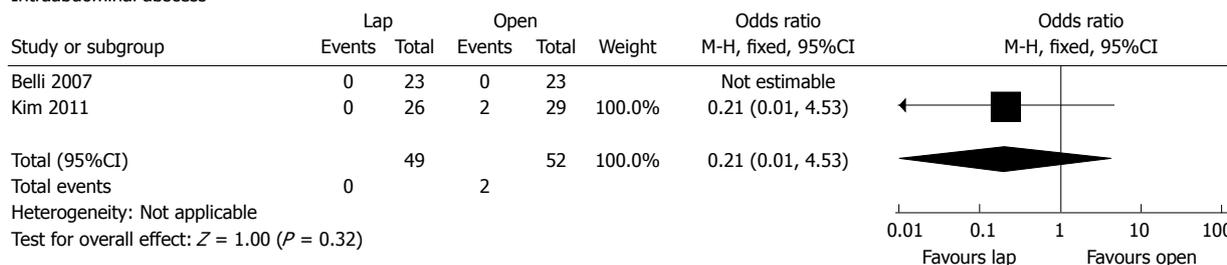
Postoperative bleeding



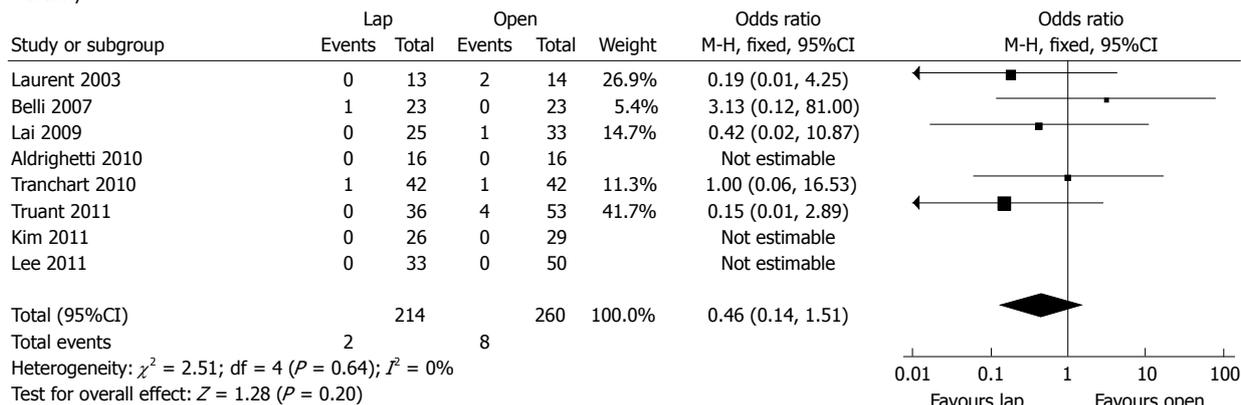
Pulmonary complications



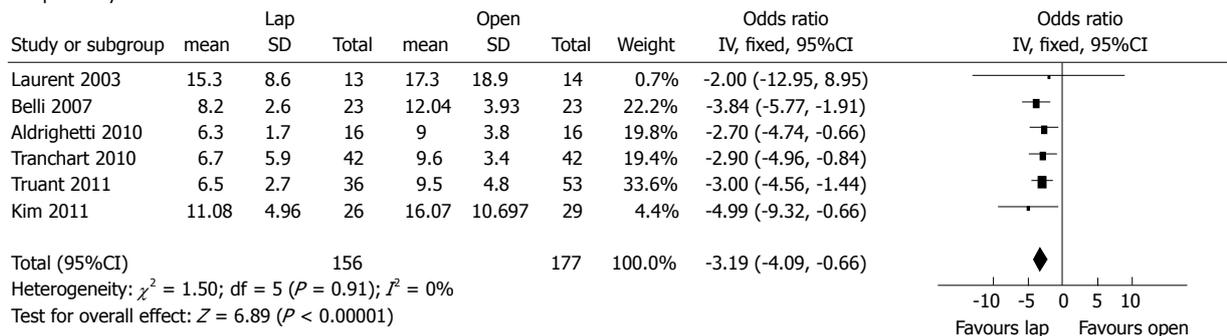
Intraabdominal abscess



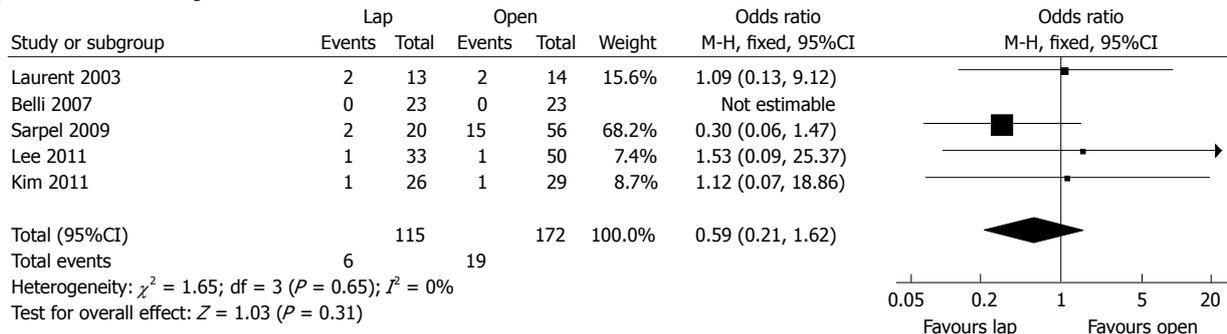
Mortality



Hospital stay



C Positive resection margins



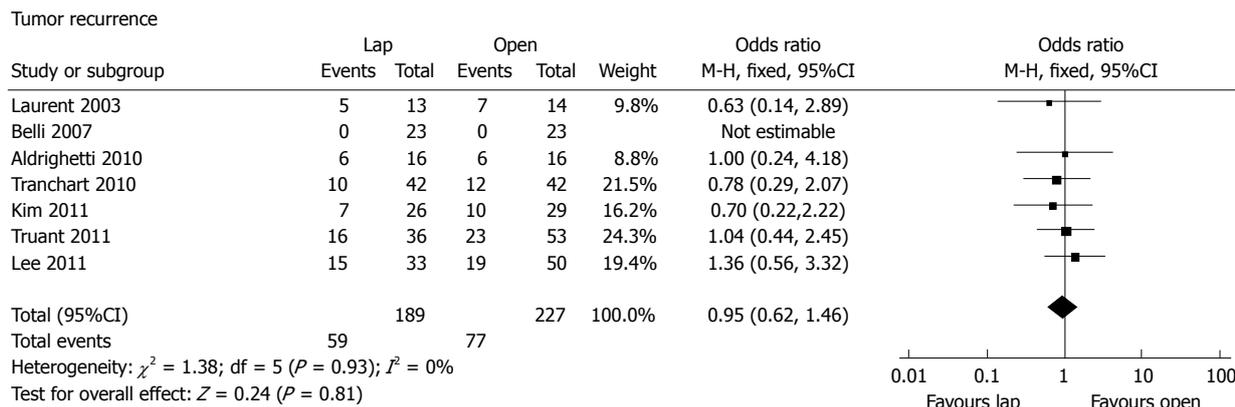


Figure 2 Forest plots demonstrating operative, postoperative and oncologic outcomes. A: Forest plots illustrating results of operative outcomes in the form of meta-analysis comparing laparoscopic vs open resection for hepatocellular carcinoma (high-quality studies only); B: Forest plots illustrating results of postoperative outcomes in the form of meta-analysis comparing laparoscopic vs open resection for hepatocellular carcinoma (high-quality studies only); C: Forest plots illustrating results of oncologic outcomes in the form of meta-analysis comparing laparoscopic vs open resection for hepatocellular carcinoma (high quality studies only). Pooled weighted mean difference or odds ratio with 95%CI was calculated using the fixed-effects or random effects model. IV: Inverse variance; M-H: Mantel-Haenszel.

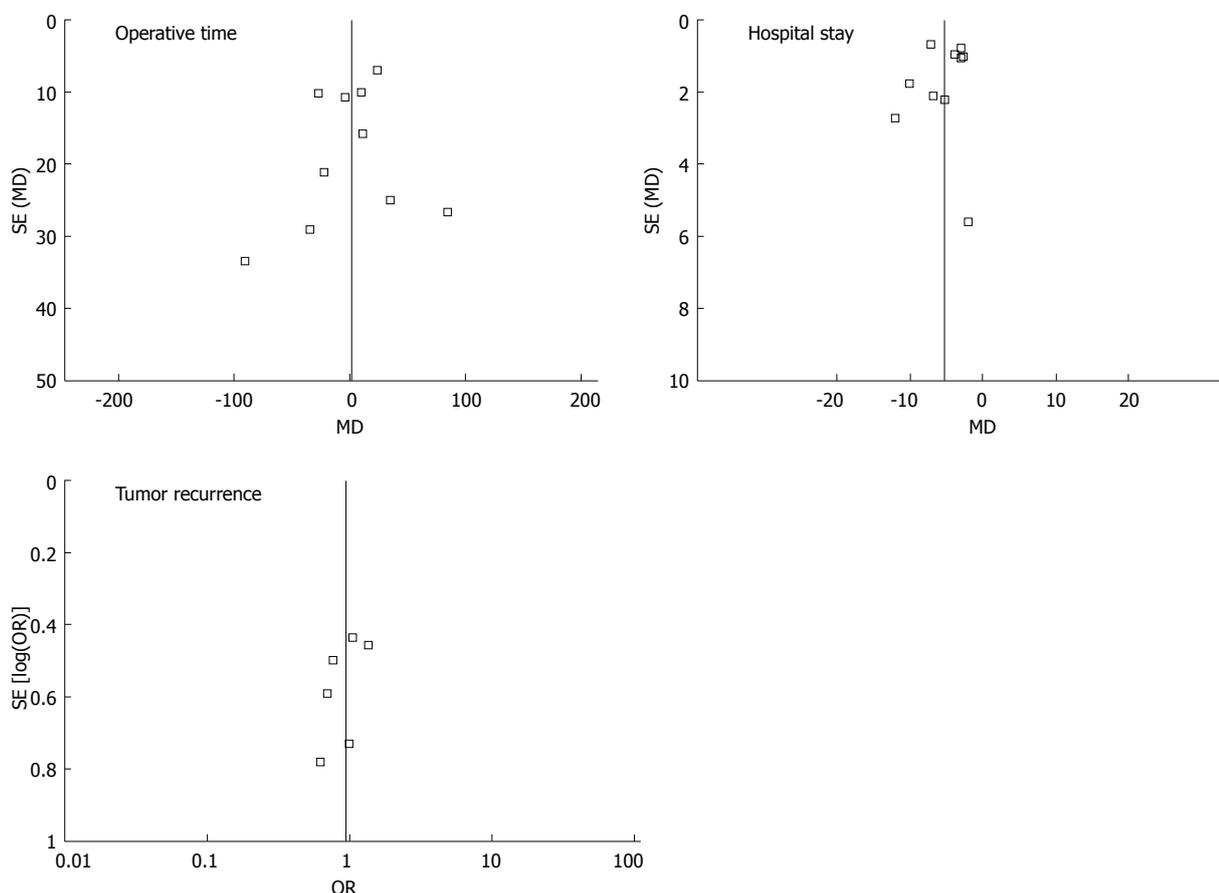


Figure 3 Funnel plot to investigate publication bias. The laparoscopic vs the open group: A funnel plot showing the operation time, hospital stay and tumor recurrence. OR: Odds ratio; MD: Mean difference.

mentation and technology, as well as surgeons' experience and learning curve^[39]. Our results demonstrate that LLR is associated with significantly less intraoperative blood loss and blood transfusion requirement, which can partly be explained by the hemostatic effect of pneumoperitoneum on the hepatic vein branches^[30,40] and also image magnification during LLR^[33]. There have been some re-

ports in literature indicating that significant intraoperative blood loss and blood transfusion are associated with recurrence and survival rates after resection of HCC^[41-43]. Hence reduced blood loss in LLR is favorable. Results from this meta-analysis also reveal a significant reduction in the postoperative hospital stay in the LLR group. These findings are consistent with laparoscopic procedures

where patients have faster ambulation, early oral intake and reduced analgesic requirements^[11,33].

There is growing evidence to suggest that LLR is associated with less postoperative morbidity particularly with regards to developing postoperative ascites and liver failure. The reduction in the incidence of postoperative ascites in LLR might be due to preservation of the abdominal wall collateral circulation, by avoiding long abdominal incisions and preservation of the round ligament, which may contain significant collateral veins, thereby reducing portal hypertension and intraoperative fluid requirements^[44]. Other favorable factors associated with LLR include less frequent mobilization and manipulation of the liver, reduced fluid requirements, decreased blood loss, early ambulation and oral food intake and reduced third space accumulation leading to hyperaldosteronism^[28,30,45-47].

Incomplete tumor resection with positive resection margins is perceived to be a potential disadvantage in LLR^[33]. However, our results reveal no significant difference in the margin positive rate between the LLR and OLR groups. Further analysis revealed no difference in recurrence between the two groups. These findings can be attributed to the use of intraoperative ultrasonography in LLR or OLR. Intraoperative ultrasonography is a sensitive tool for accurate identification of lesions and orientation of borders for non-tumorous tissue^[48,49]. The other consideration for laparoscopic resection of malignancies is the potential of peritoneal dissemination, or port-site metastasis^[50-52]. However we did not encounter any case of peritoneal dissemination or port-site metastasis in our analysis.

Although our analysis shows apparent advantages of LLR over OLR for HCC, it is important to highlight that most of the patients included in our meta-analysis underwent segmentectomy or subsegmentectomy for peripheral lesions located in the anterolateral segments of the liver. Although it is encouraging that our results have been consistent throughout the sensitivity analyses, this meta-analysis also has some limitations which should be considered when interpreting its results and warrants a discussion. Firstly, all of the studies included were non-randomized, retrospective trials, which inevitably add a degree of selection bias to the results and can lead to over/under estimation of the measured effect. Since factors such as tumor location, extent of liver cirrhosis and tumor size are important determinants of outcome, we matched the two groups based on these important factors to eliminate bias and improve the validity of our results^[53].

Secondly, we observed some heterogeneity in certain outcome measures. This might be explained by differences in surgical techniques, retrospective nature of the studies, and limited blinded outcome assessment in some of the trials. However investigation of heterogeneity using meta-regression was not possible due to small number of studies.

Thirdly, there was inconsistency in the definition of some outcomes in different studies, making it difficult

to pool the results together. Using standardized guidelines to report outcomes can potentially overcome this problem and would allow more studies to be included in meta-analyses, leading to more reliable conclusions.

Finally, it is important to note that surgeons' experience and volume of cases operated in a particular hospital may affect these outcome measures tremendously. Unfortunately, none of the studies included in this analysis provided details of these factors and therefore, we were unable to assess the effect in such settings. Future trials should carefully consider such stratification while designing their studies and interpreting their data.

In conclusion, the results of this comprehensive, high-quality up to date meta-analysis indicate that LLR is feasible and safe for the treatment of HCC. LLR should be performed in selected patients by expert surgeons in high volume centers. Further research by undertaking well designed, prospective randomized controlled trials can confirm the advantages of LLR for the management of HCC.

COMMENTS

Background

Laparoscopic liver resection (LLR) is an attractive treatment for liver benign tumor comparing with open liver resection (OLR) because of good cosmetic results and less trauma, but its role remains controversial when LLR is applied to hepatocellular carcinoma (HCC) because of a lack of high-quality randomized controlled trials in this area.

Research frontiers

In order to compare the safety and effectiveness between the LLR and OLR, the meta-analysis was used to evaluate operative, postoperative and oncologic outcomes of these two surgical methods for HCC in this study.

Innovations and breakthroughs

Although previous meta-analysis had compared the outcomes of these two surgical methods, which included a limited number of studies with fewer cases, many high-quality studies with more participants have been published since. Therefore, it is important to provide an up to date analysis of these outcomes. This meta-analysis reported that LLR had significant advantage over OLR in terms of intraoperative blood loss, blood transfusions requirement, hospital stay, postoperative ascites and liver failure compared with OLR for HCC. Meanwhile, incidences of operation time, bile leakage, postoperative bleeding, pulmonary complications, intra-abdominal abscess, mortality, positive resection margins and tumor recurrence were similar between LLR and OLR.

Applications

The results of this meta-analysis show that LLR appears to be a safe and feasible option for HCC in selected patients based on current evidence. Therefore, LLR may be an alternative treatment for HCC. However, the experience of the operating surgeon and volume of operated cases in a particular centre has to be taken into consideration.

Terminology

HCC is the fifth most common primary cancer worldwide with high malignant potential.

Peer review

The paper investigates the safety and effectiveness of LLR on HCC. The statistical analysis used in the study is appropriate and the results suggest that there are some advantages in LLR. This paper should be of interest to surgeons in the field of the hepato-biliary-pancreatic surgery worldwide.

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Small serotonin-positive pancreatic endocrine tumors caused obstruction of the main pancreatic duct

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Abstract

We report 2 cases of pancreatic endocrine tumors that caused obstruction of the main pancreatic duct (MPD). A 49-year-old asymptomatic man was referred to our institution because dilation of the MPD was revealed by abdominal ultrasonography (US). No tumor was detected by endoscopic ultrasonography, computed tomography (CT), and magnetic resonance imaging (MRI). The diameter of the MPD was > 20 mm at the body, and no dilation was noted at the head. Although malignancy was not confirmed through cytology or imaging, pancreatic cancer was strongly suspected. Pancreaticoduodenectomy was performed. Pathologi-

cal and immunohistochemical examination revealed a 5 mm × 3 mm serotonin-positive endocrine tumor. Fibrosis was present around the MPD and seemed to cause stricture. A 32-year-old asymptomatic man had elevated serum amylase, and US demonstrated dilation of the MPD. No tumor was detected by CT and MRI. Pancreatic cancer was suspected due to stricture and dilation of the MPD. Pancreatectomy of middle part of pancreas was performed. Pathological and immunohistochemical examination revealed a serotonin-positive endocrine tumor sized 5 mm × 4 mm. We report 2 cases of serotonin-positive pancreatic endocrine tumors that caused stricture of the MPD in spite of the small size of the tumor.

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Key words: Serotonin; Pancreatic endocrine tumor; Main pancreatic duct; Obstruction; Dilatation

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Ogawa M, Kawaguchi Y, Maruno A, Ito H, Nakagohri T, Hirabayashi K, Yamamuro H, Yamashita T, Mine T. Small serotonin-positive pancreatic endocrine tumors caused obstruction of the main pancreatic duct. *World J Gastroenterol* 2012; 18(45): 6669-6673 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i45/6669.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i45.6669>

INTRODUCTION

Pancreatic endocrine tumors, also known as islet cell tumors, are rare neoplasms and occur in approximately

1 of 100 000 people, representing 1% to 10% of all pancreatic neoplasms^[1-3]. Some pancreatic endocrine tumors release hormones into the blood stream that cause clinical syndromes, whereas others are non-syndromic and present as mass lesion^[4,5]. The overall prevalence of functional pancreatic endocrine tumors is reported to be approximately 10/1 000 000. In contrast, the prevalence of pancreatic endocrine tumors reported by autopsy studies is higher (0.5% to 1.5%)^[6]. Multi-detector row computed tomography (CT) plays an important role in the diagnosis and staging of both syndromic and non-syndromic pancreatic endocrine tumors. In general, syndromic pancreatic endocrine tumors are less than 3 cm in size. They are typically strongly enhanced and usually best seen on CT scans obtained during the arterial phase. Compared to syndromic pancreatic endocrine tumors, non-syndromic pancreatic endocrine tumors tend to be larger at presentation and are more likely to be cystic or necrotic^[7]. In previous studies, endoscopic retrograde pancreatography (ERP) has been used to detect main pancreatic duct (MPD) stenosis and dilation of the upstream pancreatic duct caused by pancreatic endocrine tumors^[8,9]. In addition, pancreatic endocrine tumors with serotonin production is suggested to be associated with prominent stromal fibrosis, which can extend to the MPD, causing ductal stenosis and upstream dilatation of the duct and/or upstream pancreatic atrophy^[10,11]. We recently encountered 2 cases of small pancreatic endocrine tumors that revealed serotonin-positive by immunohistochemical staining causing obstruction of the MPD.

CASE REPORT

Case 1

A 49-year-old asymptomatic man was referred to our institution because dilatation of the MPD was revealed by abdominal ultrasonography (US). However, no tumor was observed by endoscopic ultrasonography (EUS), CT, and magnetic resonance imaging (MRI). The diameter of the MPD was > 20 mm at the body and no dilation was noted at the head. Endoscopic retrograde cholangiopancreatography (ERCP) was performed; the MPD showed crab-like appearance (Figure 1). Cytology was benign. Although we could not confirm malignancy through cytology or imaging, pancreatic cancer was strongly suspected. Pancreaticoduodenectomy was performed, and pathological examination revealed a 5 mm × 3 mm tumor. Fibrosis was present around the MPD and seemed to cause stricture (Figure 2).

Immunohistochemical staining of tumor samples was positive for chromogranin A, synaptophysin, and serotonin (Figure 3). The MIB-1 (Ki-67) labeling index was less than 1%. We made the diagnosis of neuroendocrine tumor of grade 1 (NET G1).

Case 2

A 32-year-old man with no symptom had elevated serum amylase, and US demonstrated dilation of the MPD. No

tumor but MPD stenosis and dilatation was revealed by MRI. EUS demonstrated presence of a small pancreatic tumor. ERCP revealed MPD stenosis and upstream dilatation (Figure 4). Cytology was benign.

Pancreatectomy of middle part of pancreas was performed and pathological examination revealed an endocrine tumor sized 5 mm × 4 mm. Tumors with stromal fibrosis can cause stenosis of the pancreatic duct. Immunohistochemical staining of the tumor cells was positive for chromogranin A, synaptophysin, and serotonin (Figure 5). The Ki-67 labeling index was less than 1%, and we made the diagnosis of NET G1.

DISCUSSION

Pancreatic endocrine neoplasms that produce serotonin, including carcinoid tumors, account for only a small portion of pancreatic endocrine neoplasms. Compared with other well-differentiated endocrine neoplasms of the pancreas, pancreatic carcinoid tumors are associated with a higher rate of malignant behavior^[12]. These pancreatic neoplasms may have the poor prognosis since they are usually found at an advanced stage, after distant metastases have occurred, and the patient has developed carcinoid syndrome. The problem is that the mass lesion is often asymptomatic and indistinct at early stages. In addition, Shi *et al.*^[11] reported that in serotonin-producing tumors, the neoplasm was subtle or unapparent on CT images; only marked dilatation of the upstream pancreatic duct or marked atrophy of the upstream pancreas was visible. Isolated reports of an association of pancreatic carcinoid tumor with dilatation of the pancreatic duct have been described before. Nagai *et al.*^[10] reported a case of pancreatic carcinoid tumor with obstructive pancreatitis. ERP revealed MPD stenosis and dilatation of the upstream pancreatic duct. They suggested that the pancreatic carcinoid tumor obstructing the pancreatic duct might have arisen from argentaffin cells located in the MPD. However, no clear relationship between pancreatic endocrine neoplasms and pancreatic duct stenosis has been described. Takaji *et al.*^[13] reported that 3 of 4 cases showed MPD dilatation upstream of the tumor. Our 2 cases were similar to these reports; the tumors were subtle on CT or MRI images and a markedly dilated MPD was noted, and they had no symptoms as carcinoid syndrome. We decided to perform the pancreatic resection because there was a possibility of pancreatic duct dilation was caused by a tumor. As a result, we could treat the pancreatic endocrine tumor in early stage. We observed that the serotonin immunoreactivity correlated with the degree of stromal fibrosis and that stromal fibrosis caused MPD stenosis. Carcinoid tumors of the midgut, in which serotonin is the predominant hormone secreted by neoplastic cells, are usually associated with extensive fibrosis^[14]. In addition, serotonin has been shown to stimulate fibroblast mitosis in cell cultures^[15]. Recently, serotonin has been shown to play a crucial role in the progression of liver fibrosis by enhancing

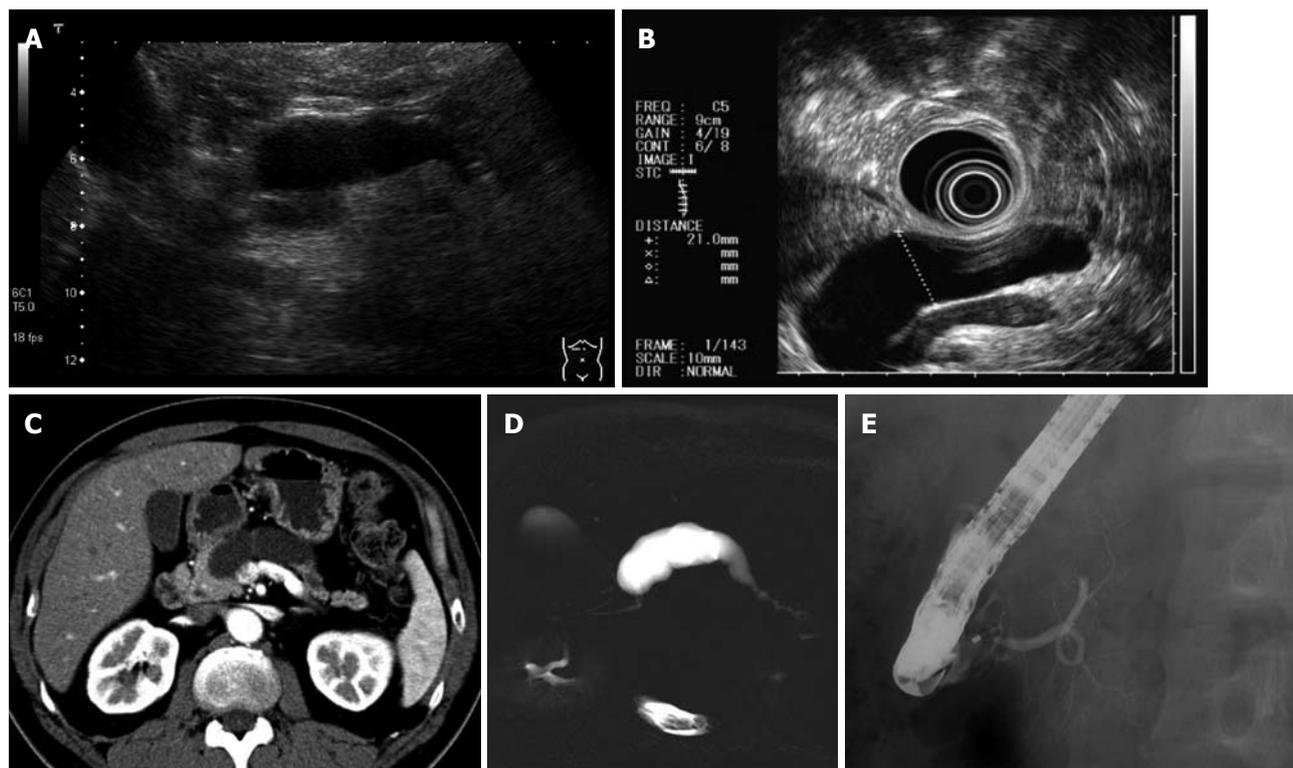


Figure 1 No tumor was detected by endoscopic ultrasonography, computed tomography and magnetic resonance imaging. The diameter of the main pancreatic duct was > 20 mm at the body. A: Ultrasonography; B: Endoscopic ultrasonography; C: Computed tomography; D: Magnetic resonance cholangiopancreatography; E: Endoscopic retrograde pancreatography.

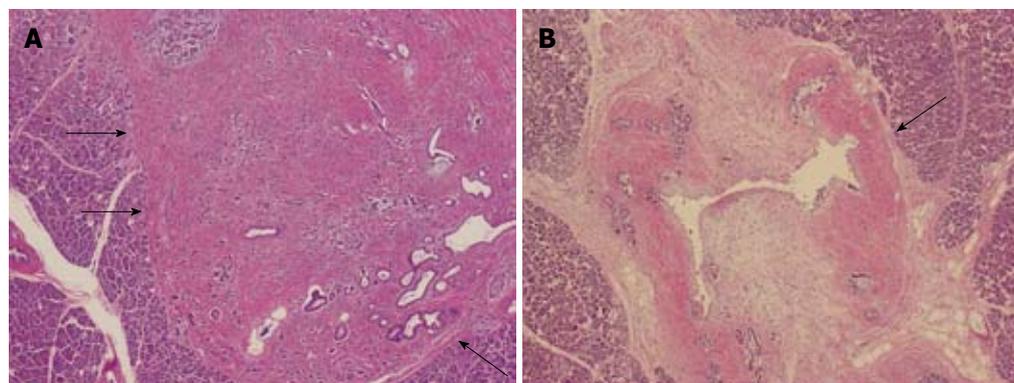


Figure 2 Pathological findings (hematoxylin/eosin staining). A: A 5 mm \times 3 mm tumor was detected (arrows); B: Fibrosis was present around the main pancreatic duct (arrow), and it seemed to cause stricture.

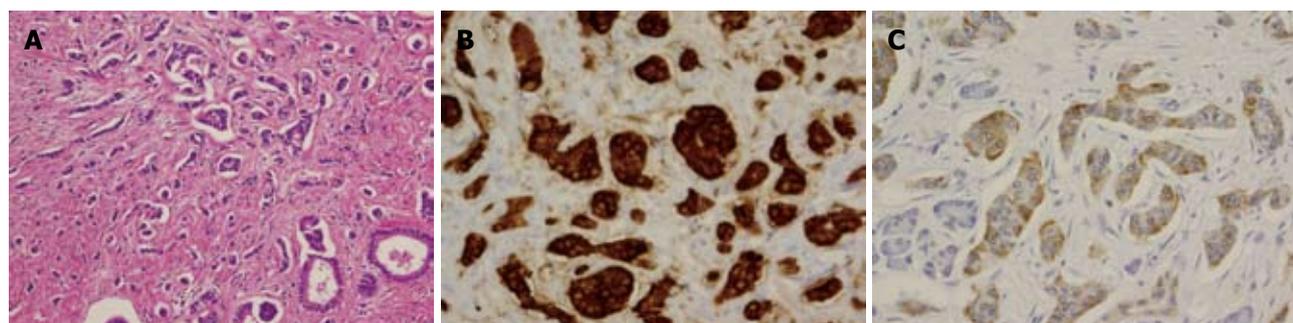


Figure 3 Immunohistochemical staining was positive for chromogranin A, synaptophysin and serotonin. A: Hematoxylin/eosin staining; B: Chromogranin A; C: Serotonin. Ki-67 labeling index was less than 1%.

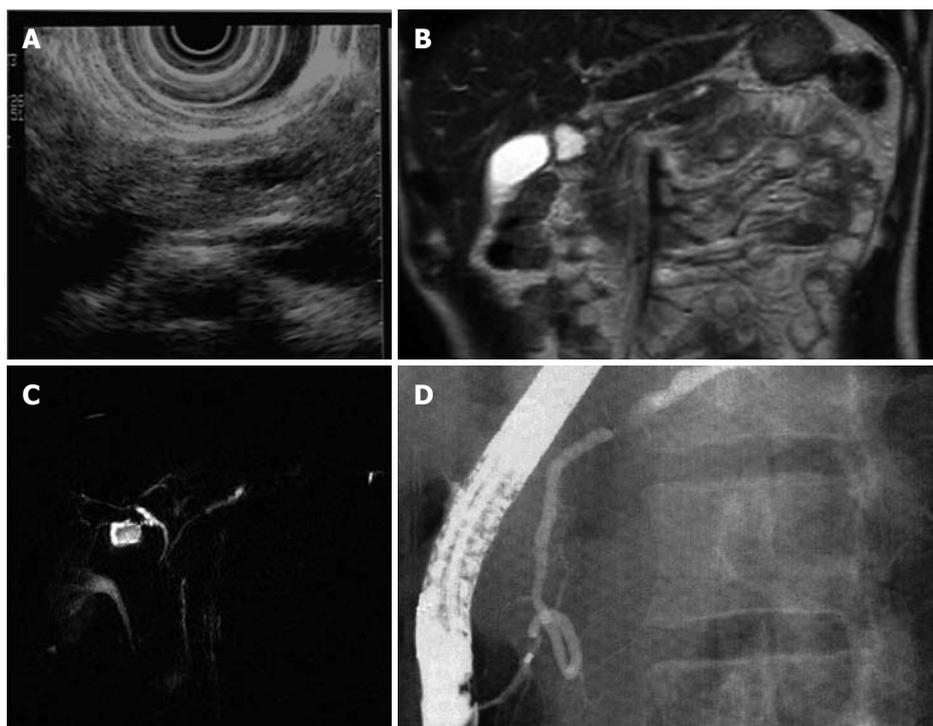


Figure 4 Dilation of the main pancreatic duct. Endoscopic ultrasonography was demonstrated presence of a small pancreatic tumor. A: Ultrasonography; B: Magnetic resonance imaging; C: Magnetic resonance cholangiopancreatography; D: Endoscopic retrograde pancreatography.

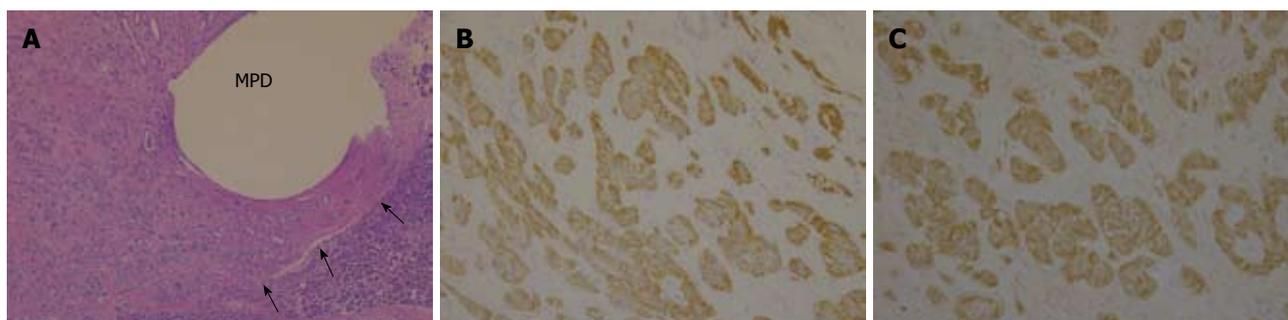


Figure 5 Pathological findings (hematoxylin and eosin staining) and immunohistochemical staining. A: A 4 mm × 5 mm tumor was detected (arrows); B: Chromogranin A; C: Serotonin. MPD: Main pancreatic duct.

the production of transforming growth factor β via selective activation of the 5-HT_{2A} serotonin receptors^[16]. Shi *et al*^[11] reported that a small portion of pancreatic endocrine neoplasms produces serotonin. Serotonin production may be associated with stromal fibrosis, and fibrosis, in turn, can cause stenosis of the pancreatic duct. In clinical practice, imaging findings of pancreatic duct stenosis that is out of proportion to the size of the causative strongly enhanced mass or that does not have an associated distinct mass are indicative of a pancreatic neoplasm. Recently, it was reported that many pancreatic NETs express high levels of somatostatin receptors, so somatostatin-receptor scintigraphy (OctreoScan) can be imaged with a radiolabeled form of the somatostatin analog octreotide. Somatostatin receptor scintigraphy has proven particularly effective for visualizing gastrinomas, glucagonomas, and nonfunctioning pancreatic tumors. However, in another study of 37 patients with a NET, MRI and CT were substantially superior to SRS for detection of liver metastases. The sensitivity of Oc-

treoScans was found to be particularly poor (< 35 percent) in lesions smaller than 1.5 cm in diameter^[17].

In conclusion, we report 2 cases of serotonin-positive pancreatic endocrine tumors that caused stricture of the MPD in spite of the small size of the tumor. It is necessary to consider that the MPD stenosis and upstream dilatation caused by the tiny pancreatic endocrine tumor as the differential diagnosis in addition to chronic pancreatitis and pancreatic cancer.

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Partial stent-in-stent placement of biliary metallic stents using a short double-balloon enteroscopy

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Abstract

Endoscopic intervention is less invasive than percutaneous or surgical approaches and should be considered the primary drainage procedure in most cases with obstructive jaundice. Recently, therapeutic endoscopic retrograde cholangiopancreatography (ERCP) using double-balloon enteroscopy (DBE) has been shown to be feasible and effective, even in patients with surgically altered anatomies. On the other hand, endoscopic partial stent-in-stent (PSIS) placement of self-expandable metallic stents (SEMSs) for malignant hilar biliary obstruction in conventional ERCP has also been shown to be feasible, safe and effective. We performed PSIS placement of SEMSs for malignant hilar biliary obstruction due to liver metastasis using a short DBE in a patient with Roux-en-Y anastomosis and achieved technical and clinical success. This procedure can result in quick relief from obstructive jaundice in a single session

and with short-term hospitalization, even in patients with surgically altered anatomies.

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Key words: Double-balloon enteroscopy; Malignant hilar biliary obstruction; Self-expandable metallic stent; Partial stent in stent; Roux-en-Y anastomosis

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INTRODUCTION

Endoscopic intervention is less invasive than percutaneous or surgical approaches and should be considered the primary drainage procedure in most cases with obstructive jaundice. Recently therapeutic endoscopic retrograde cholangiopancreatography (ERCP) using double-balloon enteroscopy (DBE) has been shown to be safe and feasible, even in patients with surgically altered anatomies^[1-4]. On the other hand, the placement of biliary stents is effective for the palliation of unresectable malignant hilar biliary obstruction in conventional ERCP^[5-8]. In particular, as we previously described, endoscopic partial stent-in-stent (PSIS) placement of self-expandable metallic

stents (SEMSs) for malignant hilar biliary obstruction has been shown to be feasible, safe and effective^[7,8], but it can be technically challenging. We report a case of a postoperative surgical patient who was managed successfully with a PSIS placement of SEMSs for malignant hilar biliary obstruction using a short DBE.

CASE REPORT

A 63-year-old male underwent total gastrectomy with Roux-en-Y reconstruction and sigmoidectomy due to simultaneous gastric and sigmoid colon cancer. Despite treatment with adjuvant chemotherapy, the patient's liver and lymph node metastases increased and caused obstructive jaundice, but no cholangitis. Computed tomography imaging showed dilation of the left intrahepatic bile duct due to liver metastasis, which occupied the right lobe (Figure 1A). For endoscopic biliary drainage, endoscopic retrograde cholangiography with a short DBE, EC-450BI5 (Fujifilm, Tokyo, Japan), was performed. The cholangiography revealed hilar biliary obstruction and a dilated left intrahepatic bile duct with tumor invasion extending to the bifurcation of the left lateral sectional bile duct branches (Figure 1B). After needle-knife sphincterotomy, a 0.035-inch guidewire was passed selectively into the left lateral superior bile duct branch (B3). The first uncovered SEMS (Zeostent 10 mm × 80 mm; Zeon Medical Inc., Tokyo, Japan) was deployed, with the proximal end in B3 and the distal end in the common bile duct. The guidewire remained in place, and the delivery system was removed. Subsequently, the wire was passed by catheter into the left lateral inferior bile duct branch (B2) through the mesh of the initial SEMS. Following balloon dilation (8 mm) at the stricture (Figure 2A), the second uncovered SEMS (Zeostent 10 mm × 100 mm) was smoothly deployed, with the proximal end in B2 through the mesh of the initial SEMS, forming a PSIS (Figure 2B). The patient was immediately relieved of jaundice and left our hospital in 7 d. He recovered enough to receive another round of chemotherapy on an outpatient basis.

DISCUSSION

In patients with surgically altered anatomy and long afferent limbs, ERCP by gastroenteroscopy, colonoscopy, or standard duodenoscopy is technically challenging and often unsuccessful because of an inability to reach the papilla or bilioenteric anastomosis. Recently, the use of a DBE or single-balloon enteroscopy has made therapeutic ERCP-including sphincterotomy, stone extraction, dilation of bilioenteric anastomotic stricture, and biliary stent placement-feasible and effective, even in patients with surgically altered anatomies^[1-4].

According to a recent report on endoscopic intervention for the relief of malignant hilar biliary obstruction, the placement of SEMSs offers advantages over plastic endoprostheses in terms of stent patency and the number of reinterventions needed^[5]. In addition, endoscopic PSIS

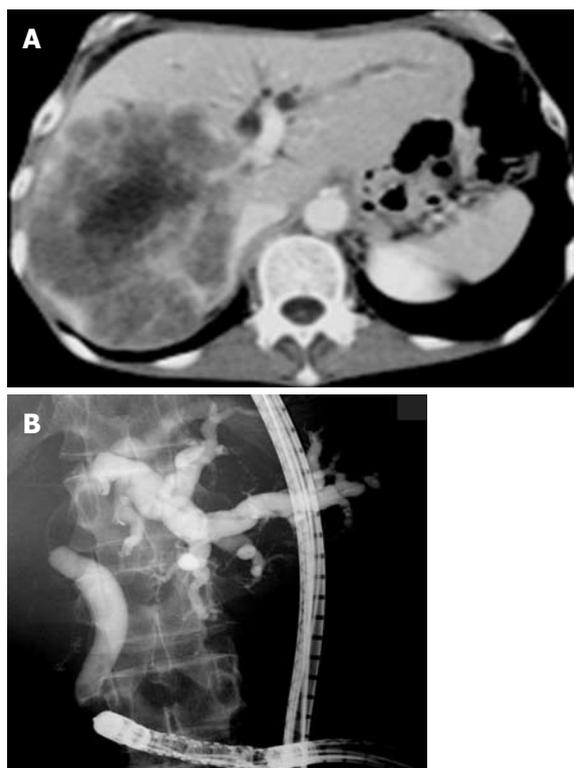


Figure 1 The hilar biliary obstruction due to liver metastasis occupying the right lobe and a dilated left intrahepatic bile duct with tumor invasion extending to the bifurcation of the lateral bile duct branch. A: Computed tomography image; B: Cholangiography.

placement of SEMSs for malignant hilar biliary obstruction has been shown to be feasible, safe and effective in conventional ERCP^[5-8]. We previously reported that this procedure is safe and effective even in cancer patients receiving chemotherapy^[8].

Therefore, in this case of a cancer patient with Roux-en-Y anastomosis, we used a short DBE to perform PSIS placement of SEMSs for malignant hilar biliary obstruction and achieved technical and clinical success. Almost all conventional accessories, including uncovered SEMS, were available, as we used a short DBE with a working channel of 2.8 mm in diameter and a 152 cm in length.

Percutaneous stent insertion for malignant obstructive jaundice had significantly higher 30-d mortality than the endoscopic method (33% *vs* 15%, $P = 0.016$) in a randomized trial^[9]. Complications related with percutaneous transhepatic biliary drainage (PTBD), including intraperitoneal hemorrhage, hemobilia, bile leakage, and pleural complications, can be avoided by using endoscopic drainage^[10]. In our cases, 2 PTBD routes would have been required for the placement of 2 SEMSs at B2 and B3, respectively. In addition, 2 sessions would have been required for the placement of the SEMSs, that is, the SEMSs are usually placed one week after the initial PTBD. The endoscopic procedure could protect our patient from the risks associated with more invasive drainage procedures, such as PTBD and surgical drainage, the latter of which is associated with high morbidity and mortality rates. Furthermore, the patient needed no further long-term hospitalization

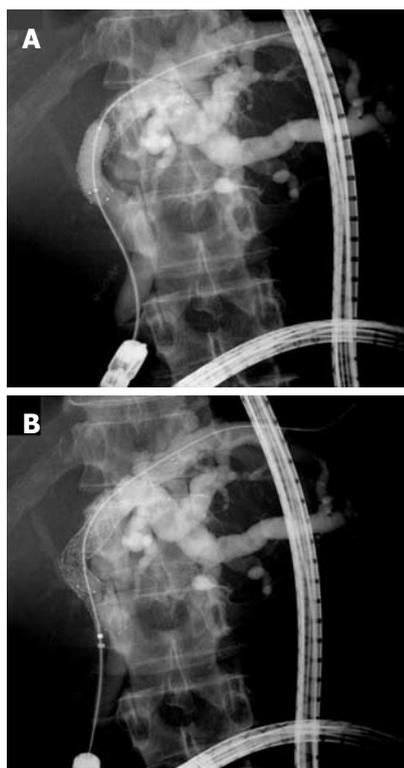


Figure 2 A partial stent-in-stent placement of biliary metallic stents using a short double-balloon enteroscopy. A: Following the placement of the first self-expandable metallic stent (SEMS), balloon dilation was performed at the stricture; B: The second SEMS was deployed through the mesh of the initial SEMS.

for the treatment of obstructive jaundice for the duration of his life. We think that this procedure is also indicated for patients in whom PTBD cannot be performed for various reasons, such as patients with severe coagulopathy, thrombocytopenia, a large amount of ascites, or an anatomically inaccessible location, e.g., patients with Chilaiditi syndrome.

In conclusion, endoscopic PSIS placement of SEMSs for the treatment of malignant hilar biliary obstruction using a short DBE was proved to be feasible and effective

in a patient with Roux-en-Y anastomosis. This procedure can result in quick relief from obstructive jaundice in a single session and with short-term hospitalization, even in patients with surgically altered anatomies.

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Plasmablastic lymphoma of the small intestine: Case report and literature review

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Abstract

Plasmablastic lymphoma (PBL) is a rare aggressive B-cell lymphoproliferative disorder, which has been characterized by the World Health Organization as a new entity. Although PBL is most commonly seen in the oral cavity of human immunodeficiency virus (HIV)-positive patients, it can also be seen in extra-oral sites in immunocompromised patients who are HIV-negative. Here we present a rare case of PBL of the small intestine in a 55-year-old HIV-negative male. Histopathological examination of the excisional lesion showed a large cell lymphoma with plasmacytic differentiation diffusely infiltrating the small intestine and involving the surrounding organs. The neoplastic cells were diffusely positive for CD79a, CD138 and CD10 and partly positive for CD38 and epithelial membrane antigen. Approximately 80% of the tumor cells were positive for Ki-67. A monoclonal rearrangement of the

kappa light chain gene was demonstrated. The patient died approximately 1.5 mo after diagnosis in spite of receiving two courses of the CHOP chemotherapy regimen. In a review of the literature, this is the first case report of PBL with initial presentation in the small intestine without HIV and Epstein-Barr virus infection, and a history of hepatitis B virus infection and radiotherapy probably led to the iatrogenic immunocompromised state.

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Key words: Plasmablastic lymphoma; Small intestine; Human immunodeficiency virus; Differential diagnosis

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INTRODUCTION

Plasmablastic lymphoma (PBL) is a distinct, aggressive B-cell neoplasm that shows diffuse proliferation of large neoplastic cells resembling B-immunoblasts with an immunophenotype of plasma cells^[1]. It was originally described in the oral cavity in the clinical setting of human immunodeficiency virus (HIV) infection, but may occur in other, predominately extra-oral sites, and these have been reflected in the current/revised 2008 World Health Organization classification^[2]. Extra-oral PBL has been reported in various locations^[3-6]; however PBL of

the small intestine was extremely rare. Here we present a rare case of PBL of the small intestine in an HIV-negative individual and review the literature.

CASE REPORT

A 55-year-old man presented with generalized abdominal pain, distension and vomiting for over a 1-mo period. He also described recent weight loss and anorexia. The above pathologic condition gradually became worse without relief of symptoms. Abdominal ultrasonography revealed multiple masses in the small intestine with extension to the bilateral adrenal glands. Radiographs and computed tomography (CT) scans of the chest, oral and peri-oral sites showed no abnormalities, and no peripheral lymphadenopathy was noted. Routine blood examination showed hemoglobin 106 g/L, white blood cell count 6.48×10^9 /L, neutrophils 64.4%, lymphocytes 15.3%, monocytes 10.2%. Serological testing demonstrated positivity for hepatitis B virus (HBV) surface antigen and HBV e-antigen, and negativity for HIV. The lactate dehydrogenase level (216 U/L) was in the normal range. Serum examination for Bence Jones' protein and rheumatoid factor were negative. The medical history was that he had a history of infection of HBV and underwent resection of squamous cell carcinoma in the maxillary sinus, followed by two courses of radiotherapy 7 mo previously.

During surgery, multiple tumor nodules were found in the intestinal wall and mesentery of the small intestine. Surrounding organs, including adrenal glands, liver, inferior vena cava and lumbar vertebrae were involved. Tumors were excised with adjacent portions of the small intestine and bilateral adrenal glands. A 55-cm long segment of the small bowel was obtained from the resection procedure. By gross examination, there was an irregular tumor nodule, volume 10 cm × 8 cm × 6 cm, in the intestinal wall surrounding the whole enteric cavity. Multiple tumor nodules ranging from 3.5 cm to 5.5 cm in diameter could be found in the adjacent mesentery. Tumor nodules in the left and right adrenal glands measured 8.5 cm × 8 cm × 4 cm and 8 cm × 5 cm × 2.5 cm respectively. The cut surface of these tumors was grey and soft. Selected tumor tissues were fixed in formalin and embedded in paraffin and cut into sections which were stained with the hematoxylin and eosin for routine histology. Additional sections of paraffin-embedded tissue were used for immunohistochemical staining and *in situ* hybridization analysis.

Histologically, the whole intestinal wall was diffusely infiltrated by malignant lymphocytes with abundant basophilic cytoplasm, eccentrically located pleomorphic nuclei, and single, centrally located prominent nucleoli (Figure 1A). The nuclei were round or oval or convoluted in shape and a large number of mitotic figures were apparent (Figure 1B). There were tumor emboli within the lymphatic vessels. Comprehensive necrosis of tumor cells was conspicuous. The tumor cells were scattered

throughout the small intestine wall into the peripheral soft tissue and adrenal glands forming tumor nodules of different sizes. Furthermore, tumor cell proliferation in lymph node sinuses was conspicuous in the mesenteric lymph nodes.

On immunohistochemistry, the neoplastic cells were diffusely positive for CD79a (Figure 1C), CD138 (Figure 1D) and CD10 (Figure 1E), and partly positive for CD38 and epithelial membrane antigen. These cells were negative for CD45, CD20, CD3, CD30, ALK-1, Bcl-2, Bcl-6, Mum-1, CD56, HMB45, S-100, CD34, CD117 and cytokeratin. The cells showed kappa light chain restriction (Figure 1F). Nuclear proliferation rate, as assessed by Ki-67 staining, was approximately 80% (Figure 1G). Epstein-Barr virus (EBV) infection was not detected by *in situ* hybridization for EBV-encoded RNA (Figure 1H) or immunohistochemistry for EBV latent membrane protein-1. On the basis of these morphologic and immunohistochemical characteristics, the pathological diagnosis of PBL was made.

After surgery, the patient was treated with two courses of a standard CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) chemotherapy regimen. Unfortunately, during the second course of chemotherapy, repeat CT scans and ultrasonography revealed tumor progression with an increase in swollen cervical lymph nodes and abdominal tumor load. The patient died of multiple organ failure due to further deterioration 1.5 mo later.

DISCUSSION

As a rare entity of non-Hodgkin B cell lymphoma, PBL was first described as a specific clinicopathologic entity by Delecluse *et al*^[7] as an aggressive B-cell lymphoma occurring in the oral cavity arising in the context of HIV infection. However, in recent years, several cases of PBL have been reported in patients without HIV infection, and several more have reported the occurrence of PBL in extra-oral sites, including the skin, subcutaneous tissue, stomach, anal mucosa or perianal area, lung, lymph node, and other regions^[3-6,8,9]. The small intestine is a rare extra-oral site of involvement in PBL patients, and only two cases in HIV-infected patients have been reported previously^[10,11]. In a review of the literature, this is the first case report of PBL with initial presentation in the small intestine and with multiple organ involvement in an HIV-negative individual.

As PBL is often associated with immunodeficiency, such as HIV infection, EBV plays an important role in the tumorigenesis of HIV-associated PBL. HIV infection creates a permissive environment for chronic EBV infection, with a subsequent latency that predisposes the EBV transformed B-cells to become malignant^[12]. There is likely a connection between HIV-induced immunosuppression and the development of EBV-associated PBL^[13]. However, recent investigation revealed that EBV infection was detected in only 17% of HIV-negative PBL cases, which suggest that this virus may not be a

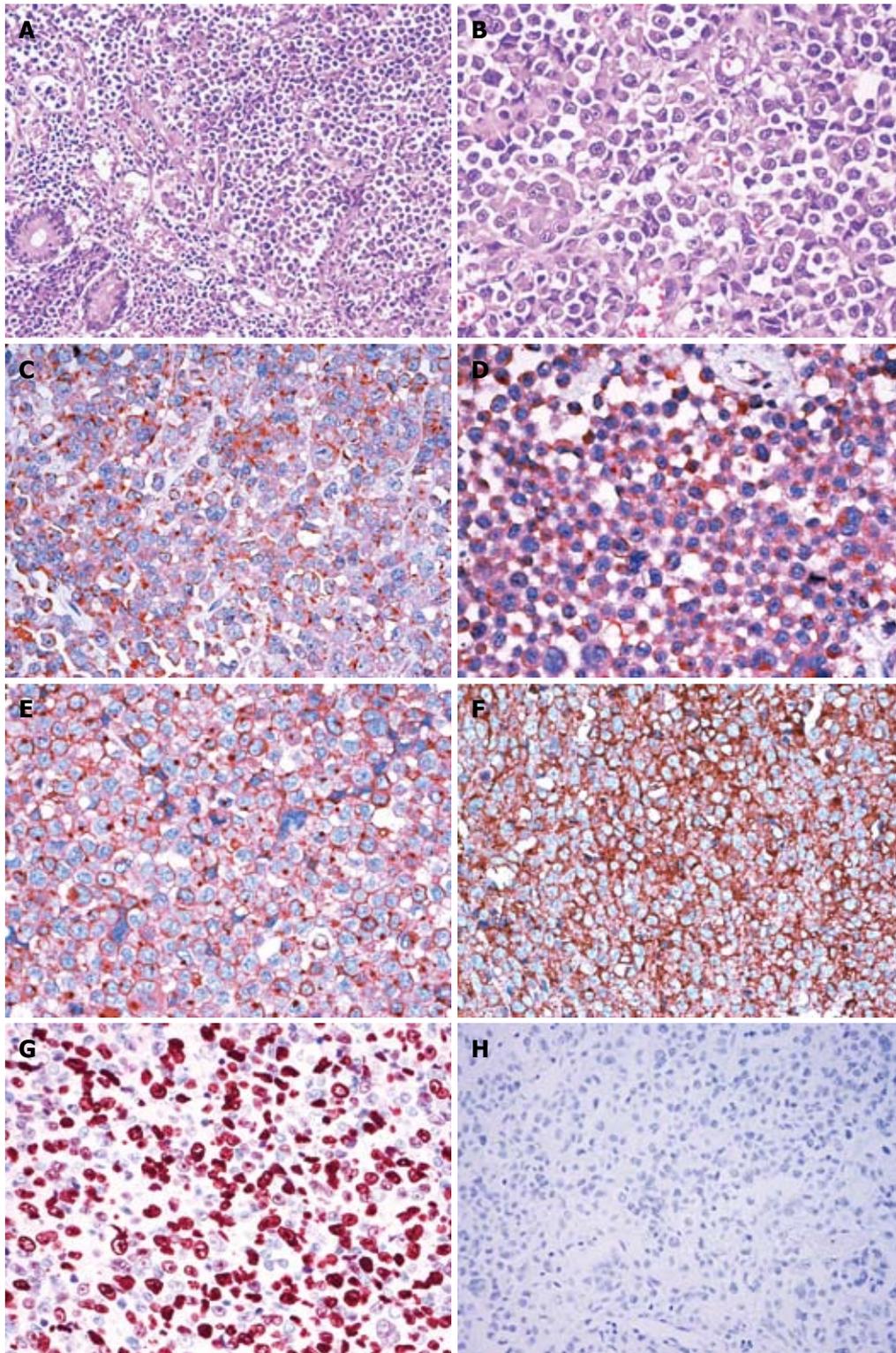


Figure 1 Plasmablastic lymphoma of the small intestine. A: Diffuse infiltration in the mucosa of the small intestinal by monotonous large atypical lymphoid cells [hematoxylin and eosin (H and E), original magnification $\times 200$]; B: These atypical cells had a plasmablastic appearance, with abundant basophilic cytoplasm, eccentrically located pleomorphic nuclei, and single, centrally located prominent nucleoli (H and E, original magnification $\times 400$); C-F: The atypical cells were diffusely positive for CD79a (C), CD138 (D), CD10 (E) and immunoglobulin light chain κ (F) (immunoperoxidase stain, original magnification $\times 400$); G: Nuclear proliferation rate as assessed by Ki-67 staining was approximately 80% (immunoperoxidase stain, original magnification $\times 400$); H: Epstein-Barr virus (EBV)-encoded RNA *in situ* hybridization for EBV shows negative staining in the nucleus of these atypical cells (original magnification $\times 400$).

unique participant in the pathogenesis of PBL, especially in patients without HIV infection^[14]. Cases of HIV-negative PBL have been mostly described after solid organ transplantation, in association with steroid therapy for autoimmune disease and some other types of immunosuppression^[15,16]. The present patient was negative for HIV, and EBV infection was not detected by immunohistochemistry or *in situ* hybridization analysis, so an HBV infection and the history of radiotherapy probably

led to the iatrogenic immunocompromised state.

The pathological differential diagnosis of PBL in the present case mainly included poorly differentiated primary or metastatic carcinoma, malignant melanoma, gastrointestinal stromal tumor (GIST), diffuse large B-cell lymphoma (DLBCL), Burkitt's lymphoma, anaplastic large cell lymphoma (ALCL), and plasmacytoma^[3]. The characteristic morphology and immunophenotype of the tumor cells in conjunction with clinical features aid

Table 1 Differential diagnosis of plasmablastic lymphoma by immunohistochemistry

Marker	Current case	PBL	DLBCL	ALCL	BL	PC	GIST	Carcinoma	Melanoma
CD45	-	±	+	+	+	±	-	-	-
CD20	-	±	+	-	+	-	-	-	-
CD79a	+	±	+	-	+	+	-	-	-
CD38	+	+	-	-	-	+	-	-	-
CD138	+	+	-	-	-	+	-	-	-
CD3	-	-	-	±	-	-	-	-	-
CD30	-	-	±	+	-	-	-	-	-
CD10	+	±	±	-	+	-	-	-	-
ALK-1	-	-	±	±	-	-	-	-	-
Bcl-2	-	-	+	-	-	-	-	-	-
Mum-1	-	±	±	-	-	+	-	-	-
EMA	+	±	-	+	-	-	-	+	-
HMB45	-	-	-	-	-	-	-	-	+
S-100	-	-	-	-	-	-	-	-	+
CD34	-	-	-	-	-	-	+	-	-
CD117	-	-	-	-	-	-	+	-	-
Cytokeratin	-	-	-	-	-	-	-	+	-
LMP-1	-	±	±	-	-	-	-	-	-
EBER	-	±	±	-	±	-	-	±	-

PBL: Plasmablastic lymphoma; DLBCL: Diffuse large B cell lymphoma; ALCL: Anaplastic large cell lymphoma; BL: Burkitt's lymphoma; PC: Plasmacytoma; GIST: Gastrointestinal stromal tumor; +: Positive; -: Negative; ±: Variable expression.

in the differential diagnosis (Table 1). Poorly differentiated carcinoma may be differentiated from PBL according to its consistent immunological staining for cytokeratin. Malignant melanoma can be ruled out by using S-100 protein and HMB45. GIST is a common kind of mesenchymal tumor in the small intestine, usually characterized by expression of CD34 and CD117. In terms of negative expression for CD20 and morphological appearance, the present case of PBL can be clearly identified with other aggressive B-cell lymphomas, such as DLBCL and Burkitt's lymphoma. Histologically, the tumor resembled extranodal ALCL; however, tumor cells in ALCL are consistently immunoreactive for CD30 and usually immunoreactive for CD3 and ALK, which is different from our case. The differential diagnosis between PBL and poorly differentiated plasmacytoma is based mostly on clinical correlations, as both have similar morphological and phenotypic features^[14]. The negative presence of serum monoclonal proteins and a high Ki-67/MIB-1 proliferation index help in the differential diagnosis from plasmacytoma in the present case.

In terms of clinical behavior, PBL is highly aggressive, with most of the patients dying in the first year after diagnosis. Most patients are at an advanced stage (III or IV) at presentation^[15]. HIV-positive and HIV-negative patients with PBL have different clinicopathological characteristics, including a better response to chemotherapy and longer survival in HIV-positive patients. HIV-negative patients had a median overall survival of 9 mo *vs* 14 mo in HIV-positive patients^[13]. Furthermore, extra-oral PBL is more commonly disseminated (57% of the patients are at stage IV) at diagnosis. Meanwhile, the loss of CD20 associated with plasmacytic differentiation and very high Ki-67 index (> 90%) conveyed a worse prognosis^[9]. In our case, the primary PBL of the small intestine had in-

filtrated multiple organs, accordingly a clinical stage IV was assigned. The HIV-negative state, extra-oral location, absent expression of CD20 and relatively high nuclear proliferation index in tumor cells collectively contributed to the more aggressive clinical course and worse outcome of this case.

In summary, we report a rare case of extra-oral PBL involving the small intestine in a HIV-negative patient, and describe the histologic and immunophenotypic findings. A diffuse infiltrative growth, rapid mitotic rate, and necrosis are consistent with the classification of PBL as a high-grade malignant lymphoma. Because PBL does not express the more common lymphoid and/or B-cell markers, it is easy to mistake them for a poorly differentiated carcinoma or sarcoma. Thus, diagnosis of PBL is challenging, particularly when it arises in extraoral locations and in immunocompetent patients. Recognition of this entity by the pathologist and clinician is important in establishing the correct diagnosis and treatment of the patients.

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Neuroendocrine carcinoma of the pancreas with soft tissue metastasis

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Abstract

Neuroendocrine carcinoma (NEC) of the pancreas is rare. We report the case of a 34-year-old man with pancreatic NEC with soft tissue metastasis. The patient presented with right upper abdominal discomfort. Computed tomography revealed a low-density heterogeneous mass in the tail and body of the pancreas that encroached on the greater curvature of the stomach and spleen. We performed exploratory laparotomy and total pancreatectomy with splenectomy and total gastrectomy. Histopathological analysis showed spindle-shaped cells with scanty cytoplasm and hyperchromatic nuclei, confirming a primary pancreatic NEC. One month after the surgery, the patient experienced leg swelling. Positron emission tomography-computed tomography revealed high uptake of fludeoxyglucose in the left leg, and the leg was amputated. Histopathological analysis confirmed metastasis of pancreatic NEC. The patient was followed up and received chemotherapy (etoposide and cisplatin). One month after amputation, the level of tumor marker neuron-specific enolase was 142.70 $\mu\text{g/L}$ and computed tomography scan revealed an aggravated metastatic lesion. The patient suffered from unbearable

pain and we treated him with odynolysis. Four months postoperatively, the patient died of respiratory failure.

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Key words: Neuroendocrine carcinoma; Pancreas; Soft tissue metastasis; Neuron-specific enolase; Positron emission tomography-computed tomography

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INTRODUCTION

Primary neuroendocrine carcinoma (NEC) of the pancreas is very rare, accounting for only 1%-1.4% of all pancreatic cancers^[1,2]. Almost all NECs of the pancreas are discovered when the tumor is fairly large (mean: 6.2 cm, range: 2.5-20 cm) and it has metastasized to several distant organs such as the liver, adrenal gland, and brain, which explains the dismal prognosis^[3]. We report a rare route of metastasis in this case.

CASE REPORT

We report a case of pancreatic NEC with soft tissue metastasis. The patient was a 34-year-old man who had no significant past medical history. He visited our hospital on January 1, 2012 with the symptom of right upper abdominal discomfort. A computed tomography (CT) scan revealed a low-density heterogeneous mass of 81

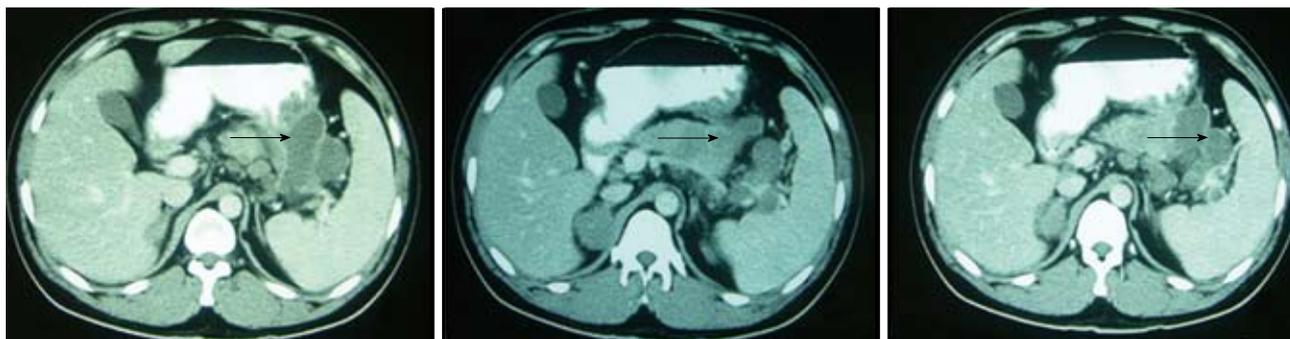


Figure 1 A computed tomography scan revealed a low-density heterogeneous mass of 81 mm × 68 mm in size in the tail of the pancreas (arrow) that invaded the greater curvature of the stomach and the spleen.

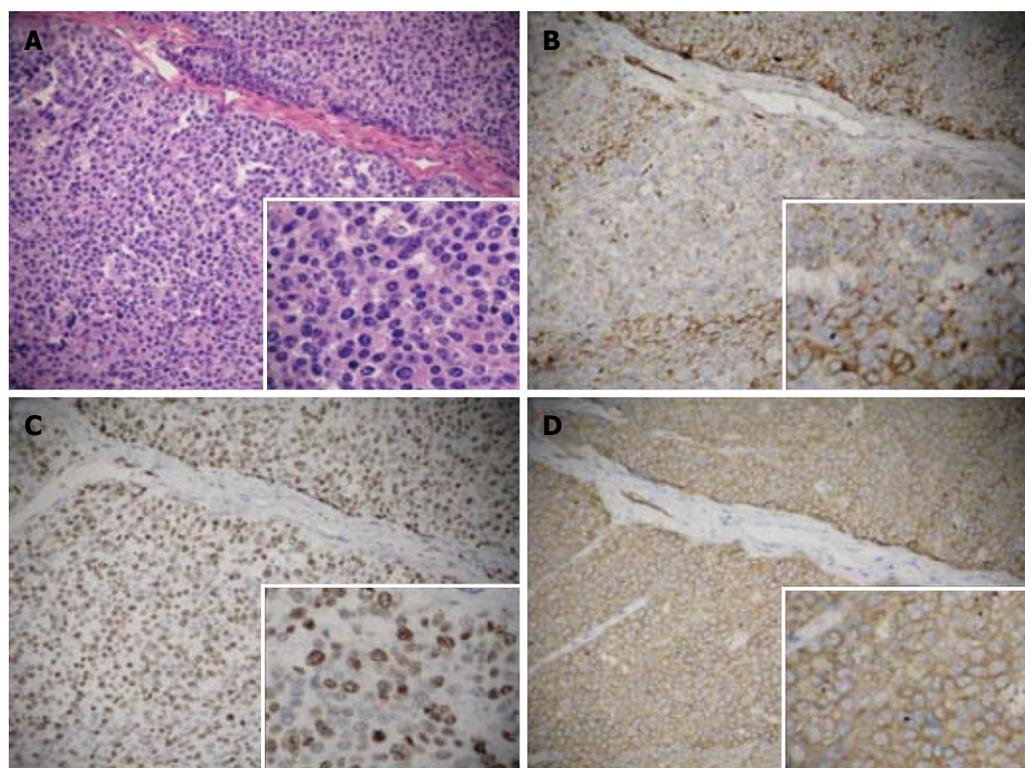


Figure 2 Pathology of the pancreas. A: Spindle-shaped cells with scanty cytoplasm and hyperchromatic nuclei (hematoxylin and eosin staining); B: Positively stained for chlorhexidine A; C: Approximately 80% of the tumor cells were positively stained for Ki67; D: Tumor cells were positively stained for synaptophysin (B, C and D: EnVision) (original magnification: ×200 and ×400).

mm × 68 mm in size in the tail and body of the pancreas that invaded the greater curvature of the stomach and the spleen (Figure 1). The laboratory findings were as follows: hemoglobin, 102 g/L; white blood cell count, 6.5×10^9 /L; platelets, 374×10^9 /L; aspartate aminotransferase, 18 U/L; alanine aminotransferase, 13 U/L; total bilirubin, 9.1 μ mol/L; direct bilirubin, 4.0 μ mol/L; serum creatinine, 59 μ mol/L; carcinoembryonic antigen, 0.86 ng/mL (normal, < 10.0 ng/mL); alpha-fetoprotein, 2.37 ng/mL (normal, < 13.40 ng/mL); and carbohydrate antigen 19-9, 101.7 U/mL (normal, < 27 U/mL). The serum neuron-specific enolase (NSE) level was 59.94 μ g/L (normal, < 17 μ g/L). Chest X-ray examination revealed no signs of primary lung cancer or metastasis. In addition, there was no evidence of liver metastasis; therefore, exploratory laparotomy was performed. During the abdominal exploration, a 1-cm mass was detected in the head of the pancreas, and an 8-cm mass was de-

tected in the pancreatic tail. The identified mass invaded the greater curvature of the stomach and the spleen. Consequently, we performed total pancreatectomy with splenectomy and total gastrectomy.

Histological examination revealed spindle-shaped cells with scanty cytoplasm and hyperchromatic nuclei. In addition, 9/12 lymph nodes were positive for metastasis. Hematoxylin and eosin staining (Figure 2) was performed on the paraffin-embedded sections. Immunohistochemical examination revealed chromogranin A and Ki-67 positivity.

One month after surgery, the patient exhibited leg swelling. Positron emission tomography-CT revealed high fludeoxyglucose uptake in the left leg and the relapse of carcinoma in both hila of the lungs (Figure 3). An orthopedist obtained a biopsy of the left leg, and the frozen section results indicated NEC. Therefore, the left leg of the patient was amputated below the knee. The



Figure 3 Positron emission tomography-computed tomography revealed high fludeoxyglucose uptake in the left leg and the relapse of carcinoma in both hila of the lungs.

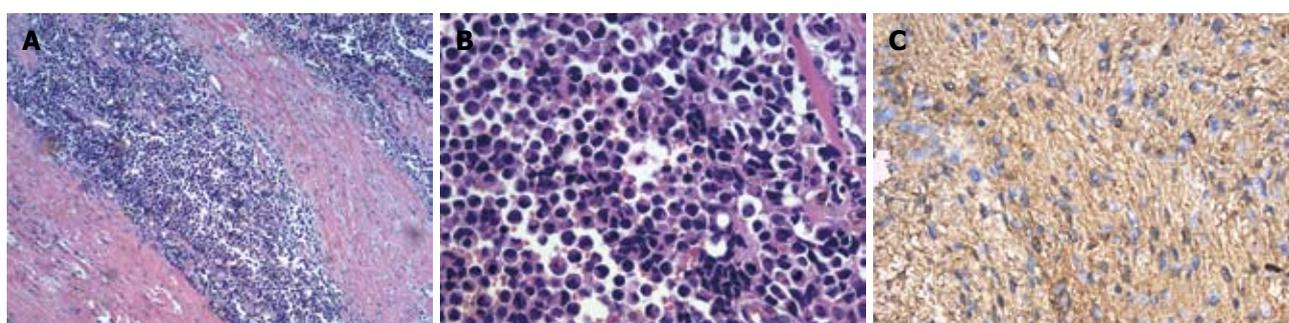


Figure 4 Pathology of the leg. A: Tumor cells displayed infiltrative growth in the striated muscle of the left leg [hematoxylin and eosin (HE) staining, original magnification: $\times 100$]; B: Tumor cells were homogeneous (HE staining, original magnification: $\times 400$); C: Tumor cells were positively stained for synaptophysin (EnVision, original magnification: $\times 400$).

postoperative pathology of the left leg was metastatic NEC of the pancreas (Figure 4). The patient was followed up, and he received chemotherapy consisting of etoposide and cisplatin.

One month after amputation, the level of the tumor marker NSE was $142.70 \mu\text{g/L}$, and a CT scan revealed an aggravated metastatic lesion. The patient reported unbearable pain, and he was treated by odynolysis. Four months postoperatively, he died of respiratory failure.

DISCUSSION

In a review of all published cases of pancreatic NEC, 91% of patients had metastases at the time of the initial diagnosis. According to the report by Vos *et al*^[4], the most common sites of metastasis are the peripancreatic lymph nodes (62%), liver (38%), lungs (14%), bone marrow (14%), bone (10%), colon (10%), and adrenal gland (10%); rarer sites of metastasis include the spleen, gallbladder, kidneys, skin, and brain.

NSE can be considered a tumor marker that can be used in the diagnosis or assessment of treatment efficacy in patients with pancreatic NEC^[5,6]. In our case, NSE was continuously aggravated.

Pancreatic NEC is a rare type of pancreatic cancer that has a poor prognosis^[7]. The clinical course is typically aggressive, often characterized by disseminated disease at presentation and poor survival. In patients with

extensive disease, a median survival as short as 2 mo has been reported, whereas in patients with limited disease, a median survival of up to 34 mo has been described^[8]. Min Sung Chung reported a case of primary pancreatic NEC with unusually long-term survival after multimodal therapy. This patient remains in good health 36 mo after surgery^[9]. In our case, the tumor extended beyond the pancreas with regional lymph node involvement. Because there was extension beyond the locoregional boundaries (extensive disease), we could perform palliative surgery. The patient in our case survived 4 mo on combined chemotherapy.

Some trials have revealed improved oncologic outcomes when patients are treated with regimens similar to those used for small cell cancers of the lungs^[10]. The regimen of cisplatin, etoposide, and radiation is generally favored for pancreatic NEC^[11]. This is the first reported case of pancreatic NEC with soft tissue metastasis. As mentioned previously, pancreatic NEC is a rare type of pancreatic cancer that has a poor prognosis. These adjuvant approaches should be considered in addition to surgery.

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Sister Mary Joseph's nodule as a first sign of pancreatic cancer

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Abstract

Sister Mary Joseph's nodule (SMJN) refers to a metastatic tumor of the umbilicus. It is a rare entity which arises from a malignancy in the intra-abdominal cavity. We herein describe a patient who presented with SMJN as his first sign of pancreatic cancer. It is an even more unusual case of SMJN. We therefore, suggest that pancreatic cancer should be included in the differential diagnosis when an umbilical mass is found. With the progress made in surgical procedures and other modalities, an early diagnosis will dramatically improve the prognosis of the patients.

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Key words: Sister Mary Joseph's nodule; Pancreatic cancer; Umbilical metastasis; Diagnosis; Management

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INTRODUCTION

Umbilical tumors are rare and 30% of them are metastatic^[1]. Sister Mary Joseph's nodule (SMJN) refers to a metastatic lesion of the umbilicus originating from intra-abdominal or pelvic malignant diseases. The most common primary sites of the metastasis are stomach, ovaries, colon, rectum and pancreas. Pancreas accounts for 7%-9% of the SMJN cases^[2]. Almost 90% of the cases arise from the body and tail of the pancreas, but not the head^[2].

We herein describe a male patient with pancreatic cancer who presented with SMJN as his first clinical sign. We also reviewed the published literatures on this disease from PubMed. Only 20 cases (including our case) of SMJN with pancreatic cancer as the first symptom and sign have been reported in English. The aim of this report is to provide new insight into the identification of pancreatic cancer presenting as SMJN.

CASE REPORT

The patient is a 40-year-old man who presented with

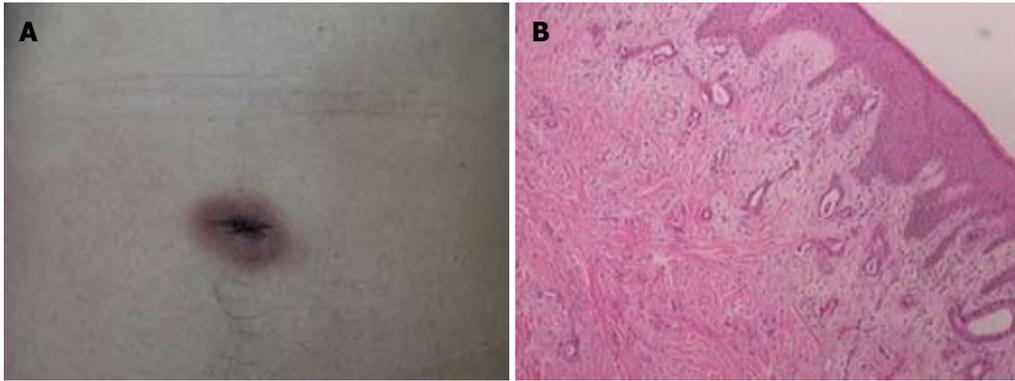


Figure 1 Appearance and pathology of umbilical tumor. A: A red nodule without ulcer appears in the umbilical region; B: Hematoxylin and eosin stain shows adenocarcinoma cell infiltration ($\times 40$).

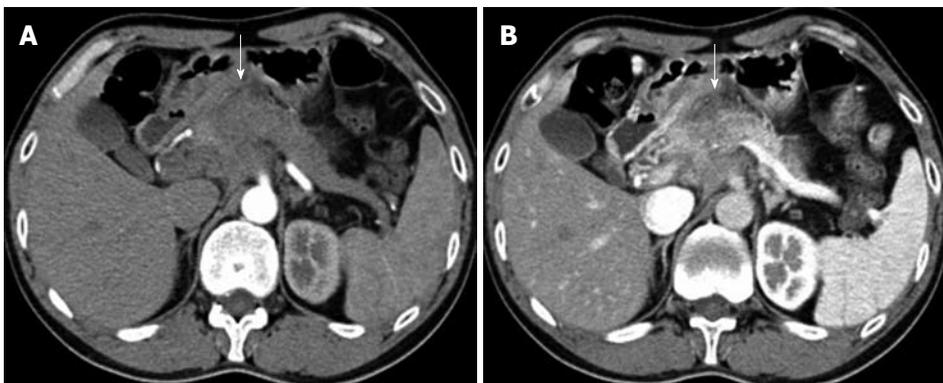


Figure 2 Abdominal contrast-enhanced multi-detector computed tomography scan. A: A mass in the neck of pancreas (arrow) is shown in arterial phase; B: The mass in venous phase.

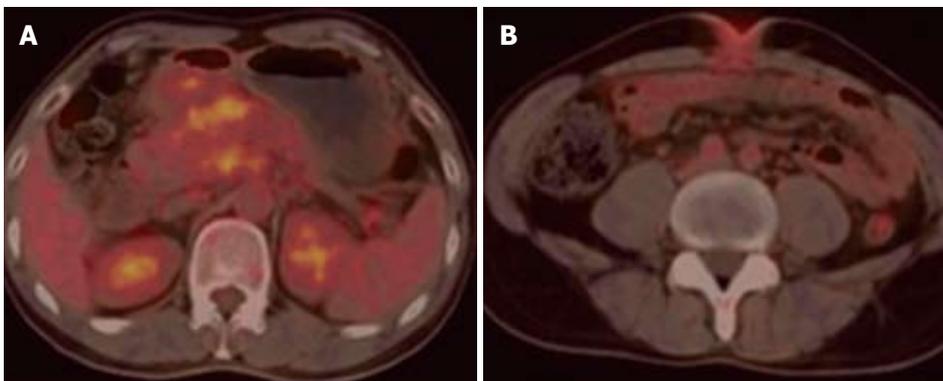


Figure 3 Positron emission tomography-computed tomography showed increased fludeoxyglucose uptake of tumors. A: Significant enhanced signal showing increased fludeoxyglucose (FDG) uptake at the site of pancreatic mass; B: Increased FDG uptake at the site of umbilical nodule.

swelling, redness and induration at umbilicus. The patient was first diagnosed with eczema at a local hospital and was treated without improvement. After six months, the patient consulted a dermatologist at our hospital. Physical examination showed an inflamed umbilicus with a nodule of 2 cm \times 1.5 cm without ulcer (Figure 1A). An umbilical skin biopsy was taken and sent for pathologic examination. The pathology was suggestive of adenocarcinoma infiltrating dermis and epidermis (Figure 1B). Immunohistochemistry (IHC) showed cytokeratin (CK) (AE1/AE3) +++, carcinoembryonic antigen +++, and CK7 +. A metastatic adenocarcinoma was suspected based on all these findings.

Serum marker of carbohydrate antigen 19-9 (CA19-9) was elevated (202.1 U/mL). Other tumor markers were all in the normal range. An abdominal multi-detector

computed tomography (MDCT) revealed a tumor at the neck of the pancreas with retroperitoneal lymph nodes enlargement as well as an umbilical soft tissue mass (Figure 2). An abdominal computed tomography (CT) angiography also showed involvement of retroperitoneal lymph nodes, superior mesenteric artery and vein, celiac trunk, proximal splenic artery, splenic vein and part of portal vein. A fluorodeoxyglucose (FDG) positron emission tomography-CT (PET-CT) scan demonstrated a mass located at the neck and body of pancreas with increased FDG uptake, with a maximal standardized uptake value (SUV) of 3.69, and thus malignancy was suspected. The umbilical mass with a maximal SUV of 2.85 was also detected and a metastatic malignancy was diagnosed by PET-CT (Figure 3).

Preoperative abdominal CT angiography showed the

tumor encasing celiac trunk and superior mesenteric artery, which, however, did not cause the occlusion or the stricture of these arteries. According to our previous experience, the patient may still have a chance of resection. An exploratory laparotomy was then performed. Mild abdominal ascites was present. A hard mass of 8 cm at the neck and body of pancreas was discovered, with invasion of celiac trunk, superior mesenteric artery, superior mesenteric vein and portal vein. Several enlarged mesenteric lymph nodes and an umbilical mass of 2 cm × 1.5 cm as well as tiny diffused seeding nodules were observed. Diaphragmatic tumor invasion was visible. A biopsy was taken and a frozen section was sent for pathology. Adenocarcinoma infiltrating the striated diaphragmatic muscle tissues was confirmed. Surgical resection was abandoned because of distant metastasis of the cancer. The patient refused chemotherapy, and died four months later.

DISCUSSION

When a clinician observes an umbilical mass, his/her differential diagnosis should include both benign and malignant tumors. Benign tumors can be caused by a number of factors, including polyps, papilloma, myoma, fibroma, hemangioma, dermoid cyst, teratoma, pyogenic granuloma, omphalith, or endometriosis^[1]. Malignant lesions account for 38% of all umbilical tumors, including both primary and secondary cancers^[1]. These can be differentiated by means of biopsy collection and histopathological examination^[3]. Cytology can not distinguish between primary and secondary malignancies^[4].

The incidence of metastatic tumor originating from intra-abdominal malignancy to the umbilicus (SMJN) is very low. The most common primary sites of SMJN in men are stomach, followed by colon, rectum, pancreas and others^[5]. In women, the most common origins are the ovaries, followed by stomach, colon, rectum, pancreas and others^[2]. Pancreas is the fourth or fifth most common primary site of SMJN. The percentage of SMJN arising from pancreas ranges from 7% to 9%, among which SMJN presenting as the first sign of pancreatic cancer is even unusual^[2,5]. To our knowledge after literature review, only 20 such cases, including our case, have been reported in English^[6-11].

The metastatic routes of the pancreatic cancer to the umbilicus are thought to be by direct invasion from the peritoneum, through lymphatic or blood vessels and along embryonic remnants^[6]. Generally, the prognosis of pancreatic cancer with SMJN is poor. The mean survival is 6-11 mo^[6]. However, with early diagnosis and surgical intervention, a prolonged survival of 18 years was reported^[6]. Currently, with the improvement of surgery, the resection rate of advanced pancreatic cancer has been increased. Moreover, with advances made in multidisciplinary therapeutic modalities including chemotherapy, radiotherapy, targeted molecular therapy, immunotherapy, and others, the survival of the patients has

also been improved.

It has been reported that resection of SMJN and the primary cancer of pancreas combined with other therapeutic modalities has improved the prognosis of the patients^[9]. Therefore, an appropriate diagnostic algorithm is very important for early detection and rational treatment.

As mentioned above, for patients with umbilical nodules as the first clinical manifestation, biopsy should be performed first if the diagnosis is uncertain^[3,4]. Almost all the cases of SMJN from pancreatic cancer reported in the literature are adenocarcinomas^[4]. IHC staining for CK 7 and 19 are very important in diagnosing adenocarcinomas of pancreas^[5]. Serum tumor markers such as CA19-9 should be examined and elevation of CA19-9 is considered as a strong evidence of the disease. Once SMJN deriving from pancreatic cancer is suspected, contrast-enhanced MDCT or magnetic resonance imaging (MRI) on pancreas should be done. Contrast-enhanced MDCT or MRI is sensitive enough to display the pancreas mass and its involvement in the adjacent organs and vessels. PET-CT is also recommended to detect other distant metastasis^[12,13]. However, PET-CT is not sensitive to the identification of tiny seeding nodules in the intra-abdominal cavity. As in this case, PET-CT failed to display intra-abdominal diffusion.

An earlier diagnosis could have caught the tumor in its early phase, hence improving the prognosis of the patient. This paper is to remind physicians and surgeons of the importance of keeping in mind pancreatic cancer as one of their initial differential diagnosis when an umbilical nodule is presented. The recommended diagnostic algorithm is hopefully useful for the early detection of pancreatic cancer which appears as SMJN as its first sign.

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Malakoplakia of the esophagus caused by human papillomavirus infection

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Abstract

Malakoplakia is a rare granulomatous disease probably caused by infection and characterized histologically by Michaelis-Gutmann bodies. We report a more rarely seen case esophageal malakoplakia in a 54-year-old woman. She presented with coughing while eating and drinking. Gastroscopy showed yellow nodules in the esophagus, and endoscopic ultrasonography showed a space-occupying lesion in the substratum of the esophageal mucosa. All findings highly resembled esophageal cancer. Histopathological examination finally identified this space-occupying lesion as malakoplakia and not cancer. Immunohistochemistry showed that she had human papillomavirus (HPV) infection in the esophagus, which indicates that infection was responsible for the malakoplakia. This is believed to be the first case of malakoplakia in the esophagus, and more importantly, we established that HPV infection was the initiator of esophageal malakoplakia.

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Key words: Malakoplakia; Esophagus; Michaelis-Gut-

mann bodies; Human papillomavirus infection

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INTRODUCTION

Malakoplakia is a rare granulomatous disease that was first described by Michaelis and Gutmann^[1] in 1902, and is characterized by Michaelis-Gutmann bodies with cytoplasmic concentric laminated inclusions^[2,3]. Malakoplakia most frequently involves the urinary tract, and less frequently, the gastrointestinal tract^[4], and the esophagus is seldom involved. We report a case of esophageal malakoplakia in a 54-year-old woman.

CASE REPORT

Clinical findings

A 54-year-old woman was referred to the gastroenterology department with complaints of coughing while eating and drinking. Her past medical history included chronic atrophic gastritis, duodenitis and rheumatoid arthritis.

On clinical examination she was pale. Her chest X-ray, electrocardiogram, blood tests and serum α -fetoprotein results were normal. Gastroscopy showed soft yellow nodules in the right wall of the esophagus, which was 23.5-25.0 cm from the cutting tooth (Figure 1A). Endoscopic ultrasonography revealed a space-occupying lesion (8.1 mm \times 5.1 mm) in the substratum of the esophageal mucosa (Figure 1B). ¹⁴C-Urea breath tests were positive for *Helicobacter pylori* (*H. pylori*).

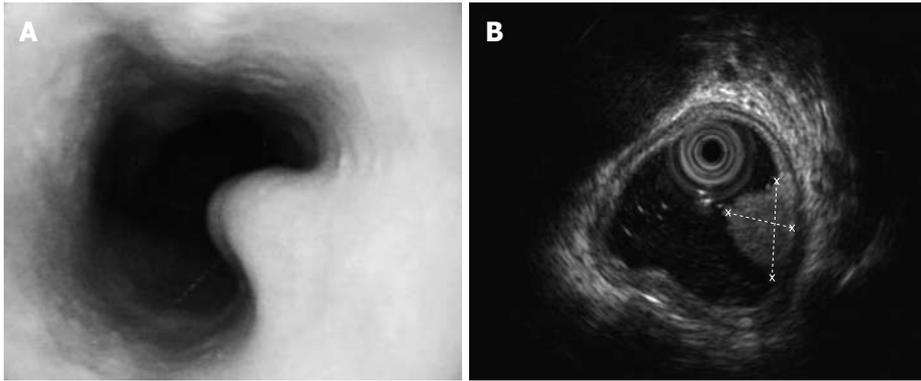


Figure 1 Soft yellow nodules protruding into the esophageal cavity. A: Gastroscopy presentation: soft yellow nodules protruding into the esophageal cavity; B: Endoscopic ultrasonography: a space-occupying lesion (8.1 mm × 5.1 mm) in the substratum sized 8.1 mm × 5.1 mm (dotted lines).

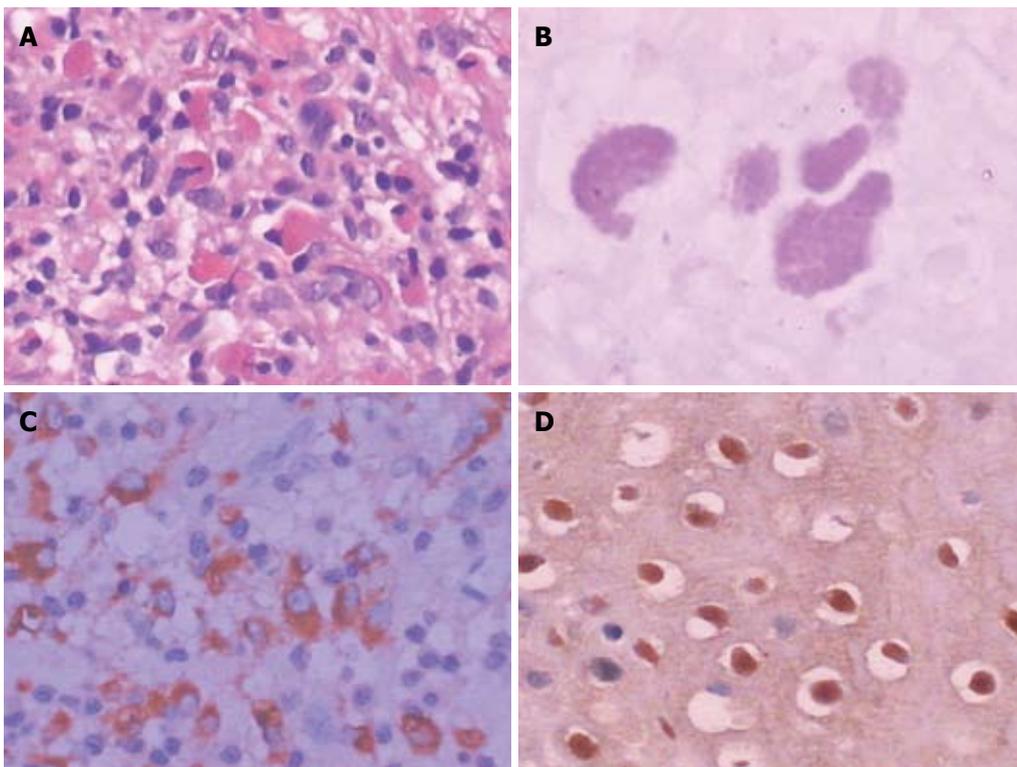


Figure 2 Michaelis-Gutmann bodies. A: Basophilic laminated inclusions in the cytoplasm (hematoxylin and eosin, ×200); B: Periodic acid-Schiff: targetoid appearance with a dense central core (×400); C: CD68 positivity by immunohistochemistry (×400); D: Esophageal squamous epithelium positive for human papillomavirus by immunohistochemistry, halo-shaped cells (×200).

Pathological findings

A local specimen of 1 cm × 1 cm was excision under gastroscopy for histopathological examination. Microscopic examination of hematoxylin and eosin stained sections showed a large amount of inflammatory cell infiltration in the substratum of the esophageal mucosa, mainly with lymphoid and histiocytic cells (Figure 2A). The presence of Michaelis-Gutmann bodies, with cytoplasmic concentric laminated inclusions of 5-15 mm confirmed the diagnosis. Follow-up examination supported this diagnosis. The Michaelis-Gutmann bodies were periodic acid-Schiff-positive (Figure 2B), and CD68-positive (Figure 2C) and human papillomavirus (HPV)-positive (Figure 2D) by immunohistochemistry, but negative for Eumycetes by hexamethylene diamine staining and *H. pylori* by Giemsa staining.

DISCUSSION

Malakoplakia is a chronic granulomatous inflammatory

disease characterized by accumulation of granular basophilic Michaelis-Gutmann bodies. These bodies are generated from histiocytes, which are positive for CD68 antibodies, as well as positive for periodic acid-Schiff stain, and exhibit a targetoid appearance with a dense central core under light microscopy^[4].

Malakoplakia has a worldwide distribution and does not have any racial, sex or age predilection^[5]. Malakoplakia most commonly affects the urinary tract, as well as the gastrointestinal system, regional lymph nodes, skin, liver, and spleen^[6-9].

The mechanism of malakoplakia is not well understood. Three postulates have been suggested. The first considers that microorganisms play a role in the pathogenesis. *Escherichia coli* infection is often found in the urinary tract^[10], *Rhodococcus equi* in the lungs^[11], and *H. pylori* in the stomach^[12]. However, there have been no previous reports of microbial infection in the esophagus. In the present case, HPV infection was identified by immunohistochemistry. We consider that HPV infection plays an important

role in development of esophageal malakoplakia. Another possibility is that abnormal immune responses are involved in the pathogenesis^[13]. Some immunosuppressive or chronic prolonged illnesses such as organ transplantation, acquired immunodeficiency syndrome, tuberculosis, sarcoidosis, and malignancy^[14] can be associated with malakoplakia. The woman in this case report suffered from rheumatoid arthritis whose pathogenesis is considered to be related to an abnormal immune response, which we consider may also have been related to her malakoplakia. The third hypothesis is an abnormal macrophage response caused by defective lysosomal function. This results in macrophages being unable to digest fully the phagocytosed bacteria, accumulation of partially digested bacteria, and generation of Michaelis-Gutmann bodies.

Malakoplakia typical presents as irregular nodules or plaque, but it also exists as widespread mucosal multinodular or polypoid lesions, or large mass lesions under endoscopy. In the present case, it presented as endoscopic nodules.

The clinical appearance of malakoplakia varies from silent nodules to various different presentations according to the organ involved. In the urinary tract it presents with lower tract irritative symptoms such as frequency, dysuria and hematuria^[15]. In the gastrointestinal system it can be clinically silent or can cause clinical symptoms such as diarrhea, abdominal pain, hemorrhage, or obstruction^[16,17]. In the respiratory system it can appear as silent nodules that mimic bronchogenic carcinoma or tuberculosis^[11]. Malakoplakia of the female genital tract usually presents with vaginal bleeding^[18]. In the present case, the patient presented with coughing while eating and drinking, which resembled esophageal cancer.

Malakoplakia is generally considered a chronic, self-limiting inflammatory disease that may undergo spontaneous regression^[19]. In the present case, despite the patient rejecting further treatment after receiving the pathological report, her symptoms disappeared and her condition did not develop. Follow-up endoscopic examinations 12 mo after resection revealed no changes in the patient's condition.

There are two therapeutic approaches to malakoplakia. Most cases have been successfully treated with antibiotics, for example, rifampicin, quinolone, and trimethoprim-sulfamethoxazole. The second approach is to attempt to correct the lysosomal defect by a cholinergic agonist, bethanechol chloride. Combination of antibiotic therapy and surgery provides satisfactory results. However, unnecessary radical surgical treatment should be avoided. The best choice depends on each specific patient. Our patient appeared to be cured by resection of the malakoplakia and showed no development during 1-year follow-up.

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 American Society of Clinical
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 Cancers Symposium
 San Francisco, CA 3000,
 United States

January 19-21, 2012
 2012 Gastrointestinal Cancers
 Symposium
 San Francisco, CA 94103,
 United States

January 20-21, 2012
 American Gastroenterological
 Association Clinical Congress of
 Gastroenterology and Hepatology
 Miami Beach, FL 33141,
 United States

February 3, 2012
 The Future of Obesity Treatment
 London, United Kingdom

February 16-17, 2012
 4th United Kingdom Swallowing
 Research Group Conference
 London, United Kingdom

February 23, 2012
 Management of Barretts
 Oesophagus: Everything you need
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 Cambridge, United Kingdom

February 24-27, 2012
 Canadian Digestive Diseases Week
 2012
 Montreal, Canada

March 1-3, 2012
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 Paris, France

March 7-10, 2012
 Society of American Gastrointestinal
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 San Diego, CA 92121, United States

March 12-14, 2012
 World Congress on
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 Omaha, NE 68197, United States

March 17-20, 2012
 Mayo Clinic Gastroenterology and
 Hepatology
 Orlando, FL 32808, United States

March 26-27, 2012
 26th Annual New Treatments in
 Chronic Liver Disease
 San Diego, CA 92121, United States

March 30-April 2, 2012
 Mayo Clinic Gastroenterology and
 Hepatology
 San Antonio, TX 78249,
 United States

March 31-April 1, 2012
 27th Annual New Treatments in
 Chronic Liver Disease
 San Diego, CA 92121, United States

April 8-10, 2012
 9th International Symposium on
 Functional GI Disorders
 Milwaukee, WI 53202, United States

April 13-15, 2012
 Asian Oncology Summit 2012
 Singapore, Singapore

April 15-17, 2012
 European Multidisciplinary
 Colorectal Cancer Congress 2012
 Prague, Czech

April 18-20, 2012
 The International Liver Congress
 2012
 Barcelona, Spain

April 19-21, 2012
 Internal Medicine 2012
 New Orleans, LA 70166,
 United States

April 20-22, 2012
 Diffuse Small Bowel and Liver
 Diseases
 Melbourne, Australia

April 22-24, 2012
 EUROSON 2012 EFSUMB Annual

Meeting
 Madrid, Spain

April 28, 2012
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May 3-5, 2012
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 SGNA: Society of Gastroenterology
 Nurses and Associates Annual
 Course
 Phoenix, AZ 85001, United States

May 19-22, 2012
 2012-Digestive Disease Week
 San Diego, CA 92121, United States

June 2-6, 2012
 American Society of Colon and
 Rectal Surgeons Annual Meeting
 San Antonio, TX 78249,
 United States

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 Pancreatic Cancer: Progress and
 Challenges
 Lake Tahoe, NV 89101, United States

July 25-26, 2012
 PancreasFest 2012
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Neurogastroenterology and Motility
 Meeting
 Bologna, Italy

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September 8-9, 2012
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 Bowel Disease
 La Jolla, CA 92093, United States

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 Surgery
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 American Association for the Study
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 Boston, MA 02298, United States

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 Advances in Inflammatory Bowel
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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

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID: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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- 9 Outreach: Bringing HIV-positive individuals into care. *HRS A Careaction* 2002; 1-6 [PMID: 12154804]

Books*Personal author(s)*

- 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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- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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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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Write as mean \pm SD or mean \pm SE.

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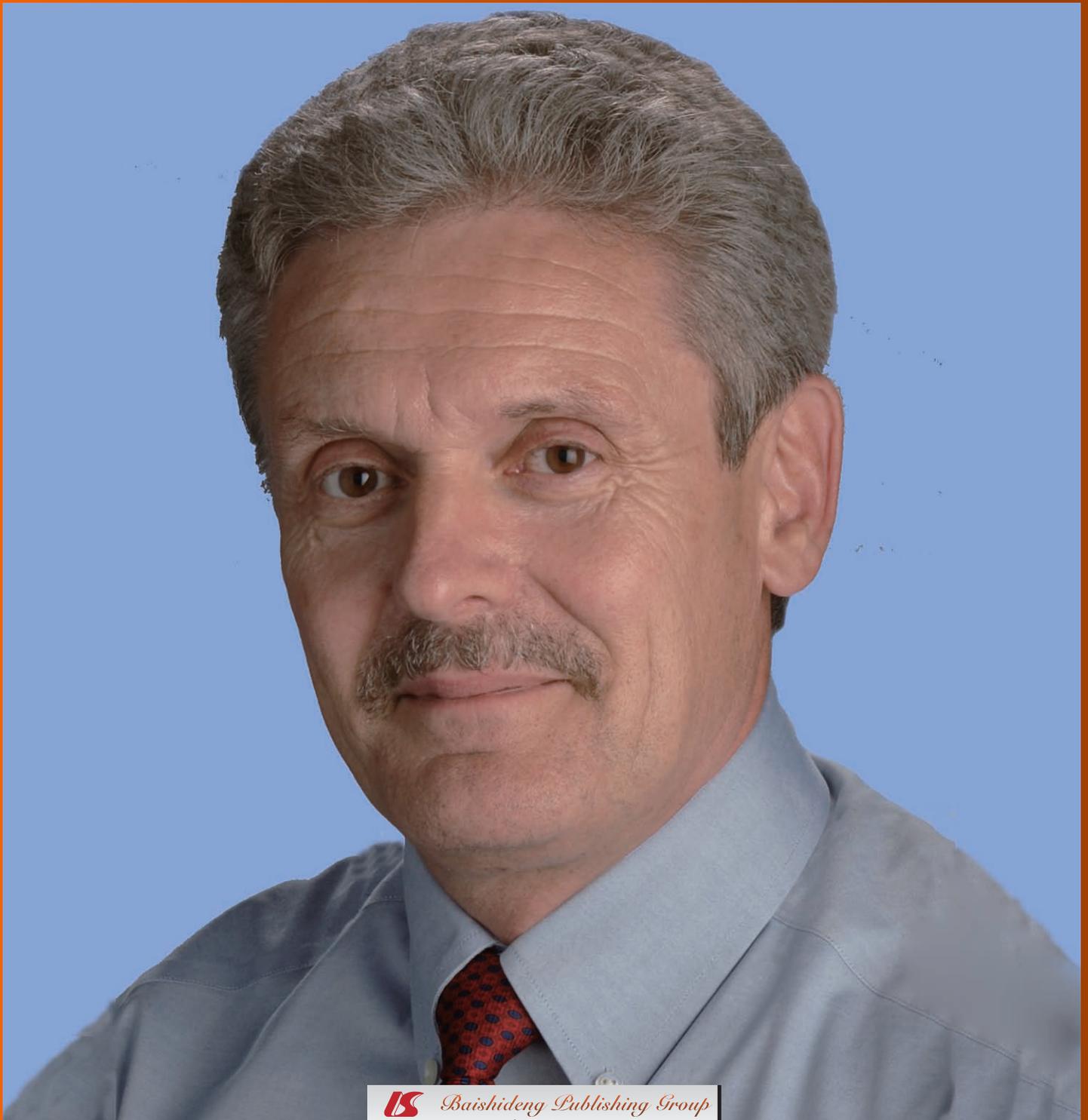
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Prevention and treatment of hepatic encephalopathy: Focusing on gut microbiota

Matteo Garcovich, Maria Assunta Zocco, Davide Roccarina, Francesca Romana Ponziani, Antonio Gasbarrini

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Abstract

The gut flora plays an important role in the pathogenesis of the complications of cirrhosis. Hepatic encephalopathy (HE) represents a broad continuum of neuropsychological dysfunction in patients with acute or chronic liver disease and/or porto-systemic shunting of blood flow and it manifests with progressive deterioration of the superior neurological functions. The pathophysiology of this disease is complex, as it involves overproduction and reduced metabolism of various neurotoxins, particularly ammonia. Management of HE is diversified and requires several steps: elimination of precipitating factors, removal of toxins, proper nutritional support, modulation of resident fecal flora and downregulation of systemic and gut-derived inflammation. This review will provide an overview of gut barrier function and the influence of gut-derived factors on HE, focusing on the role of gut microbiota in the pathogenesis of HE and the recent literature findings on its therapeutic manipulation.

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INTRODUCTION

Humans have been proposed to be a “meta-organisms” consisting of a huge number of bacterial cells that are metabolically and immunologically integrated with somatic cells^[1]. This interaction is especially important in the gastrointestinal tract, where the commensal bacteria, known as intestinal microbiota, are an integral component of human gut physiology and, together with the intestinal mucosa, form an important barrier against pathogens. Gut homeostasis and physiology are closely linked to the liver since it receives the intestinal blood content through the portal system and influences intestinal functions through bile secretion into the lumen^[2]. Thus, alterations of gut barrier seem to play an important role in the pathogenesis and progression of liver damage^[3]. Understanding of both partners in this normal gut-liver interaction is critical to the development of new therapeutic modalities to treat or prevent liver disease and its complications. This review will provide an overview of gut barrier function and the influence of gut-derived factors on hepatic encephalopathy (HE), fo-

cusing on the role of gut microbiota in the pathogenesis of HE and the recent literature findings on its therapeutic manipulation.

HOST-MICROBIOTA CROSS-TALK

The human intestine provides residence to more than 10^{14} bacteria, a number which is 10 times the number of somatic cells in the human body^[4,5]. Microorganisms start colonizing the gut immediately after birth and are characterized by a succession of different population until a stable, adult microbiota has been established. In this bacterial community anaerobes are more abundant than aerobes and the majority of the species are from the genera *Bacteroidetes* and *Firmicutes*^[6]. Bacterial density and types differ substantially from lower small intestine to distal colon, and are regulated by physiological conditions. The specific populations also vary among individuals and in the same individual during periods of illness or dietary changes^[7]. Microarray analysis of intestinal transcriptional responses and molecular taxonomic methodologies have greatly increased our understanding of the gut microbiota composition, activities and functions^[1,8,9]. In a healthy individual the host/microbiota relationship is characterized by a homeostatic symbiosis, in which the host provides nutrients and a stable environment and, in turn, the microbiota ensures optimal epithelial functioning.

GUT-LIVER AXIS

Receiving most of its blood supply from the intestine through the portal circulation, the liver is exposed to gut-derived toxins, including bacteria and bacterial products, and must be prepared to react against these potential systemic pathogens. For this purpose it contains a large number of resident immune cells including macrophages, dendritic cells, lymphocytes, natural killer cells. These cells act together with other non-parenchymal cells like endothelial and stellate cells to produce an organized response to these potentially highly inflammatory factors^[10,11]. The role of immune cells during inflammatory response or chronic liver injury and the potential impact of gut-derived toxins on these processes have been extensively studied^[2]. In particular bacterial overgrowth and altered intestinal permeability result in high plasmatic levels of bacterial endotoxins, such as lipopolysaccharide (LPS), peptidoglycan, and various lipopeptides also termed pathogen-associated molecular patterns. Endotoxemia could be responsible for initiation of the liver damage, through its interaction with specific recognition receptors, the toll like receptors on the surface of immune cells. These receptors contribute to adaptive immune response and regulation of inflammation and represent a link between intestinal flora changes, endotoxemia, and liver damage^[12]. Liver cirrhosis is characterized by several abnormalities of both the systemic and local immune systems and in particular by

reduced phagocytic activity of Kupffer cells^[13]. A great deal of evidence indicates that patients with cirrhosis could present increased intestinal permeability^[14]. This is related to structural changes that occur in the presence of portal hypertension and hypertensive enteropathy: an altered oxide-reductive state with consequent oxidative damage of the brush border membrane and the overproduction of nitric oxide resulting in tight junctions expansion and cytoskeleton destruction^[15]. Altered intestinal permeability, together with bacterial overgrowth and immune dysfunction are associated with the migration of bacteria or their products from the gut to mesenteric lymph nodes or to other organs, a process known as bacterial translocation (BT). This phenomenon, in association with the presence of vascular shunts, is responsible for increased circulating levels of LPS. Blood concentration of bacterial endotoxin directly correlates with the severity of liver disease and participates in the initiation of a complex series of mechanisms that lead to the development of cirrhosis complications^[16]. In particular, the main consequences of portal hypertension and BT are the occurrence of infections and HE.

Bacterial infections are present in about 15%-47% of patients with liver cirrhosis and are especially related to Gram-negative bacteria. The most frequent are spontaneous bacterial peritonitis (SBP), urinary tract infections, pneumonia, pleural empyema and sepsis. It has been shown that patients with SBP have a higher prevalence of small intestinal bacterial overgrowth (SIBO)^[17] and altered intestinal permeability^[18] than patients without SBP. Ammonia and other toxic substances derived from the gut, in the presence of portal and systemic shunts as well as of reduced liver clearance capability, represent the pathogenic mechanisms of HE as described in the following paragraph.

GUT MICROBIOTA AND HE

HE is a reversible neuropsychiatric disorder associated with liver dysfunction after exclusion of other potential causes of brain disease^[19] and is characterized by poor survival^[20]. Main features are disturbances in cognitive function, personality and behaviour with a wide spectrum of neuropsychiatric abnormalities that range from mild impairment of cognitive function and consciousness to coma. There are two types of HE named overt and minimal HE (OHE, MHE). The first is present in 30%-45% of patients with cirrhosis and in 10%-50% of patients with transjugular intrahepatic portosystemic shunt^[21]. OHE can be diagnosed clinically through a constellation of signs and symptoms by several scoring systems^[22]. The most widely used are the West-Haven criteria (Table 1) which are based on neurological examination and specific questionnaires to detect mental status changes. In addition, OHE can be further divided into episodic or persistent depending on time course and clinical behaviour: episodic HE remains below the clinical detection level among different episodes, whereas

Stage	Consciousness	Intellect and behaviour	Neurological findings
0	Normal	Normal	Normal examination ¹
1	Trivial lack of awareness	Impaired attention span; altered sleep; euphoria	Mild asterixis
2	Lethargic	Disoriented; inappropriate behaviour depression	Asterixis; slurred speech
3	Somnolent but arousable	Gross disorientation; bizarre behaviour	Muscular rigidity/clonus hyper-reflexia
4	Coma	Coma	Decerebrate posturing

¹If impaired psychomotor testing, then minimal hepatic encephalopathy.

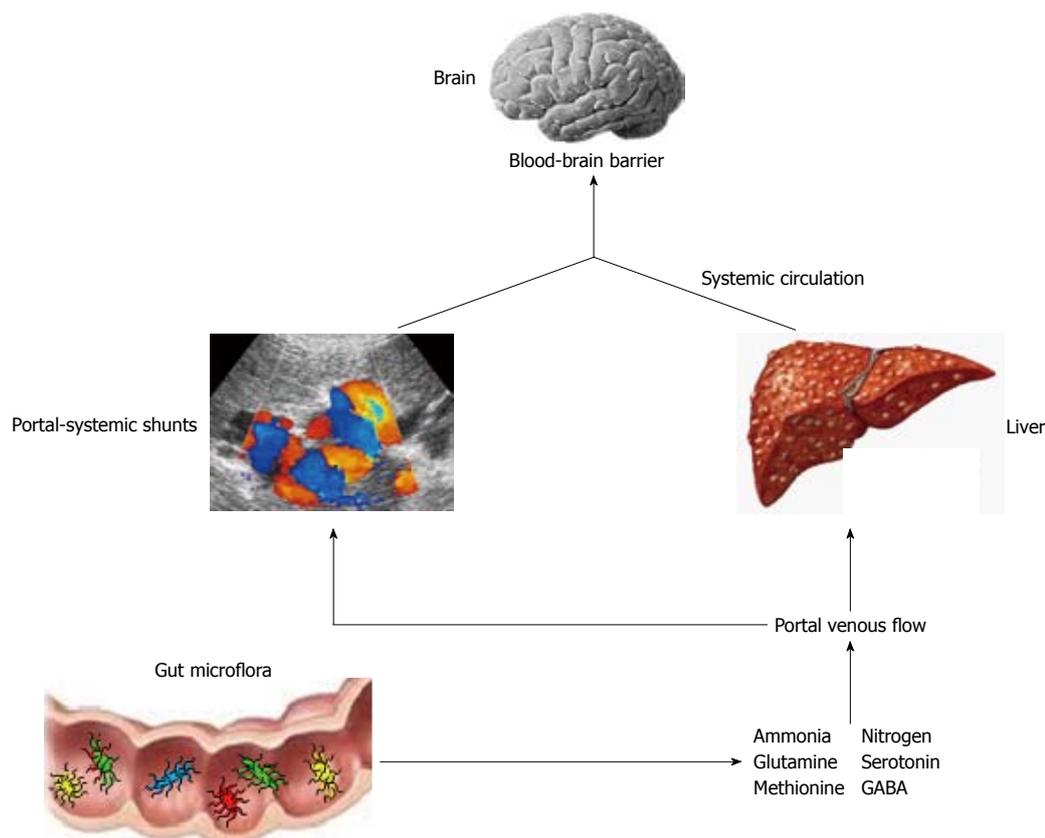


Figure 1 Pathophysiology of hepatic encephalopathy in liver cirrhosis. GABA: γ -aminobutyric acid.

persistent HE is always clinically evident^[23]. Prevalence for MHE ranges from 30% up to 80%, probably because there is still no accepted gold standard for diagnosis of MHE and the majority of the screening tests are time-consuming and cumbersome to perform^[23,24]. It is characterized by alterations of psychometric and neurophysiological tests in otherwise asymptomatic patients^[25]. This condition is considered a preclinical stage of OHE and is associated with poor quality of life, high risk of traffic violations and accidents and increased progression to OHE^[26-28]. The diagnosis of MHE aims to detect attention deficits and processing speed and is based on different batteries of tests. Among them, the portal systemic encephalopathy (PSE) syndrome test, that includes number connection test A (NCT A), number connection test B (NCT B), line tracing test errors and time, serial dotting test and digit symbol test, has been validated in different countries and is recommended by

the Working Group on HE^[29]. Although the pathogenesis of this disorder is not well understood, a key role is played by circulating gut derived toxins of the nitrogenous compounds with a resultant neuroglial injury in the setting of a systemic inflammatory milieu (Figure 1). In particular, the currently accepted hypothesis is that endogenous neurotoxic substances escape from catabolism by the liver, due both to the impaired function of the cirrhotic liver and to the presence of portal-systemic shunts. These substances circulate in the systemic blood flow, reach the brain through the blood-brain barrier and results in different severity of cerebral impairment^[30]. Several factors have been shown to precipitate HE, including gastrointestinal bleeding, electrolyte imbalances, infection, and medications such as sedatives and diuretics. They work by increasing the underlying inflammatory milieu, increasing toxins production or reducing the threshold for mental status decline, or a combination of

the above. Ammonia, mercaptans, phenols, short and medium-chain fatty acids and benzodiazepine-like compounds have all been found to be elevated in cirrhotic patients with HE. The majority of these toxic substances are produced in the intestine by the bacterial flora, and are absorbed into the portal venous flow. Thus, gut microflora contribute to the pro-inflammatory state of cirrhosis even in the absence of overt infection^[31]. In addition, cirrhotic patients have a substantial alteration of the gut microecology with a high prevalence of SIBO and delayed oro-cecal transit time (OCTT)^[17,32-36]. The latter has a multifactorial etiology. First, it could be due to the presence of autonomic neuropathy that has been demonstrated as an independent predictor of reduced intestinal motility in this kind of patients^[37]. Moreover, patients with autonomic dysfunction were found to have a higher incidence of new onset of HE^[38]. Second, a delayed OCTT may be associated with metabolic alterations that occur in patients with portal hypertension and portal-systemic venous shunting. Finally, SIBO itself may lead to delayed OCTT since it has been shown a significant improvement of intestinal motility after antibiotic therapy^[36]. There might be also a link between the cognitive impairment in cirrhosis with inflammation and specific bacterial taxa. For example, recent evidence showed how interleukin (IL)-23 system and innate immune response were highly correlated with several bacterial families in patients with cirrhosis and HE, and how there was a direct correlation between cognition, Porphyromonadaceae and Alcaligenaceae families^[39]. Based on these considerations, the manipulation of gut flora could be useful in the treatment of HE.

MODULATION OF GUT MICROBIOTA FOR THE MANAGEMENT OF HE

Treatment goals and options are dependent on the stage and acuity of HE^[23]. Once the diagnosis of OHE is confirmed, an extensive search for potential precipitating factors should be instituted along with treatment of OHE. As previously described, the leading causes are gastrointestinal bleeding, sepsis, dehydration resulting from diuretics, diarrhoea or vomiting, transjugular intra hepatic porto systemic shunting, constipation and the use of sedative and narcotic drugs. Their treatment can reverse OHE in most cases. However, when it is not possible to identify a precipitating factor despite an exhaustive search, specific treatment for OHE should be instituted. Most therapies for HE focus on treating episodes as they occur and are directed at reducing the nitrogenous load in the gut, an approach that is consistent with the hypothesis that this disorder results from the systemic accumulation of gut-derived neurotoxins in patients with impaired liver function and portosystemic shunting^[40]. Therefore, the majority of therapeutic options currently in use are directed towards the gut. Prebiotics are non-digestible food ingredients that act by directly stimulating the growth of bacterial strains potentially beneficial to

the host like *Bifidobacteria* and *Lactobacilli*, thereby indirectly reducing the influence of potentially more harmful resident flora (i.e. urease-producing species). They come in the form of indigestible fibers and have shown benefit for the management of HE, particularly MHE, both as prebiotics and when used in combination with probiotics (in which case they are termed synbiotics)^[41,42]. Probiotics are living non pathogenic microorganisms that are thought to exert an effect in HE by reducing intestinal ammonia production by enterocyte glutaminase and reduce BT, modulate gut permeability and modulate pro-inflammatory responses. Furthermore, probiotics bypass the small bowel and get fermented by colonic bacteria to form lactic, acetic, and butyric acids, and gas (mainly hydrogen); any resultant prokinetic effect may increase the expulsion of ammoniogenic bacteria^[43]. Probiotics have been studied for the treatment of HE and have shown some benefit, mostly in the setting of minimal disease^[24,44,45]. The bacterial species that appear to be most successful include *Lactobacilli* and *Bifidobacteria*. Probiotics may also improve overall liver function, perhaps by reducing translocation and subsequent endotoxemia and by ameliorating the hyperdynamic circulation^[41]. To quantify unambiguously the beneficial and harmful effects of any probiotics at any dosage, a recent meta-analysis identified seven randomized trials for the treatment of acute or chronic HE. The authors of this Cochrane review assessed a range of outcomes including death, recovery, adverse events, and quality of life. There was no benefit of probiotics shown for any of the primary outcomes including mortality. On the other hand, there was a significant difference in secondary outcomes such as lowering of plasma ammonia concentration compared with no treatment. The authors concluded that this finding is of questionable importance, not recommending the use of probiotics for patients with HE until further randomized clinical trials are undertaken^[46]. Non-absorbable disaccharides, such as lactulose and lactilol, have traditionally been considered the first-line drug therapy for lowering the production and absorption of ammonia^[47]. These substances are metabolized by the intestinal bacteria to acetic and lactic acid. The consequent acidification of the colonic contents creates a hostile environment for the survival of intestinal bacteria involved in the production of ammonia and facilitates the conversion of NH₃ to non-absorbable NH₄⁺. Moreover, their cathartic effects cause an increased in faecal nitrogen excretion^[48]. The non-absorbable disaccharides have been used for decades with anecdotal and clinical trial experience^[49]. However, side effects of lactulose therapy, including cramping, diarrhoea and flatulence, result in frequent non-compliance^[40]. Overdosage may also result in severe diarrhoea, electrolyte disturbances and hypovolaemia that, if severe enough, may itself precipitate encephalopathy symptoms. In spite of their anecdotal usefulness, in the last years the true efficacy of the disaccharides for this indication has been questioned. As the use of lactulose pre dated randomized controlled trials, a comprehensive meta-analysis

endorsed by the Cochrane Collaboration did not find any significant difference in outcomes in patients treated with and without lactulose^[50]. Based on a critical analysis of available published literature, Als-Nielsen *et al*^[50] concluded that the evidence in favour of utilizing non-absorbable disaccharides in HE did not meet the current minimum criteria for adequacy. In fact, although in some cases the administration of lactulose was associated with improvement in mental status, it is difficult to assess the reason for improvement since precipitating factors were simultaneously being corrected. On the other hand newer clinical studies suggest benefits with lactulose conferring improved neuropsychometric and quality of life scores^[47]. Also, two recently published meta-analysis on the clinical efficacy and safety of lactulose in patients with MHE, provide substantial evidence for the beneficial effects of non-absorbable disaccharides^[51,52]. Therefore, at the present time, there is a lack of sufficient evidence to completely dismiss the use of non-absorbable disaccharides for the treatment of HE, while compliance and cost effectiveness should be carefully balanced against clinical outcomes. Antimicrobial agents have long been utilized as an alternative treatment option for patients intolerant or unresponsive to non-absorbable disaccharides due to their ability to inhibit ammonia production by intestinal bacteria. Neomycin is the most commonly used antimicrobial for HE, but despite the legitimate theoretical rationale for its use, there is a paucity of clinical data to support this practice^[48,53]. Moreover, the occurrence of serious adverse effects such as nephrotoxicity and ototoxicity limit their use to relatively short periods of time. Other antimicrobials, including metronidazole and vancomycin, have been studied to a more limited extent than neomycin^[48]. However, long-term use of metronidazole has been associated with neurotoxicity in patients with cirrhosis, whereas the risk for enteric bacteria resistance preclude the routine use of vancomycin for HE. Thus, with the potential for serious adverse effects and the lack of demonstrated clinical benefit, the routine management of HE with conventional antibiotics should be questioned. Rifaximin is a poorly absorbed synthetic antibiotic with a broad spectrum of antibacterial activity, against aerobic and anaerobic Gram positive and Gram-negative organisms^[54]. As a derivative of rifamycin, it similarly works by blocking bacterial RNA synthesis. However, rifaximin has an additional pyridoimidazole ring that allows for high concentrations in the gastrointestinal tract and minimal systemic drug absorption.

Due to its low rate of systemic bioavailability, the safety profile of rifaximin appears to be superior to that of systemic antibiotics, particularly for patients with liver disease, making it suitable for long-term use. Moreover, the risk of bacterial resistance appears to be lower with rifaximin than with systemic antibiotics because bacteria outside the gastrointestinal tract are not exposed to appreciable selective pressure^[55]. The safety and efficacy profiles of rifaximin as treatment of overt HE have been extensively explored in several clinical trials^[48]. The results of controlled double-blind studies demonstrated

that rifaximin was more effective than non-absorbable disaccharides in the treatment of acute HE: mental state, electroencephalogram irregularities and PSE-index were all significantly improved in the rifaximin group^[56-59]. While rifaximin was well tolerated, adverse events, including flatulence, diarrhoea, nausea and anorexia, were reported by patients in the disaccharides group. A recent study found a reduced hospitalization rate during rifaximin therapy compared with that of lactulose^[60]. Once again, the HE grade was significantly lower and patients compliance was significantly higher in the rifaximin group. Further data suggest that rifaximin is at least as effective as neomycin in decreasing plasma ammonia levels and in improving the clinical symptoms related to HE with fewer clinically significant adverse events during a 21 d treatment period^[61]. Based on these findings, the Cochrane review recommends the use of rifaximin in the treatment of acute HE^[50]. Until recently, there has not been any conclusive evidence to support routine use of pharmacological prophylaxis to prevent future recurrence in patients who have recovered from an acute episode of HE. However, the prevention of episodes of HE is an important goal in the treatment of patients with liver disease, especially since symptoms of overt encephalopathy are debilitating and decrease the ability for self-care, leading to frequent hospitalizations, and a poor quality of life. A recent clinical trial has been conducted to evaluate the efficacy of rifaximin as secondary prophylaxis of overt HE^[62]. This double blind, placebo controlled, multicentre trial randomized 299 patients with a recent history of recurrent, overt HE to receive either rifaximin or placebo for a period of 6 months. The majority of the patients in both groups were also maintained on concomitant lactulose therapy. During the study period, an acute episode of HE occurred in a significantly lower percentage of patients in the rifaximin group (22.1%) than in the placebo group (45.9%), with a hazard ratio (HR) of 0.42.

Furthermore, there was a significantly reduced risk of hospitalization in the rifaximin group when compared with placebo: 13.6% *vs* 22.6% of patients respectively with a corresponding HR of 0.50. No significant difference in the incidence of adverse events was found between the two groups. The authors concluded that the addition of rifaximin to a standard lactulose regimen may offer advantages in terms of decreasing risk of both acute HE episodes as well as hospitalizations when compared with lactulose alone. Overall, this pivotal study expands previously reported findings of the efficacy of rifaximin in the treatment of overt HE and demonstrates a clinically relevant benefit of rifaximin as pharmacological prophylaxis of HE. The protective effect of rifaximin was confirmed by an extension trial performed on the same patients to assess the efficacy of rifaximin in maintaining remission over time^[63]. The results of this preliminary study support a possible long-term protective pharmacological effect. In addition, an ancillary analysis of data from the same trial to assess the effect of rifaximin on health-related quality of life

(measured *via* the Chronic Liver Disease Questionnaire) provided evidence that rifaximin improves perception of daily well being and quality of life outcomes in all domains adversely affected by underlying liver diseases^[64]. Therefore at this time, both lactulose and rifaximin can be used to prevent recurrent episodes of OHE. Ambivalence remains as concerning the treatment of MHE. It is clear that MHE has a major impact on quality of life and should be promptly diagnosed and treated. However, for many years, various treatment modalities including lactulose, probiotics and dietary manipulation, have been studied with unconvincing evidence. Limits on the use of antibiotics have been exceeded by the results of the randomised ischaemic mitral evaluation trial^[65], which showed unequivocally that rifaximin improves psychometric test performance scores as early as after 2 wk of treatment. The same patients experienced, also, a significant improvement in health related quality of life, which was strongly correlated with the improvement in cognitive functions. The authors speculated that there are two possible mechanisms by which rifaximin could lead to an improvement in MHE: first by reducing the ammonia-producing bacteria in the gut^[61], second by decreasing BT and inflammation.

The results of the RIME trial represent an important step in the establishment of rifaximin as an effective and safe treatment for MHE and are in agreement with the findings of a more recent study which demonstrates improved driving skills following rifaximin treatment in patients with MHE^[66]. In this double-blind placebo controlled trial, patients randomized to rifaximin had a significant reduction in the number of total driving errors, and specifically speeding tickets and illegal turns, on a driving simulator, compared with those on placebo. Driving requires balance and integration of different cognitive functions, such as attention, adequate reaction time, visuo-motor coordination and can be considered a practical interpretation of the cognitive domains affected in MHE^[67]. Since patients on rifaximin presented increased plasmatic levels of the anti-inflammatory cytokine IL-10, the authors speculated that rifaximin could act by regulating local intestinal immunity and inflammation. The same authors provide in another paper a cost effective analysis based on a Markov model of progression from cirrhosis without MHE, to MHE and to OHE focused on motor vehicle crashes as an objective endpoint. The results of this analysis indicate that diagnosis of MHE followed by lactulose therapy could result in substantial societal cost savings by preventing major motor vehicle crashes among MHE patients. In contrast, because of its high monthly cost, treatment with rifaximin is unlikely to generate overall cost savings unless the rifaximin monthly cost is substantially reduced^[68].

CONCLUSION

Different chronic liver diseases are associated with alterations of the intestinal microbiota, gut barrier dysfunction

and translocation of bacteria or bacteria-derived antigens into the systemic circulation. The same processes, in turn, exacerbate liver disease leading to enhanced tissue damage and development of complications such as systemic infections, phosphate buffered saline, hepato renal syndrome and portal-systemic encephalopathy. Modulation of the gut microbiota may represent a good therapeutic target for the prevention and treatment of different complications associated with liver cirrhosis. In particular, due to its low rate of systemic bioavailability and a good tolerability profile, the non-absorbable antibiotic rifaximin could be an 'ideal' antimicrobial for selective targeting at the gastrointestinal tract in this kind of patients. As reviewed, at present this antibiotic is recommended for the treatment of acute HE. Furthermore, recent literature appears to support a favourable benefit-risk ratio for rifaximin both in the prevention of overt HE and in the treatment of minimal HE.

However, additional high-quality clinical trials are needed to more clearly define the effectiveness of long-term or periodic treatment with rifaximin on gut microbiota modulation in cirrhotic patients with HE.

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Glycogenotic hepatocellular carcinoma with glycogen-ground-glass hepatocytes: A heuristically highly relevant phenotype

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Abstract

Glycogenotic hepatocellular carcinoma (HCC) with glycogen-ground-glass hepatocytes has recently been described as an allegedly "novel variant" of HCC, but neither the historical background nor the heuristic relevance of this observation were put in perspective. In the present contribution, the most important findings in animal models and human beings related to the emergence and further evolution of excessively glycogen storing (glycogenotic) hepatocytes with and without ground glass features during neoplastic development have been summarized. Glycogenotic HCCs with glycogen-ground-glass hepatocytes represent highly differentiated neoplasms which contain subpopulations of cells phenotypically resembling those of certain types of preneoplastic hepatic foci and benign hepatocellular neoplasms. It is questionable whether the occurrence of glycogen-ground-glass hepatocytes in a glycogenotic HCC justifies its classification as a specific entity. The typical appearance of ground-glass hepatocytes is due to a hypertrophy of the smooth endoplasmic reticulum, which is usually associated with an excessive storage of glycogen and frequently also with an expression of the hepatitis B surface antigen. Sequential studies in animal models and observations in humans indicate that glycogen-ground-glass hepatocytes are a facultative, integral part of a characteristic cellular sequence commencing with focal hepatic glycogenosis potentially progressing to benign and malignant neoplasms. During this process highly differentiated glycogenotic

cells including ground-glass hepatocytes are gradually transformed *via* various intermediate stages into poorly differentiated glycogen-poor, basophilic (ribosome-rich) cancer cells. Histochemical, microbiological, and molecular biochemical studies on focal hepatic glycogenosis and advanced preneoplastic and neoplastic lesions in tissue sections and laser-dissected specimens in rat and mouse models have provided compelling evidence for an early insulinomimetic effect of oncogenic agents, which is followed by a fundamental metabolic switch from gluconeogenesis towards the pentose-phosphate pathway and the Warburg type of glycolysis during progression from preneoplastic hepatic glycogenosis to the highly proliferative malignant phenotype.

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Key words: Acquired focal hepatic glycogenosis; Inborn hepatic glycogenosis; Hepatic preneoplasia; Hepatic neoplasia; Early metabolic aberrations; Progression-linked metabolic switch

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INVITED COMMENTARY ON HOT ARTICLES

The glycogenotic hepatocellular carcinoma (glycogenotic

HCC) with glycogen-ground-glass hepatocytes has recently been described as a “novel variant” of HCC by Callea *et al*^[1]. Relating the excessive storage of glycogen (glycogenosis) to a complete absence of glucose-6-phosphatase activity as measured by a microbiobiochemical approach, the authors referred to the long known finding of a focal hepatic glycogenosis (FHG) as an early event in experimental hepatocarcinogenesis^[2], continuing “however, ground-glass cells have not been reported in FHG”. In contrast to this statement, the characteristic hepatocellular phenotype addressed was not only explicitly described for the first time in preneoplastic and neoplastic lesions in rodents^[3,4] and in humans^[5,6] (Figure 1) decades ago but has also been proven to be heuristically highly relevant in numerous publications since then. Glycogenotic ground-glass hepatocytes (GGH) predominate in subpopulations of many focal precancerous hepatocellular lesions, particularly in preneoplastic FHG and benign hepatocellular neoplasms such as hepatocellular adenomas and focal nodular hyperplasia, but may also occur in more or less extended subpopulations of HCC as observed in various species, including non-human primates and human beings^[7,8]. However, glycogen-GGH hardly ever account for whole neoplasms. This also applies to the glycogenotic HCC depicted by Callea *et al*^[1] in which the GGH are mixed with “clear” (glycogenotic) cells without ground-glass features. It is, hence, questionable whether glycogenotic HCC with glycogen-GGH should be considered a specific entity as proposed by Callea *et al*^[1]. Extensive investigations in models of chemical, viral, and hormonal hepatocarcinogenesis and some observations in humans suggest that FHG with and without GGH indicates a critical early metabolic aberration in the pathogenesis of benign and malignant hepatocellular neoplasms^[7,8].

Animal models of hepatocarcinogenesis

The observations in humans were preceded by several seminal findings in animal models of hepatocarcinogenesis as repeatedly reviewed^[2,9]. In their pioneering electron microscopic investigations in rats continuously exposed to 3-methyl-dimethylaminoazobenzene, Porter *et al*^[10] detected a hypertrophy of the smooth endoplasmic reticulum in many hepatocytes, and addressed its light microscopic counterpart as “hyaline degeneration”^[11]. The authors related this characteristic subcellular change to a decreased rather than an increased storage of glycogen and felt “that only cells which, through mutation, lose the normal tendency to differentiate for glycogenesis will survive and so will be selected out for continued growth and differentiation”^[10]. However, investigations in other models of rat hepatocarcinogenesis, employing both continuous or limited (stop model) exposure to N-nitrosomorpholine at various dose levels and time schedules^[3,4], or ethionine for up to approximately 20 wk^[12] revealed that hypertrophy of the smooth endoplasmic reticulum is often associated with an excessive storage of glycogen, the smooth membranes forming either a typical network or peculiar lamellar complexes which often

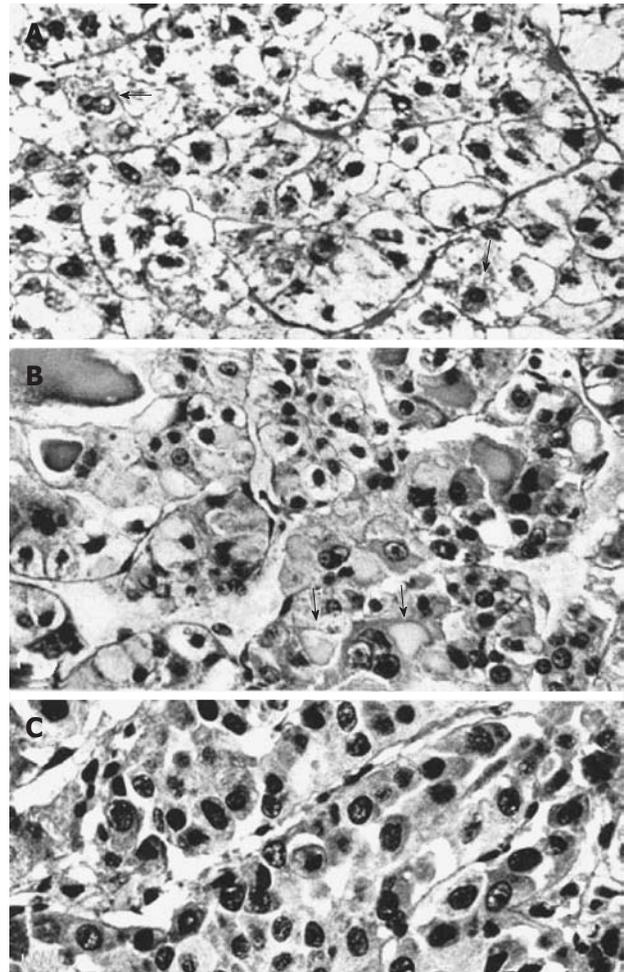


Figure 1 Light micrographs of portions from human hepatocellular neoplasms with and without glycogenosis. A: Clear-cell hepatocellular adenoma consisting predominantly of glycogenotic cells. In some cells (arrows) there is a reduction of glycogen and focal increase in cytoplasmic basophilia; B: Highly differentiated hepatocellular carcinoma (HCC) composed of a mixed population of clear (glycogenotic) cells, acidophilic cells (ground-glass hepatocytes, arrows), and some glycogen-poor, basophilic cells; C: Poorly differentiated, glycogen-free, basophilic HCC. All: Hematoxylin and eosin stain, x 460, from Bannasch *et al*^[6].

show a close spatial relationship with glycogen particles (Figure 2) but may also be free of glycogen forming “fingerprints”^[4]. Steiner *et al*^[12] designated the complexes of the smooth endoplasmic reticulum associated with glycogen as “glycogen-bodies”, and speculated that these formations indicate a “resistance” to the carcinogen, reflecting a reactivation of the glycogen-storing ability after an early loss of glycogen in response to toxicity. In contrast to both of these considerations, many studies on experimental hepatocarcinogenesis in different species revealed that FHG composed of glycogenotic clear and/or acidophilic cells, the latter showing a pronounced hypertrophy of the smooth endoplasmic reticulum (corresponding to glycogenotic GGH), regularly occur in a multi-centric fashion in early stages of neoplastic development induced in small rodents by a variety of chemicals^[3,4,9,13]. More recently, typical glycogenotic GGH were also found after chronic infection of woodchucks with hepadnaviridae^[14-16], in hepatitis B virus (HBV)-transgenic mice^[17], and during hormonal hepatocarcinogenesis in

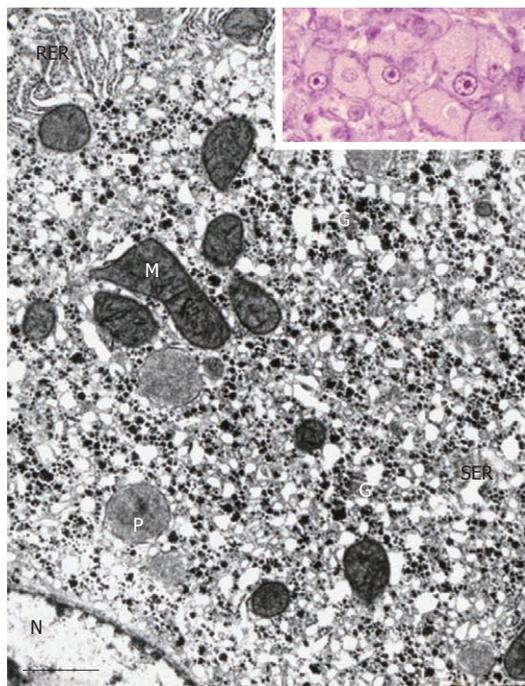


Figure 2 Portion of a glycogenotic acidophilic hepatocyte (corresponding to glycogen-ground-glass hepatocyte) induced in rat liver by N-nitrosomorpholine. Note abundant α - and β -glycogen particles (G) in close spatial relationship with large network complexes of proliferated smooth endoplasmic reticulum (SER). Rough endoplasmic reticulum (RER), mitochondria (M), peroxisomes (P), and nucleus (N). Inset: Group of acidophilic hepatocytes as seen under the light microscope. Transmission electron microscopy, lead citrate. Bar: 1 μ m.

diabetic animals after intrahepatic transplantation of pancreatic islets in rats and mice^[18,19].

As demonstrated particularly in stop models of hepatocarcinogenesis in rats exposed to N-nitrosomorpholine or thioacetamide, glycogenotic hepatocytes with and without hypertrophy of the smooth endoplasmic reticulum may persist for weeks and months after withdrawal of the carcinogen, and may persist as almost entire populations of hepatocellular adenomas (“hyperplastic nodules”, “neoplastic nodules”) and in subpopulations of highly differentiated HCCs^[4,20,21]. These observations are neither compatible with the idea of a preferential cellular survival by loss of differentiation for glycogenesis^[10] nor with the notion of a “resistance” of these cells to carcinogen^[12,22,23]. The functional significance of the persistent hypertrophy of the smooth endoplasmic reticulum has remained obscure, but many findings suggest that the organelle represents a metabolic compartment whose increase is a facultative consequence of the disturbed carbohydrate metabolism characterizing the acquired hepatocellular glycogenesis^[4,24].

Independent of the observation of an acquired FHG produced in rat liver by N-nitrosomorpholine^[5], Gössner *et al*^[25] described a focal reduction in the activity of glucose-6-phosphatase in rats exposed to N-nitrosodiethylamine. A causal relationship between this enzyme deficiency and the accumulation of glycogen in preneoplastic FHG and “hyperplastic liver nodules” has been suggested by a number of authors^[3,4,26,27]. This conclusion

appears to be supported by the well known high risk of children suffering from an inborn hepatic glycogenosis type I, especially type I a (von Gierke), due to a genetically fixed deficiency of the glucose-6-phosphatase, to develop hepatocellular adenomas and carcinomas when passing through adolescence^[2,9,28]. This interpretation is in line with the recent finding that the targeted deletion of liver glucose-6-phosphatase in a knock-out mouse model results in hepatic glycogenosis and steatosis, and eventually also in multiple hepatocellular adenomas in all animals beyond 18 mo of age^[29].

It is important to realize, however, that correlative cytochemical studies in rodent models of hepatocarcinogenesis have shown that the focal decrease of glucose-6-phosphatase activity in FHG is regularly combined with decrease or increase in the activity of many other enzymes^[7,8,30], especially enzymes of the carbohydrate metabolism^[2,24,31]. In addition, over-expression of the key enzyme of *de novo* fatty acid synthase has been described in FHG in the N-nitrosomorpholine stop-model^[32]. In rats exposed to N-2-fluorenylacetylamine, Williams *et al*^[33] demonstrated that the preneoplastic glycogenotic clear cell foci are resistant to the storage of iron.

For a long time the cause of these complex metabolic alterations in FHG remained elusive. More recently, however, histochemical, microbiological and molecular biochemical studies on FHG and advanced preneoplastic and neoplastic liver lesions in tissue sections and laser-dissected specimens obtained from small rodents exposed to chemical carcinogens or oncogenic viruses provided evidence for an early insulin-like (insulinomimetic) effect of these agents^[7,24,34-36]. This notion has been corroborated by a number of studies on hormonal hepatocarcinogenesis induced in diabetic rats and mice by local hyperinsulinemia^[18,19,32,37-39].

The phenotype of preneoplastic FHG is not stable, but undergoes dramatic changes during progression to the benign and/or malignant neoplastic phenotype^[3,4]. Collectively, all types of specific focal hepatocellular lesions appearing during the preneoplastic phase in rodents have been termed foci of altered hepatocytes, and have been widely used as early indicators of neoplastic development in toxicologic pathology^[40-42]. The characteristic sequence of cellular changes starting with FHG follows an ordered pattern, passing through intermediate or mixed cell foci composed of glycogenotic, intermediate, and glycogen-poor basophilic (ribosomeric) cell types, the latter corresponding to the typical cell type in poorly differentiated HCCs^[2,4,24]. Detailed morphological analysis of intermediate cell types at the light- and electron microscopic level has suggested that the hypertrophied smooth endoplasmic reticulum is usually transformed into rough endoplasmic reticulum by the addition of ribosomes during this phenotypic conversion^[4,9,20]. Frequently, the intermediate cells show an accumulation of neutral fat, often leading to a combination of glycogenosis and steatosis^[2,20]. Evidence of this sequence of cellular changes was originally provided by light- and electron-microscopic studies in rats

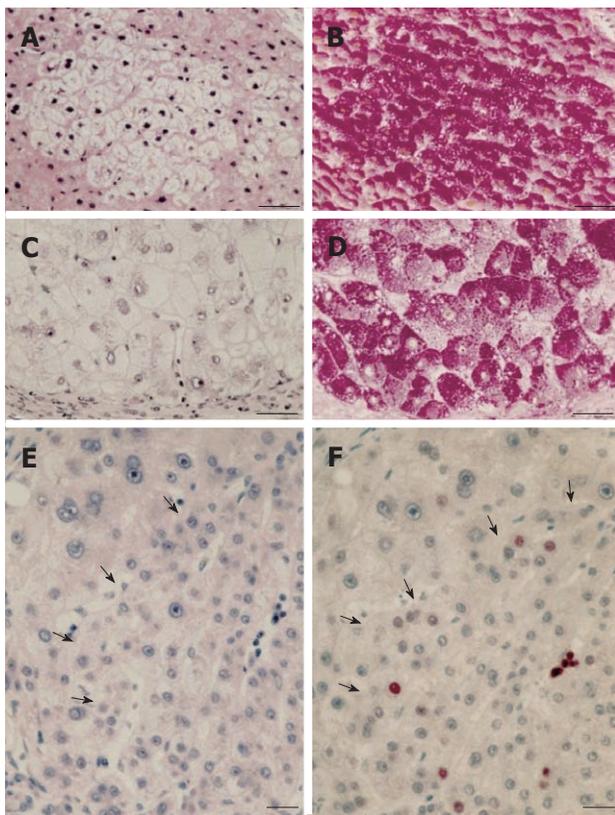


Figure 3 Human focal (A, B) and nodular (C, D) hepatic glycogenosis, and more advanced mixed cell populations (E, F) with intrafocal small cell change. A, B: Hepatic vein. Perivascular glycogenolytic (clear cell) focus in an hepatocellular carcinoma (HCC)-bearing liver with hepatitis B virus (HBV)-associated cirrhosis, demonstrated in serial sections with hematoxylin and eosin (A) and periodic acid schiff (PAS) (B)-reaction counterstained with orange G and iron hematoxylin (Tri-PAS). Bar: 100 μ m; C, D: Glycogenotic (clear cell) nodule in an HCC-free liver with HBV-associated cirrhosis demonstrated in serial sections with hematoxylin-eosin (C) and Tri-PAS (D). Bar: 100 μ m; E, F: Hematoxylin and eosin (E), proliferating cell nuclear antigen (F) immunostaining. Increased cell proliferation (arrows) in a mixed cell focus with less pronounced glycogen storage (not shown) and with intrafocal small cell change in liver with cryptogenic cirrhosis, demonstrated in serial sections. Bar: 50 μ m, from Su *et al*^[68].

exposed for the lifetime or for limited time periods to N-nitrosomorpholine^[3,4]. And it has since been substantiated by a series of morphometric studies^[43-45] and by similar observations in other rodent models of hepatocarcinogenesis elicited by several “genotoxic” and “non-genotoxic” chemicals^[7,41], by local hyperinsulinemia^[18], by hepadnaviridae^[14,15], and by oncogenic transgenes^[17,46]. Most recently, multiple FHG, more advanced types of foci of altered hepatocytes, hepatocellular adenomas and HCC indicative of such a sequence, including an intermediate steatosis, were also observed in a knock-out mouse model with a reduced expression of the mitochondrial protein frataxin, which is responsible for the inherited neurodegenerative disease Friedreich’s ataxia in humans^[47].

The conversion of the highly differentiated glycogenotic clear or acidophilic to the de-differentiated glycogen-poor, basophilic (ribosome-rich) phenotype is associated with a fundamental metabolic switch characterized by a reduction in gluconeogenesis, an activation of the pen-

tose phosphate pathway and the Warburg type of glycolysis as detailed elsewhere^[7,8,24], and by an ever increasing cell proliferation which is inversely related to the gradual reduction of the glycogen initially stored in excess^[48]. Based on these observations, Kopp-Schneider *et al*^[49] developed the so-called color-shift model of hepatocarcinogenesis considering epigenetic changes in parenchymal colonies rather than multiple successive genomic mutations in single cells as the main cause of neoplastic cell conversion induced in the liver by exogenous oncogenic agents. The importance of epigenetic events in chemical hepatocarcinogenesis has been discussed by several authors previously^[7,50], and has been emphasized in recent years by Pogribny *et al*^[51,52].

Human hepatocarcinogenesis

In human pathology, the predominance of clear (glycogenotic) cells (Figure 1A and B) in a minor proportion of HCCs^[6,53], comprising about 8% in 150 cases studied by Buchanan *et al*^[54], and in many hepatocellular adenomas^[6,53-56] is well known. A favorable prognosis of the clear-cell variant of HCC has been reported^[57]. Sasaki *et al*^[58] described two cases of clear-cell HCC associated with hypoglycemia and hypercholesterolemia, and postulated a disturbed glucose metabolism of the tumor tissue, directed to lipogenesis and/or glucogenesis. Acquired FHG has been considered a preneoplastic condition in humans^[6]. This idea was supported by the fortuitous observation of FHG (clear-cell foci) in HCC-bearing livers of children suffering from different disorders^[59,60], in women after long-term use of oral contraceptives^[61], in about 12% of 95 males studied in a consecutive autopsy series in Finland^[62], in patients with Crohn’s disease treated over years with azathioprine which is apparently also responsible for associated HCC development^[63-67], and in a variety of other chronic liver diseases prone to develop HCC^[8,24,53,68,69]. Special cases are patients with genetic hemochromatosis endowed with a high risk of developing HCC, which frequently show FHG excluding iron similar to the iron-resistant FHG observed in experimental hepatocarcinogenesis in rodents^[8,53,69-73].

Particularly relevant are systematic histochemical and histological investigations on the phenotype and proliferation kinetics of foci and nodules of altered hepatocytes in more than 150 explanted and resected human livers with and without HCC^[68,74,75]. The results suggest that foci of altered hepatocytes are proliferative preneoplastic lesions, mixed cell foci (frequently with “small cell change”) being more advanced than FHG (Figure 3), potentially transforming into nodules of altered hepatocytes, highly differentiated HCC containing glycogenotic clear and ground-glass hepatocytes (Figure 4), and eventually also glycogen-poor, basophilic HCC (Figure 1C)^[68]. In keeping with these findings, clear-cell change, steatosis and small cell change have been considered histological features predicting malignant transformation in non-malignant hepatocellular nodules^[76]. Analysis of clonality and chromosomal aberrations in nodules of altered hepatocytes microdissected from cirrhotic livers revealed

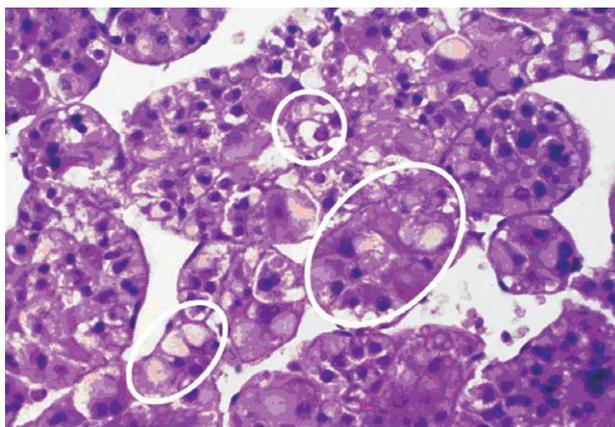


Figure 4 Portion of a human trabecular hepatocellular carcinoma with mixed populations, including clear (glycogenotic) cells (small upper white circle), acidophilic (glycogenotic) ground-glass cells (somewhat larger white circle at the bottom, left), transitions from ground-glass hepatocytes into basophilic cell populations (largest white circle in the middle, right), and basophilic cell populations in the middle and at the bottom right (not marked by circles). Hematoxylin and eosin.

a loss of chromosomal inactivation mosaicism in three large “regenerative” nodules and in all (12) nodules of altered hepatocytes with small cell change, indicating their neoplastic nature^[77]. Even among 60 nodules of altered hepatocytes without small cell change, almost 50% (29) were shown to be monoclonal, whereas FHG and 14 “regenerative” nodules were found to be polyclonal. Interestingly, Cai *et al*^[78] using a similar approach to analyze focal nodular hyperplasia, the pathogenesis and neoplastic nature of which has been debated for decades^[53], found that this lesion, as a whole, is polyclonal, but represents a cluster of nodules of altered hepatocytes, some of which are monoclonal harboring chromosomal aberrations as in hepatocellular adenomas. The lack of genomic alterations in fatty and clear-cell changes in HCC and precursor nodular lesions in cirrhotic livers emphasized by some authors^[79] is in line with the polyclonal nature of many of these lesions^[77], but in view of the increasing evidence for a decisive role of epigenetic events in the development and progression of human HCC^[80] the findings by Laurent *et al*^[79] do not argue against a preneoplastic nature for these cellular changes.

A pronounced hypertrophy of the smooth endoplasmic reticulum was discovered in biopsies from cirrhotic livers and liver cell carcinomas at the light and electron microscopic level (Figure 1B) almost half a century ago, and related to aberrations of glycogen metabolism including glycogenesis from the very beginning^[5,6]. The altered hepatocytes (Figures 1B and 4) were designated as “acidophilic” (or “eosinophilic”). Later on, Popper *et al*^[81,82] found frequent association of this phenomenon with the expression of the hepatitis B surface antigen (HBsAg) localized in the lumen of hypertrophied smooth endoplasmic reticulum (Figure 5), and coined the term “ground glass hepatocyte” which has become an important diagnostic entity in chronic liver diseases elicited by HBV and has dominated the literature since

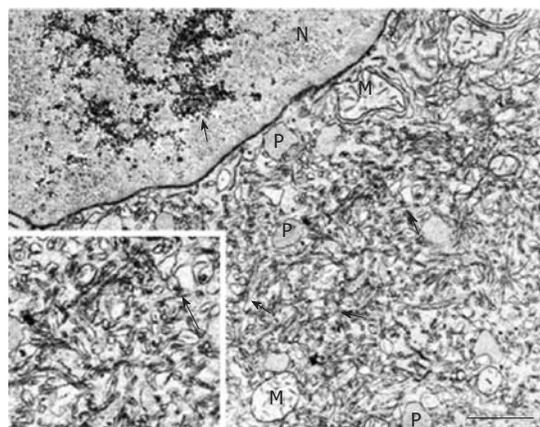


Figure 5 Portion of a ground-glass hepatocyte from a human liver with hepatitis B virus-associated cirrhosis, showing an accumulation of glycogen granules (long arrow) in the nucleus (N) and abundant smooth endoplasmic reticulum containing filamentous hepatitis B surface antigen (short arrows). M: Mitochondria; P: Peroxisome. Transmission electron microscopy, lead citrate. Bar: 1 μ m.

then^[83]. Wills^[84] described HBsAg-free “ground glasslike hepatocytes” exhibiting “glycogen bodies” in liver biopsies from an immunosuppressed (azathioprine, prednisone, clonidine, frusemide) renal transplant patient, similar to those observed in experimental chemical hepatocarcinogenesis^[4,12].

Irrespective of the expression of HBsAg, glycogenotic hepatocytes showing hypertrophy of the smooth endoplasmic reticulum (corresponding to GGH) have often been observed in human FHG, hepatocellular adenomas, and HCC and considered preneoplastic or highly differentiated neoplastic phenotypes^[6,53,68]. In 30 specimens of HBV-associated cirrhosis, GGH were identified in 17 of 25 showing HBsAg expression^[85] (Figure 5). Without mentioning any particular relationship to glycogen, others described GGH containing pre-S mutants in chronic HBV infection, postulating that they represent preneoplastic lesions^[83]. Wisell *et al*^[86] depicted partially persisting “glycogen pseudoground glass hepatocytes” in 12 patients immunosuppressed for numerous indications, but detected neither viral particles nor hypertrophy of the smooth endoplasmic reticulum under the electron microscope. Similar observations were reported by Bejarano *et al*^[87] but no efforts were made in either of these studies to directly compare GGH in serial sections of defined focal lesions at the light and electron microscopic level. Different types of altered hepatocytes, which superficially resemble glycogenotic GGH appearing during hepatocarcinogenesis, have also been observed in several other diseases but will not be further discussed in this context^[83,86,87].

An intriguing form of acquired hepatic glycogenesis was discovered by Mauriac *et al*^[88] in a child with poorly controlled insulin-dependent diabetes type 1. The excessive storage of glycogen resulted in hepatomegaly and was associated with growth retardation, delayed puberty, and a cushingoid face (named after Harvey Williams Cushing). Many additional cases resembling Mauriac’s

syndrome, especially with respect to hepatic glycogenosis, have subsequently been described^[89-91]. Torbenson *et al*^[91] emphasized that this “glycogenic hepatopathy” is an underrecognized complication of diabetes mellitus. In addition to children with insulin-dependent diabetes, hepatomegaly due to glycogen storage has also been recognized in adults afflicted by non-insulin-dependent diabetes type 2 with poor glycemic control^[91-93]. To the best of my knowledge neither GGH nor a relationship of glycogenic hepatopathy to the evolution of HCC in patients suffering from diabetes mellitus has hitherto been described, but it might be timely to take a closer look into this possibility.

The high risk of diffuse glycogenosis characterizing inborn hepatic glycogen storage diseases, particularly glycogen storage disease type I due to glucose-6-phosphatase deficiency, developing into hepatocellular adenomas potentially progressing to HCC has been well established^[2,9,28,94] since the first description of a case by Mason *et al*^[95]. In the meantime, hepatocellular neoplasms are now known to also be occasionally found in other types of glycogen storage disease, namely glycogenoses type III (amylo-1,6-glucosidase deficiency), type IV (α -1,4-glucan: α -1,4-glucan-6-glycosyl transferase deficiency) and type VI (phosphorylase deficiency, Hers disease)^[94,96-99]. I am not aware of any explicit report of GGH in inborn glycogenoses, but from the findings outlined it is obvious that a more detailed comparison of the molecular, metabolic, and morphological aspects of hepatocarcinogenesis in inborn and acquired (focal) hepatic glycogenosis should help to further elucidate the pathogenesis of hepatocellular neoplasms, and facilitate development of appropriate measures for the prevention and therapy of this frequently fatal disease. From a diagnostic point of view it appears to be of great advantage to use the characteristic changes in hepatocellular glycogen content during hepatocarcinogenesis as simple “superficial” histochemical markers of complex basic aberrations at the molecular and metabolic level.

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Targeting late SV40 factor: Is the achilles heel of hepatocarcinogenesis revealed?

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Abstract

Hepatocellular carcinoma (HCC) is a dreadful cancer and a major cause of death among patients with chronic liver disease and cirrhosis. The apparent alterations in a diversity of intracellular pathways found in HCC has set the rationale for developing molecular-directed drugs that simultaneously inhibit multiple pathways, such as the multi-kinase inhibitor Sorafenib. However, recently this concept has been challenged by showing that HCC is heavily dependent on a single oncogene designated late SV-40 factor (LSF), a transcription factor that is over-expressed in liver cancer cells and that its expression is strongly correlated with tumor grade and aggressiveness. Furthermore, using an intensive screening for drugs that inhibit LSF activity, Grant *et al* have found a molecule designated factor quinolinone inhibitor 1 that can specifically block the ability of LSF to bind its target promoters, resulting in a massive death of HCC cells both *in vitro* and *in vivo*. The innovative findings of HCC representing "oncogene addiction" to LSF and the ability of a single molecule to block the activity of this oncogene resulting in tumor abolishment are encouraging and provide us with the hope that the "Achilles heel" of HCC has been found.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in men and the seventh in women, and its incidence has been sharply rising during the last two decades^[1]. Whereas patients with localized disease can benefit from curative therapeutics modalities, such as liver resection, liver transplantation or radiofrequency ablation, until recently little could be offered to patients with advanced disease, whose survival was often measured in a few months^[2].

MULTIPLE PATHWAYS ARE DISTORTED IN HEPATOCELLULAR CARCINOMA

Disruption of a variety of cellular pathways, as well as mutations in tumor suppressors and oncogenes have been described in patients with HCC^[3]; mutations in the genes encoding for *TP53* and β -catenin, amplifications of the vascular endothelial growth factor (VEGF) and Cyclin D1 (*CCND1*) genes, silencing of E-Cadherin tumor suppressor and activation of the myelocytomatosis viral oncogene are a partial list. Indeed, the more effort

invested in elucidating molecular mechanisms underlying HCC, the clearer it became that the complexity and diversity of such mechanisms make the development of potential gene-targeted drugs a highly complicated task.

SORAFENIB-THE FIRST APPROVED MOLECULAR-DIRECTED DRUG FOR HEPATOCELLULAR CARCINOMA

In 2008, Llovet *et al*^[4] have published the results of a phase 3 multi-center placebo control trial showing that the multi-kinase inhibitor Sorafenib prolongs survival in patients with advanced HCC. The rationale for using a multi-kinase inhibitor that simultaneously blocks Raf, VEGF, platelet-derived growth factor and c-Kit signaling is to “cover” as much diverse signaling pathways involved in hepatic carcinogenesis as possible. However, although the introduction of this first molecular-directed drug is a major breakthrough in the field of HCC, the results are still far from optimal as reflected by the only modest improvement in life expectancy of 2.8 mo on average in the price of often-severe adverse events^[5,6].

LATE SV40 FACTOR-AN ESSENTIAL ONCOGENE IN HEPATOCELLULAR CARCINOMA

Recently, Yoo *et al*^[7] has recognized the Late SV40 factor (LSF) transcription factor as a central oncogene in HCC. LSF, which is induced in the liver via inflammatory cytokines, has a major role in DNA synthesis by transcriptionally activating the rate-limiting enzyme in pyrimidine synthesis, thymidylate synthase, as well as other target genes important for DNA synthesis and cell survival. LSF inhibition results in constraining DNA synthesis, which ultimately leads to cell death. Interestingly, Sarker's group has found that LSF protein is significantly over-expressed in HCC cells as compared to normal human liver cells. Furthermore, the degree of LSF expression revealed a significant correlation with the stage and grade of the tumor. Most importantly, whereas over expression of LSF pushed the tumor towards a more aggressive phenotype, LSF inhibition resulted in abrogation of tumor growth and its metastatic potential, both *in-vitro* and *in-vivo*. Interestingly, among the various genes induced by LSF following its binding to their promoters, *SPP1* gene encoding for the osteopontin (OPN) protein has been found to be induced to the greatest extent. Indeed, the importance of OPN in promoting hepatic carcinogenesis was recently emphasized by a study showing that OPN is much more sensitive than the traditional alpha-fetoprotein as a marker for early HCC^[8]. Whatsoever, Sarker's work as well as a later work by Fan *et al*^[9] strongly indicated that LSF is an essential oncoprotein required for the maintenance and propagation of liver cancer, making it a potentially ideal target for HCC treatment. Furthermore, the merit of these findings lies

in the fact that although multiple genes and signaling pathways are impaired in HCC, the cancerous liver cells are heavily dependent on a single oncogenic protein, the transcription factor LSF, for their survival. This phenomenon, designated “oncogene addiction”, has been recognized in various cancers in the last few years, making the oncogene to which a particular cancer is addicted to an ideal target for anti-cancer therapy^[10,11].

FACTOR QUINOLINONE INHIBITOR 1 SPECIFICALLY INHIBITS LATE SV40 FACTOR ACTIVITY RESULTING IN ABOLISHMENT OF HEPATOCELLULAR CARCINOMA

However, how can one translate experimental abolishment of LSF achieved mainly by dominant negative constructs or knockdown strategies to a drug that inhibits LSF function *in-vivo* and that can be easily delivered to the liver?

A study recently published in the PNAS provided an unexpected and exciting solution to this problem^[12]. By screening for small molecules that could block the interaction of LSF to its DNA binding sites along the genome, Grant *et al*^[12] have revealed a small molecule named factor quinolinone inhibitor 1 (FQI1) that inhibits LSF DNA binding activity both *in-vitro* and *in-vivo*. Functionally, treating HCC cells with FQI1 results in a massive apoptosis of HCC cells whereas normal hepatocytes remain intact. FQI1 treatment results in a robust activity *in-vivo*, as well, reflected by inhibition of tumor growth in mouse HCC xenografts. Importantly, no toxicity was observed in FQI1 treated animals, as evaluated by animals' general well being and by careful examination of various non-hepatic tissues that remained intact following treatment. Noteworthy is the observation that tumors from FQI1-treated animals expressed LSF at similar levels to those of control mice, whereas the expression of a central LSF target gene, OPN, as well the proliferative activity of the tumor were dramatically reduced. This observation reflects the inhibition of LSF activity as a transcription factor by blocking its binding to DNA, rather than reducing its level following FQI1 treatment. Furthermore, the close correlation between the concentrations of FQI1 required for inhibition of LSF transactivation and those required for proliferation inhibition strongly suggest that FQI1 specifically targets LSF and does not share a general non-specific anti proliferative activity.

FQI1 AS AN EMERGING ANTI HEPATOCELLULAR CARCINOMA DRUG-PROMISES AND CHALLENGES

The originality and the importance of the aforementioned studies are dual. First, the identification of a single oncogene serving as a cellular transcription factor to

which HCC is “addicted” and completely dependent on. This finding may completely change the current concept of using drugs inhibiting multiple alternated cellular targets^[5], to a strategy that specifically inhibits a particular target that is essential for HCC maintenance and propagation. Second, in contrast to what was formally considered as an almost impossible target for drug therapy, the efficiency of FQI1 strongly indicates that targeting the activity rather than the level of a transcription factor is an effective and specific mechanism for an anti-cancer drug.

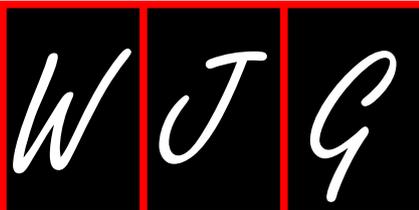
Further studies should address potential caveats and open question remaining before implementing those findings to an efficient anti-HCC drug. The bioavailability of FQI1 in human subjects following treatment should be carefully checked and the long-term consequences in terms of adverse effects should be monitored. The potential for tumor resistance due to mutations in the LSF DNA binding domain is certainly there and should be taken into consideration. In this regard, the combination of the current molecular-targeted drug, Sorafenib, with LSF inhibitors has the potential to minimize the risk of cancer cells “escaping” their oncogene addiction to LSF. Last but not least, the validity of LSF role in HCC should be ascertained for the various etiologies of HCC, including viral, metabolic and toxic.

In summary, the introduction of a small molecule that specifically inhibits the activity of an oncogene on which HCC heavily depends seems as stimulating news to the field. Time will tell whether, similar to the Greek mythology, LSF represents the Achilles heel of HCC, an innovation that can ultimately lead to defeating this deadly cancer.

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Thinking in three's: Changing surgical patient safety practices in the complex modern operating room

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Abstract

The three surgical patient safety events, wrong site surgery, retained surgical items (RSI) and surgical fires are rare occurrences and thus their effects on the complex modern operating room (OR) are difficult to study. The likelihood of occurrence and the magnitude of risk for each of these surgical safety events are undefined. Many providers may never have a personal experience with one of these events and training and education on these topics are sparse. These circumstances lead to faulty thinking that a provider won't ever have an event or if one does occur the provider will intuitively know what to do. Surgeons are not preoccupied with failure and tend to usually consider good outcomes, which leads them to ignore or diminish the importance of implementing and following simple safety practices. These circumstances contribute to the persistent low level occurrence of these three events and to the difficulty in generating sufficient interest to resource solutions. Individual facilities rarely have the time or talent to understand these events and develop lasting solutions. More often than not, even the most well meaning internal review results in a new line to a policy and some rigorous enforcement mandate. This approach routinely fails and is another reason why these problems are so persistent. Vigilance actions alone have

been unsuccessful so hospitals now have to take a systematic approach to implementing safer processes and providing the resources for surgeons and other stakeholders to optimize the OR environment. This article discusses standardized processes of care for mitigation of injury or outright prevention of wrong site surgery, RSI and surgical fires in an action-oriented framework illustrating the strategic elements important in each event and focusing on the responsibilities for each of the three major OR agents-anesthesiologists, surgeons and nurses. A Surgical Patient Safety Checklist is discussed that incorporates the necessary elements to bring these team members together and influence the emergence of a safer OR.

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Key words: Complex adaptive systems; Wrong site surgery; Retained surgical items; Retained foreign objects; Retained foreign bodies; Surgical patient safety; Surgical fires; Safety checklist

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INTRODUCTION

Operating rooms (ORs) used to be just complicated places, but the modern OR has changed. No place epitomizes the complexity of health care delivery better than the OR where there is the routine interface of heterogeneous, variously trained personnel using high technology

equipment while providing service to an unconscious, anesthetized patient. In fact it is most helpful to think of the modern OR as a complex adaptive system that consists of (1) heterogeneous interdependent decision making agents; (2) who interact frequently with each other; and (3) develop a characteristic called emergence which arises when the whole actually begins to perform better than the sum of its parts^[1]. That is through the learning and interdependence of the heterogeneous agents a larger, higher functioning system is created. Anyone who has worked in a highly functioning OR over time can understand how this entity can evolve but also how rare and difficult it is to achieve.

Today it is expected that the delivery of surgical care will be (1) knowledge-driven (safe, effective); (2) patient-centered; and (3) system-based (timely, efficient and equitable)^[2]. Hospitals and the surgeons who operate within them are also learning that if the care is not of high value, that is high quality surgical outcomes at low total costs of care, there will be low or no reimbursement for their effort^[3]. Thus begins another step in the journey to stimulate hospitals and surgeons to accelerate improvement in the quality of surgical care and increase efforts to prevent errors and treatment complications. Multiple stakeholders have begun to reengineer or design, adopt and implement new OR practices and refined communication methods in order to reduce errors and waste. The specific surgical patient safety events are uncommon but the effort needed for prevention requires innovative solutions and illustrate the difficulty in changing OR culture (e.g., shared customary beliefs, values and behaviors).

The three main preventable surgical patient safety events which occur within the OR are (1) the surgical wrongs; (2) retained surgical items (RSI); and (3) surgical fires. The incidence of all three combined may affect at most 5000 patients a year (approximately 2000 wrong surgery events, 2000 RSI, 500 surgical fires) in the United States. National sentinel event disclosure is disparate and depends on state-based and regulatory agency voluntary reporting. Data on close-calls, which are likely to be more frequent, remain sketchy. No matter the total number of events, we do know that the number is greater than zero and therefore these are still not “never events”.

It has been tempting to use a “person approach” (e.g., the forgetfulness, inattention, ignorance, carelessness of individuals) in the analysis of why these events occur but we know that a “system approach” (e.g., analysis of the conditions under which individuals work and how defenses failed) that incorporates human factors (e.g., the interaction of human abilities, expectations and limitations) while harder to design and implement is likely to be more successful^[4]. These safety problems are reflective of the culture of safety in the OR rather than on patient or case characteristics. The people who work in the OR make it safe and it is important to know that there are subtle behaviors that aren't well quantified

but are highly valued that arise from the development of expertise. Expertise is the result of knowledge and experience. The behaviors are intuitive and have been recognized as characteristics of “safe people”^[5]. These feelings and intuitions are very important to nurture and develop but do have a quality of mystery to them but it is highly desirable for an organization to value expertise and develop “safe people”. When hospitals address these safety issues and there is culture change there may be positive consequences which decrease or prevent other more common perioperative occurrences such as surgical infections and venous thromboembolic events. ORs have not yet been characterized as highly reliable organizations (akin to nuclear reactors or aircraft carriers) so there is much room for improvement.

The current projects developed to help hospitals take action to prevent wrong site surgery, RSI and surgical fires focus on specific OR practice and communication problems. Unsafe OR practices are usually highly variable and efforts to standardize multi-stakeholder processes of care and develop specific communication modes are shared core elements in prevention strategies. Practice change and communication improvements are often viewed disparagingly as “soft” approaches which lack rigorous data and experimental validation however making sense of the rare and unexpected requires different tools and approaches^[6]. Increasingly, event reporting and analysis of close calls have informed failure modes and collective learning on how to manage unsafe acts which is just as important as preventing them. To change OR culture requires change in the practices of the people who are engaged in the work. To change their practices you have to change the relationships with whom they work and the environment in which they work.

Finally, a surgical patient safety checklist which is a simple, widely applicable, inexpensive, unifying tool, can be used by all OR personnel to enforce the individual safe practices which have programmatically been developed and enhance inter-personnel communication^[7]. We describe how one can be used to teach and enforce safer practices and address serious and avoidable surgical complications^[8].

WRONG SURGERY

The three types of wrong surgery are (1) wrong patient surgery, where the performance of an operation or procedure is carried out on a person other than the one for whom the procedure was intended; (2) wrong procedure surgery, is the performance of a procedure other than that intended or indicated for a specific patient; and (3) wrong site surgery where a planned procedure is performed at the wrong place, part, level, side or site^[9]. Wrong site surgery is a type of wrong procedure surgery and is the most common occurrence of the three^[10]. All three of these events are usually the result of mistakes in correctly applying identification procedures.

The Joint Commission (IJC) has been at the fore-

front of the wrong site surgery issue since 1998 when the first Sentinel Event Alert newsletter on the subject was printed. Subsequently TJC published a follow-up alert in 2001, in 2003 the first Wrong-Site Surgery Summit was held and in 2004 the Universal Protocol was launched. In spite of all of these efforts, it has most recently been estimated that wrong site, wrong procedure and wrong patient cases still occur more than 40 times a week in the United States^[11].

The essential elements of the Universal Protocol mandate that OR teams must (1) complete a preoperative verification process; (2) mark the operative site; and (3) take a “time out” immediately before starting the procedure. The verification process is to ensure that the correct patient has been consented and is present for the operation and all relevant documents and studies are available before the procedure is performed.

The most important elements are that the three major primary operative sources: the history and physical, the consent, and the official OR schedule are in exact agreement. If there are discrepancies the patient is considered the primary source for rectification. Patient verification and identification practices are usually performed by nursing and anesthesia personnel with requirements that at least one of these stakeholders must remain in constant attendance with the patient while being brought into the OR once patient identification has been performed. The site marking is within the domain of the surgeon and must be performed before the patient is brought into the OR and should be visible while the patient is prepped and draped. The mark must be visible within the operative field and referenced during the time out. Recent alcohol based prep solutions frequently diminish the site mark and practices must be established to use different inks or have a process in place whereby the person who performed the prep can refresh the diminished mark with a sterile surgical marker to ensure that it will be able to be seen before an incision is made. The time out is truly a team based activity called by the surgeon, monitored and orchestrated by the circulating nurse with responses from anesthesia and any other providers. The time out is usually performed just before the incision is made and all team members have to stop all activities. At the time out the three questions are answered: (1) Who is this patient? (2) What operation is to be performed? and (3) Is this the correct site mark, using secondary confirmatory evidence present on an armband or on the operative consent and unanimity from all stakeholders is required. Many facilities have checklists and scripted procedures with hard stops (e.g., the scalpel isn't passed until the time out is completed) to enforce compliance. All questions are supposed to be asked in a non-intimidating environment where all staff feel equal in their contribution to safe patient care. These conditions have been challenging to meet.

While the Universal Protocol as a directive was comprehensive and widely mandated the implementation and effectiveness of the protocol was variable^[12]. Review

of wrong site surgery cases reveal that multiple errors occurred along the OR journey and multiple defenses failed which might have caught the mistakes^[5]. Inadequate standardization of practices and poor training and enforcement of practices provide the latent factors which exist that sets OR personnel up for failure. More than just the directive to use the Universal Protocol appears to be needed and hospitals need guidance on how to effectively make the process work.

To meet this goal, in July 2009 TJC started an eight hospital demonstration project using a Robust Process Improvement method that is a fact based, systematic and data-driven problem solving methodology^[13]. Project teams are developed for observational audits looking to discover specific risk points and contributing factors that lead to close calls and wrong site surgery events. The timeframe begins at the time of scheduling a surgical procedure and ends with confirmation that the intended operation was correctly performed.

Preliminary results from TJC project have shown that in 39% of cases, errors were introduced in the verification step that increased the risk of a wrong site surgery event^[11]. Usually these errors involved inadequate information about the patient and scheduling confusion. Identifying this failure mode lead to the development of standardized ways of collecting and having the information accessible. Site marking and time out practices have been contributory but were not as frequent a source of errors as the initial verification process^[5]. Further refinements to improving this practice will be available at TJC Targeted Solutions Toolkit which will provide a step-by-step process to measure performance^[13].

RETAINED SURGICAL ITEMS

A RSI occurs when surgical material, usually a sponge, needle, instrument or miscellaneous small item or device fragment is inadvertently left inside a patient. Retained surgical item is the preferred term rather than retained foreign body or object since bullets, shrapnel or ingested objects can be retained but aren't the result of surgical error. An RSI is a surgical patient safety problem and results from problems in unreliable surgical OR practices and problems with communication between OR stakeholders^[14].

Reports of RSI have relied on mandatory state and voluntary regulatory agency reporting requirements which vary as does the definition of what is considered retention. The National Quality Forum's (NQF) definition is most frequently referenced and in 2011 all NQF Serious Reportable Events were reviewed which included the issue of surgical retention. The new directive excludes reporting of unremovable items intentionally left at the judgment of a surgeon and most importantly addressed the definition of when an item is considered retained after surgery. Many states construed “after surgery” to mean closure of the incision, which meant even in cases where the sponge or surgical item was

discovered to be missing while the patient was on the OR table, and was removed in the OR without delay or harm, if the wound had been closed these cases were reported as a RSI. With the new 2011 definition, this area has been corrected and the operation ends after all incisions or procedural access routes have been closed in their entirety, devices have been removed and if relevant, final surgical counts have concluded and the patient has been taken from the operating/procedure room^[15]. In 2011 an item is considered to be retained only if found after the patient is out of the OR or procedure room. This change alone may lead to decreased numbers of RSI cases.

The most common RSI is the cotton gauze surgical sponge which has been found in the abdomen/pelvis, chest and increasingly in the vagina. Retained sponges occur after any type of surgical procedure and the risk of retention is unrelated to the number of sponges used during an operation. Sponges have been retained when only 10 sponges were used and small biopsy or skin incision made. This finding has led to policy changes which should require that surgical sponges must be accounted for in all cases in which surgical sponges are used and an incision is made^[16]. There should be no determinates for case exclusion because of size of the incision or length of the case.

Traditional means of accounting for sponges has relied on the longstanding practice of counting. Observational audits and focused reviews of cases of retained sponges has shown that the practice of counting sponges is highly variable between ORs and even within rooms in the same OR suite. This variation leads to sources of error and with increased demands of the OR environment, frequent change of shift between nursing personnel and multiple diversions the common practice of counting has proven unreliable. Examination of cases of retained sponges have often revealed that the sponges were counted during the procedure but no one knows where the error occurred or how the sponge was retained. In fact, overall about 80% of retained sponge cases occur in the setting of a correct count^[17]. That is at the end of the case the counts were called correct. In about 20% of cases of retention there was a known incorrect count at the end of the case, something was missing, yet the patient still got out of the OR without finding the sponge. The missing item is subsequently discovered sometime later. This occurs because of knowledge and communication problems usually at the interface with radiology, the taking and interpretation of xrays or with inadequate follow-up at the next level of care to determine if a sponge has been retained. Because of these observations about the counting of sponges a revised manual sponge management practice was developed by the NoThing Left Behind[®] Surgical Patient Safety Project^[17]. The practice is called Sponge ACCOUNTing that uses the adjunct of plastic blue-backed hanging sponge holders and wall mounted dry erase boards posted in each OR on which to record in a standardized manner the surgical counts.

Sponge ACCOUNTing was developed to standardize and improve the transparency of the practice of sponge management. The guiding principles have outlined three important steps to ensure that all sponges used in the procedure have been accounted for with important unique actions for each OR stakeholder during the case and a team based “show me” step at the debriefing or end of the case. The nursing essence is (1) that all sponges are only used in groups of 10 and each plastic sponge holder contains 10 pockets; (2) at the natural pause point which occurs at the time wound closure begins, it is the job of the surgeon to perform a methodical wound exam (MWE), not just a swish or sweep of the wound, but to do the best job possible to look for and remove any sponges (or other items not intended to remain in the patient) and give them to nursing staff; and (3) the last step is at the end of the case when the skin is closed and all of the sponges (the used and unused sponges) must be off the field and in the pockets of the plastic hanging sponge holders. Since there are 10 pockets per holder and sponges are managed only in multiples of ten there should be one holder for each 10 sponges and there should be no empty holder pockets at the end of the case. The team based activity is for the surgeon to say “show me the holders” so they can see that there are no empty pockets or for the nurse to say to the surgeon “here let me show you”, there are no empty pockets. Who shows who doesn't matter, it's that there is a visual confirmation that all sponges have been accounted for.

There are now three technological adjuncts for sponge management (1) a device which counts 2D matrix labeled sponges; (2) a device which detects radiofrequency tagged sponges; and (3) devices which can count and detect RadioFrequency IDentification (RFID) chip embedded sponges. The essential components of each device are a distinct type of detection element attached to a surgical sponge and a distinct compatible electronic readout system.

The computer assisted sponge counting system consists of two-dimensional matrix labeled sponges and a scanning device that can read the labels^[18]. The matrix label is scanned in with a handheld or table mounted scanner as the sponges are put on the sterile field and then each sponge is scanned out when the sponges are removed from the table. The matrix labels are embedded onto surgical sponges of various sizes and each sponge has a unique identifier that enables the scanner to count different types of sponges. The sponges are counted maintaining “line of sight” for each sponge. In order to account for all sponges at the final count, the sponges must be removed from the patient and individually passed under the scanner. The scanner has no capacity to “read-through” the patient and detect the presence of a matrix labeled sponge. In the event of a missing sponge an X-ray is used to determine if it is in the patient. This system is in place in many hospitals and most recently the experience from the Mayo Clinic has been reported^[19]. After 18 mo of use the device accurately

counted all sponges in all cases throughout the hospital and there were no cases of retained sponges.

The radiofrequency detection system consists of sponges that have a small passive radiofrequency tag sewn into a pocket on each sponge and a handheld wand or mat which contain the antennae and detection system^[20]. The tag is 4 mm × 12 mm and is recognized as only a yes or no signal. The tag is detected when the handheld wand or mat is activated and the computer console presents a visual and audible signal that a sponge has been detected. The system does not distinguish between sponge types or number of sponges. The signal readout will be the same intensity if there are one or five sponges. In the event of a missing sponge the mat can be activated to determine if the sponge is in the patient or the wand can be used to wand the patient or scan the trash to find the sponge. This system does not count sponges so at this time is usually used in conjunction with a manual counting practice such as Sponge AC-COUNTing. Some hospitals have adopted mandatory wand protocols to be used for specific types of cases, others require wand in lieu of using xray to find a missing sponge. There is an early trial from the University of North Carolina in the assessment and safety of this device and after 24 mo they have not had any retained sponges^[21].

RFID systems have a unique RFID chip sewn into each type of sponge and a separate computer console with a scanning bucket into which used sponges are placed^[22,23]. Each sponge has a specific RFID chip and thus sponges of different types pooled together can be distinguished and counted. Unopened packages of sponges are placed on a front panel of the console to be electronically counted-in and the sponges are then opened and placed on the sterile field. Used sponges can be put directly into the bucket or into plastic-bag lined kick buckets and the entire plastic bag full of sponges then placed into the scanning bucket. The sponges will all be individually counted-out. If there is a missing sponge it can be detected with a wand. All of these devices are undergoing clinical assessments in different hospitals around the world.

With the continued improvement in the prevention of retained sponges, reports of retained miscellaneous small items and unretrieved device fragments are increasing^[17,24]. The preventive strategy for these types of items are not applicable only to the OR since retained guidewires, sheaths and catheters are found after interventional vascular, cardiac and radiological procedures performed in various sites throughout a hospital. In addition various types of providers now must develop standardized processes to account for all of the items and parts of devices at the conclusion of invasive procedures. This is new territory for interventional radiologists and cardiologists and practices originating in the OR can be shared with these other clinical groups to help speed accountability.

In the OR, small miscellaneous items or broken parts or pieces of tools and material used during an operation

when found to be missing may require a clinical decision to leave the item behind because the risk of removal may cause greater harm than leaving the object behind. If a small item or un-retrieved device fragment is intentionally left behind, the patient must be informed. Leaving fragments and small objects in patients can be prevented because often human factors are involved in why the devices or objects break or fracture. Identifying early when an item is missing or broken means best practices in the OR will involve the coordination of actions between the surgeon and the surgical technologists who must become the content experts on the equipment. If something is missing or broken the scrub person has to recognize the defect and speak up so actions to find the missing part can be undertaken. Retained whole instruments are exceedingly rare and previous reports that indicated instruments as the second most common item most certainly included small devices and fragments in this category rather than the separate class in which they are now considered. Inside or out of the OR, standardized practices have to be established that are transparent, widely applicable and simple to perform so multiple providers at the completion of every case, delivery or invasive procedure can be sure there is “NoThing Left Behind”.

SURGICAL FIRES

OR fires occur near or around a patient but a surgical fire is a fire that occurs in or on a patient and includes airway fires^[25]. Surgical fires are the rarest of the three surgical patient safety events with an estimate from a 2009 study suggesting that between 500-600 fires a year occurred in the United States^[26]. This national estimate was based on data from the Pennsylvania safety event reporting system which is the best available evidence. Surgical fires are dangerous, lethal and preventable but are so infrequent that most surgeons are painfully unaware of what preventive measures need to be taken.

A surgical fire requires the classic constellation of three components, “the fire triad”: (1) an oxidizer; (2) an ignition source; and (3) fuel. The breadth of elements and circumstances that exist in each of these components are underappreciated and make it difficult to easily standardize or develop simple practices^[27,28]. For example, possible fuels sources can include almost anything on the patient or in a surgical field: tracheal tubes, sponges, drapes, gauze, alcohol or other volatile compound containing solutions, oxygen masks, nasal cannulae, hair, fat, dressings, gowns, gastrointestinal tract gases, suction catheters, cable coverings, gloves and packaging materials. The ignition sources can be from almost any energy generating device and all differentially function or have the potential to cause injury based upon the oxidizer enriched atmosphere.

In 2007 the American Society of Anesthesiologists published a practice advisory for the prevention and management of OR fires which provides a complex algorithm that outlines a risk assessment approach and action plans for surgical providers^[25]. In 2009, further

recommendations were promulgated for oxidizer management and communication guidelines to follow for prevention^[26]. These efforts are thorough but the end results are still highly complex and detailed and because of the rarity of surgical fires it remains difficult to engage providers. Otolaryngology surgeons are the most familiar with surgical fire prevention measures because they frequently operate with ignition sources near the airway, within a highly oxidized atmosphere however all types of surgeons need to learn and adopt safer surgical fire preventive strategies because all types of cases actually provide risky conditions. To this end a provider oriented accountability approach for surgical fire management has been developed which assigns responsibility for action to specific stakeholders. This protocol teaches providers to know their role in preventing fires and respond quickly should a fire occur^[29,30]. The roles for prevention are based on the domains of control for each of the elements in the fire triad. Anesthesiologists have the control and direct ability to act on the management of oxidizers which includes oxygen and nitrous oxide. Nurses have control and direct ability to monitor the fuel sources and provide immediate remedy to extinguish flames with saline. The Surgeon has control over the ignition source and is responsible for assessing the environment before starting or using electrocautery.

Surgical fire prevention requires constant situational awareness because of the changing nature throughout an operation of the availability of different fuels, oxidizer status and ignition sources. Information exchange between the anesthesiologist, nurse and surgeon are key. If a fire breaks out rapid responses from all three stakeholders are important and some suggest fire drills should be conducted for high risk cases so team members are adequately primed to respond appropriately. If there is the risk of an airway fire the anesthesiologist must keep the surgeon aware of oxidizer concentrations and atmospheric conditions and be ready to stop all airway gases and remove the tracheal tube. The surgeon must have thoughtful control and use of cutting devices while nursing personnel in the scrub position must be ready with saline and wet sponges. All of these coordinated actions will have to take place in seconds in order to save the patient from extensive injury. To this end, surgical fire case-specific risk assessment strategies are useful activities and allow all providers to be primed at the time and ready to go rather than relying on memory or remote training. Surgical fire risk assessment can be incorporated into part of the operative briefing and safety checklist so each case can be assessed and necessary steps taken before the case starts to make sure all team members know what to do and are on the same page^[8].

SURGICAL PATIENT SAFETY OR CHECKLIST

Common to the prevention of surgical patient safety events and mitigation of harm is the need to have all

team members prepared to address events as they occur. The nature of preparedness involves having knowledge and the appropriate skills and tools available. Simulation training and drills are ways in which many industries maintain preparedness. The use of checklists as an adjunct to the pre-existing skill set of team members who come together has also been important to make sure all necessary tools and practices are performed correctly.

In 2009 a surgery safety checklist was first introduced which was beyond what was traditionally used by nursing services to make sure the patient was prepared for surgery. This 19 item checklist was divided into three parts: (1) sign in activities; (2) time-out activities; and (3) sign-out activities which covered the recommended practices the World Health Organization (WHO) had developed to ensure the intraoperative safety of surgical patients worldwide^[7]. The elements were simple and were intended to enhance inter-professional communication and prevent surgical events that may result from poor teamwork and inadequate surgical operative preparation. This checklist was adopted and disseminated by the Institute for Healthcare Improvement and has been in place in more than 3900 hospitals around the world^[31]. Each facility is encouraged to develop a surgical safety checklist that will conform to their site-specific conditions but at the minimum the original 19 points are strongly encouraged to remain. Many surgical subspecialties e.g., ophthalmology have customized the original WHO safety checklist to their needs and recent work has been started on the development of crisis checklists for the operating room which aim to guide providers to perform the correct actions in intraoperative crises^[32]. Implementation of some form of a surgical safety checklist has been widespread and the benefits of checklist use continue to be evaluated.

There is no one surgical safety checklist that is “the best” but there are many which provide the necessary information for all stakeholders to come together and make sure the correct patient is being operated on, the correct operation is known and the operating team is properly prepared to perform that operation safely. A surgical patient safety checklist should help surgical providers address the preventative solutions to prevent the wrongs, RSI and surgical fires at a minimum^[33]. An example of a surgical patient safety checklist that covers this territory is included in the reference section^[34]. The checklist is used three times during a patient’s operation (1) as an aid during a pre-operative briefing that is conducted when the patient is first brought to the OR; (2) during the time-out; and (3) as a reporting tool during the de-briefing that is held at the end of the case to discuss what problems occurred and also what went well.

During the pre-operative briefing patient elements are reviewed, such as procedure verification and imaging, antibiotic administration, venous thromboembolism prophylaxis management, beta-blocker use, allergy history and blood availability but more importantly the surgical team puts together a surgical fire risk assessment score.

If the fire risk is considered high, based on a simple 3 point scoring system, then each of the three stakeholders in fire prevention go over what they are supposed to do as outlined on the back of the checklist. In this way the circulating nurse and surgical technologist, the surgeon and the anesthesiologist discuss what maneuvers and actions will be taken during that specific high risk case. During the time-out the three essential questions are answered using the checklist as a memory jogger and confirming primary source information in the computer or on the patient's armband. These actions prevent the occurrence of a wrong site surgery. At the de-briefing, all surgical items are accounted for, pathology specimens are reviewed to determine that they are correct and labeled correctly and any equipment or instrument problems are noted with specific referral units noted who are to take action for remediation thus preventing RSI.

CONCLUSION

The three surgical patient safety events, wrong site surgery, RSI and surgical fires can be prevented and even if prevention strategies fail mitigation of patient harm can be accomplished with application of consistent safety practices and effective communication. Effective communication includes the use of a comprehensive surgical patient safety checklist which brings all surgical providers together for at least a few moments to have a shared mental model for the patient's surgical care. The three major stakeholders working together in our complex OR environment are nurses, surgeons and anesthesiologists who face ever increasing work demands and knowledge requirements. It is not humanly possible to master all the knowledge domains for all safe practices for these three events, let alone all the other things that are necessary for the performance of an uncomplicated procedure. However, thought modules, checklists and assignment of areas of expertise and responsibility can be helpful in getting humans to work effectively and efficiently together. Of the three surgical patient safety events, it is not a stretch to see that there are knowledge domains which can drive improved performance and the three domains of expertise are possessed by each of the three major stakeholders. The content experts for wrong site surgery are surgeons, for RSI it is nurses and for surgical fires it is anesthesiologists.

Prevention of wrong site surgery is highly dependent on preoperative practices of patient identification and patient preparation in which the surgeon's knowledge and preparation of the primary documents and materials falls within their care domain. It is the surgeon who has determined what operation is to be performed, has marked the operative site and has seen the patient at some time prior to their arrival in the OR. During the surgeon activated time-out confirmatory actions are conducted in concert with the use of a checklist to become the primary means of wrong site surgery preventive actions. The prevention of RSI strongly depends on actions under the control of the scrub person and

circulating nurse who track and account for all the surgical material which is used. The use of a reliable sponge management practice in concert with a MWE is essential to prevent retained surgical sponges. The nurse also provides the interface with other stakeholders such as radiology should intra-operative x-rays be needed. For prevention of surgical fires it is the anesthesiologist who monitors oxygen status and has the situational awareness to alert and direct actions of the other stakeholders in the event of a fire. This is not to say that the other stakeholders don't have roles and responsibilities but when providers of care come together to perform a surgical case there is a team leader. The role of team leader is actually not fixed and immutable. It will have to change as the circumstances of the operation change and the team leader interestingly enough should be the person who is the content expert of that situation as it arises.

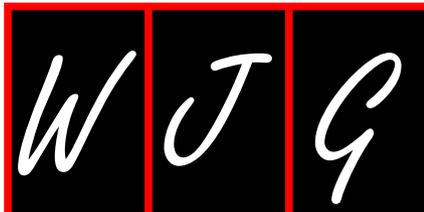
It is not possible for everyone to know everything about all things. It is possible for the content experts of each of their domains to be present, willing and able to share their knowledge and communicate effectively with other personnel. Rather than thinking one person has to know it all and do everything, thinking in threes can help all providers put a vast amount of information into some useable context where it will be up to the three major stakeholders to work together consistently in every case to prevent the three major surgical patient safety events.

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Modern treatment of gastric gastrointestinal stromal tumors

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Abstract

Gastrointestinal stromal tumors (GIST) are rare mesenchymal smooth muscle sarcomas that can arise anywhere within the gastrointestinal tract. Sporadic mutations within the tyrosine kinase receptors of the interstitial cells of Cajal have been identified as the key molecular step in GIST carcinogenesis. Although many patients are asymptomatic, the most common associated symptoms include: abdominal pain, dyspepsia, gastric outlet obstruction, and anorexia. Rarely, GIST can perforate causing life-threatening hemoperitoneum. Most are ultimately diagnosed on cross-sectional imaging studies (i.e., computed tomography and/or magnetic resonance imaging in combination with upper endoscopy. Endoscopic ultrasonographic localization of these tumors within the smooth muscle layer and acquisition of neoplastic spindle cells harboring mutations in the *c-KIT* gene is pathognomonic. Curative treatment requires a complete gross resection of the tumor. Both open and minimally invasive operations have been shown to reduce recurrence rates and improve long-term survival. While there is considerable debate over whether GIST can be benign neoplasms, we believe that all GIST have malignant potential, but vary in their propensity to recur after resection and metastasize to distant organ sites. Prognostic factors

include location, size (i.e., > 5 cm), grade (> 5-10 mitoses per 50 high power fields and specific mutational events that are still being defined. Adjuvant therapy with tyrosine kinase inhibitors, such as imatinib mesylate, has been shown to reduce the risk of recurrence after one year of therapy. Treatment of locally-advanced or borderline resectable gastric GIST with neoadjuvant imatinib has been shown to induce regression in a minority of patients and stabilization in the majority of cases. This treatment strategy potentially reduces the need for more extensive surgical resections and increases the number of patients eligible for curative therapy. The modern surgical treatment of gastric GIST combines the novel use of targeted therapy and aggressive minimally invasive surgical procedures to provide effective treatment for this lethal, but rare gastrointestinal malignancy.

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Key words: Gastrointestinal stromal tumors; Laparoscopic resections of gastrointestinal stromal tumors; Imatinib mesylate; Gastrectomy

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INTRODUCTION

Gastrointestinal stromal tumors (GIST) are rare mesenchymal tumors that occur throughout the smooth muscle layer of the gastrointestinal (GI) tract^[1]. GIST represent less than 1% of all GI tract malignancies. The most common location of these tumors is the stomach (70%), small bowel (20%-30%), small intestine and colon/rec-

tum (10%). Uncommonly, they can arise within the greater omentum, esophagus, appendix, and gallbladder. Most cases are sporadic, and affect men slightly more frequently than women (54% *vs* 46%)^[2]. The annual incidence in the United States has remained stable at 3000 to 4000 cases per year. The world-wide age-adjusted annual incidence rates range from 6.8 to 14.5 cases per million and vary between countries of origin. The median age at diagnosis is 58 years of age, but GIST have been reported in newborns and adolescents^[3]. GIST can range in size from several millimeters to over 30 cm in diameter. Tumor diameter appears to significantly influence biologic behavior, as small GIST may remain indolent for many years and large, “massive” GIST have higher rates of recurrence and associated metastases^[2].

Historically, smooth muscle sarcomas were classified as leiomyosarcomas^[4]. The development of malignant GIST requires the transformation of the interstitial cells of Cajal, pacemakers of the GI tract, to a malignant phenotype through activating or gain of function mutations in the *c-KIT* proto-oncogene^[5]. GIST are spindle cell neoplasms that usually retain the ultrastructural characteristics of smooth muscle cells, but have immunohistochemical staining for *c-KIT*, *CD-34*, smooth muscle actin, desmin and S-100^[6]. Approximately 70% of GIST are spindle cell type neoplasms; the minority are epithelioid (20%) or mixed cell type (10%)^[7]. Hirota *et al*^[5] first described the novel mutation in the *KIT* tyrosine kinase receptor gene in 1998. Since this landmark discovery, most “leiomyosarcomas” have been reclassified as GIST. *KIT* is located on chromosome 4q11-q12 and functions as a transmembrane receptor for its ligand, stem cell factor. In the non-cancerous state, this ligand binds to the extracellular portion of the receptor to induce homodimerization and downstream activation of its cell-signaling pathways^[8]. Wild-type *c-KIT* normally regulates cellular differentiation, growth, and survival. Approximately 80%-90% of GIST harbor mutations in the *KIT* genome. Mutations in the platelet-derived growth factor receptor (*PDGFR α*) occur in 5%-10% of *c-KIT*-wild type GIST. *DOG1* mutations may help identify GIST with wild type *c-KIT* and *PDGFR*^[9]. Mutated *KIT* receptors induce ligand-independent, unregulated activation of the downstream cell signaling pathways which collectively results in a loss of normal cell adhesion, differentiation, and proliferation to promote tumorigenesis. Exon 11 mutations in the *KIT* gene cause constitutively activated receptors leading to unregulated autophosphorylation of the intracytoplasmic tyrosine kinases^[10]. *KIT* mutations in exons 9, 13, 17 are less common and have been associated with more aggressive tumor behavior.

Biologically, gastric GIST tumors grow locally within the stomach (intra- or extraluminal expansion) and eventually obtain the capability to metastasize *via* hematogenous routes to the solid viscera (liver, small bowel, lungs) and peritoneal cavity. Tumors can also spread along the smooth muscle planes within the stomach or can rupture into the peritoneal cavity causing sarcomatosis. Complete surgical resection is thought to be the only curative

treatment for GIST. The recent use of cytostatic agents, such as imatinib mesylate, in patients with metastatic disease has been associated with durable recurrence-free survival^[11]. This important observation suggests that overall survival may not be the most important endpoint to consider when making treatment decisions. Radical gastrectomy is seldom required for extirpation of these tumors^[3]. In contradistinction to gastric adenocarcinoma, where it is essential to obtain at least five-centimeter proximal and distal margins, GIST tumors can be effectively treated by a complete gross resection of the tumor^[1]. Given the infrequency of lymphatic metastases, regional lymphadenectomy is not indicated. Minimally-invasive operations are now frequently used to treat gastric GIST. Retrospective series suggest that these techniques may reduce perioperative stress and are associated with lower rates of postoperative complications, shorter hospital stays and equivalent recurrence rates (Table 1)^[12-16].

EPIDEMIOLOGY AND DIAGNOSIS

Miettinen *et al*^[3] published the largest retrospective series of gastric GIST that reviews 1869 cases seen at the Armed Forces Institute of Pathology from 1970-1996. The vast majority of cases occurred in patients over 40 years of age and the median tumor diameter was 6 cm (range: 0.5-4.4 cm). Most gastric GIST had spindle-cell or epithelioid differentiation. Over 90% of these neoplasms had mutations in the *c-KIT* gene; *PDGFR* mutations were more frequently identified in epithelioid tumors. The metastatic potential of these tumors strongly correlated with their size and rate of mitotic activity. Unfavorable prognostic factors included proximal tumors (gastric cardia and gastroesophageal junction), the presence of coagulative necrosis, ulceration, and invasion deep to the mucosal layer.

Most GIST are incidentally diagnosed during evaluations for nonspecific GI symptoms, such as pain, nausea and vomiting, and weight loss^[2]. Tumor hemorrhage commonly occurs when large tumors develop an ischemic, punctate ulcer (Figure 1A). Usually, the bleeding can be temporized using endoscopic sclerotherapy or electrocautery techniques. Seldom is it necessary to take patients urgently for surgical resection with intractable hemorrhage (Figure 1B and C). Often these patients can be stabilized with medical and endoscopic therapy and have elective operations to extirpate these tumors. Intraperitoneal tumor rupture with hemoperitoneum and tumor dissemination is a difficult clinical problem that is associated with a significant risk of intraperitoneal sarcomatosis.

Computed tomography (CT) scanning is the most widely used and effective staging modality^[2]. Multiphase detector can localize the tumor within the stomach and remains a very sensitive technique to detect distant metastasis (at least ≥ 1 mm in diameter) within the liver or lungs; small volume intraperitoneal disease is often only detected on diagnostic laparoscopy and is respon-

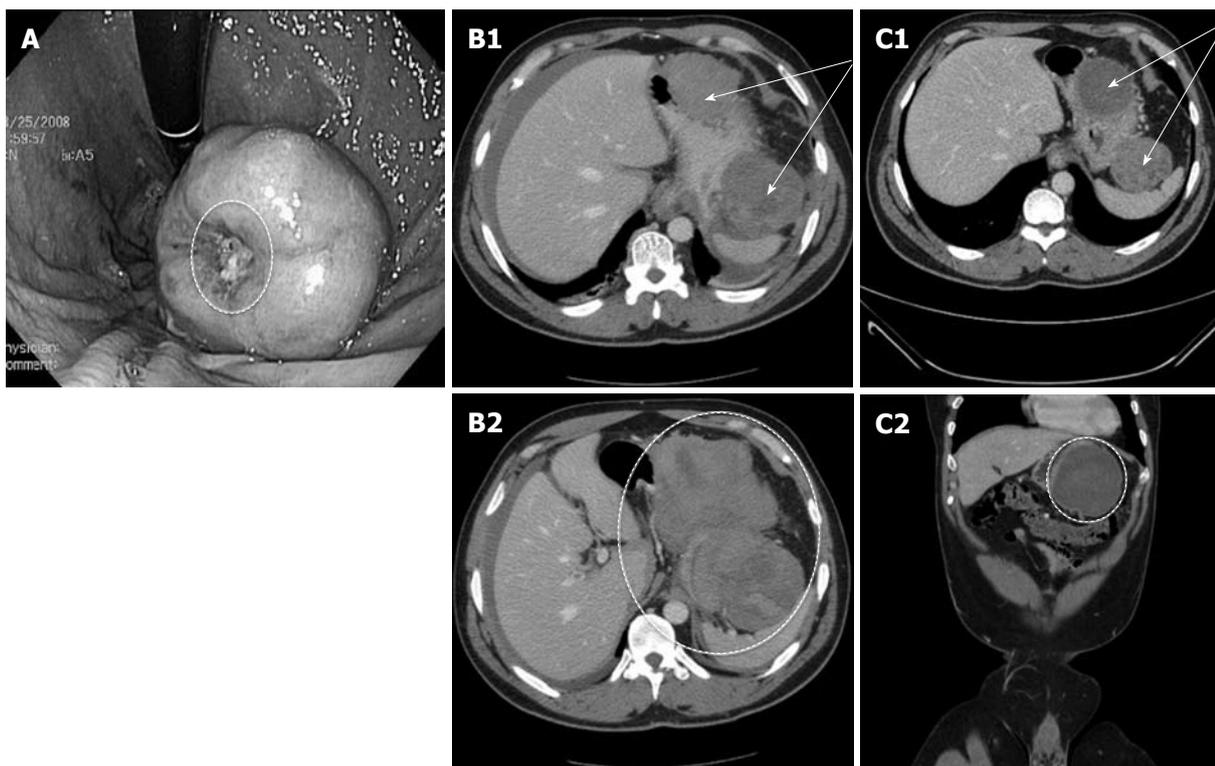


Figure 1 Clinical images of complicated gastrointestinal stromal tumors. A: Large intraluminal gastric gastrointestinal stromal tumors (GIST) with punctate central ulceration. The bleeding ulcer was treated endoscopically with sclerotherapy and electrocautery (cauterized tissue; white oval). The patient had an interval resection electively without additional hemorrhage from the tumor; B: Acute presentation of a patient with a ruptured gastric GIST with hemoperitoneum. These images represent contrast-enhanced computed tomography (CT) scan from a patient with a large extraluminal gastric GIST along the greater curvature of the stomach. B1 demonstrates axial CT images of the bi-lobed tumor with irregular borders (arrows); B2 shows additional axial images at the caudal extent of gastric tumor with layering of blood in the splenic recess (oval). He was diagnosed with hemoperitoneum and was resuscitated with packed red blood cells, fresh frozen plasma, and platelets; the patient was on antiplatelet therapy at the time of admission. He stabilized and had an upper endoscopy/ultrasonography for tissue diagnosis and to plan definitive treatment; C: Ruptured gastric GIST following conservative management. Contrast-enhanced CT images following a six-week period of conservative management of the patient with ruptured gastric GIST. C1 demonstrates the more organized bi-lobed tumor with distinct borders (arrows); C2 shows coronal images of the organized hemorrhagic component within the splenic recess after a period of observation (oval). Ultimately this patient had an interval open subtotal gastrectomy for a high-grade GIST.

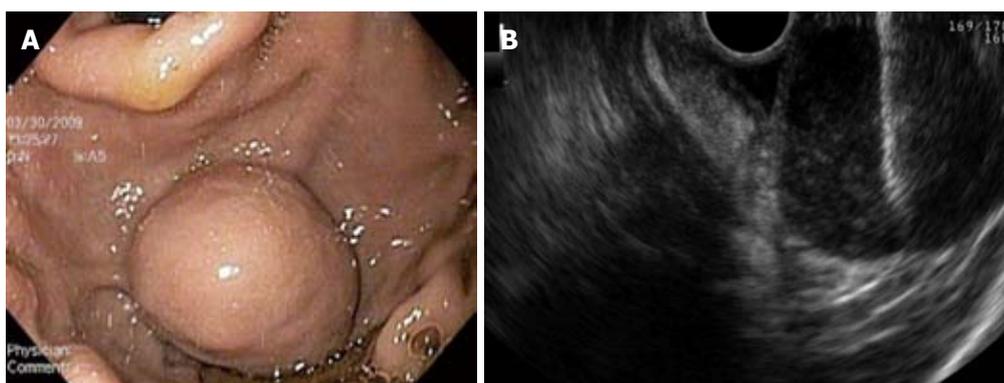


Figure 2 Endoscopy ultrasound images with fine needle aspiration biopsy. A: A 3 cm × 3 cm submucosal intraluminal mass within the gastric cardia; B: This Endoscopy ultrasound image shows the fine needle aspiration biopsy needle (horizontal white line in upper right corner of image) puncturing the submucosal gastric gastrointestinal stromal tumors.

sible for the reported 10%-15% of false negative rate with dynamic CT. Magnetic resonance imaging (MRI) is an acceptable alternative to CT for patients with renal dysfunction or in whom the risk of cumulative ionizing radiation may be prohibitive. Positron emission tomography (PET) remains an experimental test that may be useful in confirming distant metastatic disease and de-

termining the response to neoadjuvant targeted therapy. PET scans usually indicate tumor responsiveness to imatinib mesylate within days to weeks of induction therapy.

Upper endoscopy (EGD) with ultrasonography (EUS) is an essential diagnostic modality to acquire tissue for diagnosis, usually by fine needle aspiration (FNA) or core-needle biopsy (Figure 2). In addition, EUS is accu-

Table 1 Summary of large-series (> 35 cases) of minimally-invasive resections for gastric gastrointestinal stromal tumor

Ref.	Location	MIS/ GIST	Proximal tumors <i>n</i> (%)	Size (cm)	Operative time (min)	Compli- cations <i>n</i> (%)	Conversion to open surgery <i>n</i> (%)	LOS (d)	R0 resection rate	Intermediate/ high risk GIST <i>n</i> (%)	Recurrence rate <i>n</i> (%)	Median F/U (mo) (range)
Sasaki <i>et al</i> ^[16]	Japan	45 ¹ /37	6 (13)	3.2 (1.6-7.4)	100 (30-240)	1 (2)	1 (2)	NR	100	9 (24)	0	74 (1-81)
Sexton <i>et al</i> ^[14]	Germany	112/61	7 (11)	3.8 (± 1.8)	151.9 (± 67.3)	10 (16.4)	1 (2)	3.9 (± 2.2)	98	15 (25)	3 (5)	15 (0-103)
Wilhelm <i>et al</i> ^[15]	Germany	93/63 ³	36 (39)	2.6 (0.3-6.5)	90.7	7 (7.5)	6 (6.5)	7.3	100	8 (13)	0	40 (2-99)
Otani <i>et al</i> ^[13]	Japan	60	36 (60)	3.6 (1.8-15.0)	141/188 ⁴	NR	0	7.2	100 ²	17 (28)	2 (3)	53
Novitsky <i>et al</i> ^[12]	NC	50	17 (34)	4.4 (± 2.0)	135 (± 56)	4 (8)	0	3.8 (± 1.6)	100	14 (28)	4 (8)	36 (4-84)
Total (for GIST)		271	102 (38)				8 (3)			63 (23)	9 (3)	

¹Forty-five laparoscopic operations and 37 confirmed gastrointestinal stromal tumors (GIST); ²no positive margins, but one patient had a laparoscopic resection in the setting of distant metastatic disease; ³ninety-three consecutive patients, including 62 GIST; there was 1 laparoscopic-assisted endoscopic resection, 55 laparoscopic wedge resections, and 34 transgastric resections; ⁴the mean operative time was 141 for laparoscopic operations and 188 min for laparoscopy-assisted operations. NC: North Carolina; *n*: Number of patients in each series; MIS: Minimally invasive operations; Proximal tumors: GIST at gastroesophageal junction or within gastric cardia; Size: Median pathologic tumor size; Complications: Surgical morbidity; LOS: Length of hospital stay; NR: Not reported; R0 resection: Gross and microscopically-negative margins; F/U: Follow-up.

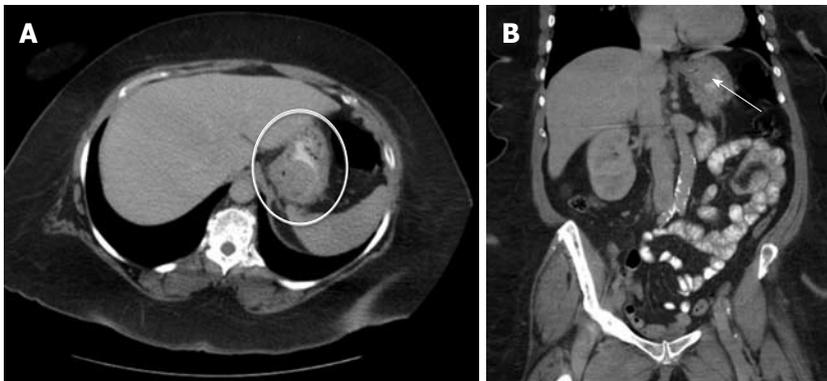


Figure 3 Gastroesophageal junction gastrointestinal stromal tumors. A: An axial computed tomography image of a gastric gastrointestinal stromal tumor (white oval) located along the posterior wall of the gastroesophageal junction (GEJ); B: Coronal images of the tumor (white arrow) show its proximity to the GEJ.

rate in determining the depth of penetration and origin of these neoplasms and also allows one to potentially consider a hybrid endoscopy/laparoscopic resection^[17]. The published National Comprehensive Cancer Network (NCCN) guidelines outline the recommended principles of tissue sampling for GIST (<http://www.nccn.org>). Since most GIST are soft, fragile, well-encapsulated tumors, indiscriminate biopsies increase the risk of tumoral hemorrhage and rupture. This is associated with higher rates of tumor recurrence and/or intraperitoneal dissemination. The decision to perform a preoperative or pretreatment biopsy should be individualized and only performed when the results of the sampling would definitively influence the choice of treatment^[18]. Biopsy is mandatory for all locally-advanced gastric GIST that will be treated with pre-resection neoadjuvant targeted therapy. Careful review of the acquired tissue by experienced GI histopathologists and use of comprehensive immunohistochemical staining for c-KIT and other markers is essential to confirm the diagnosis. Given the accuracy and real time localization of these tumors, EUS-guided biopsy is generally preferable to CT- or ultrasound-guided FNA biopsy techniques^[2,19].

EGD/EUS can identify the key anatomic relation-

ships of the tumor to the gastric wall layers. GIST at the gastroesophageal junction (Figure 3), pylorus and along the posterior wall of the stomach represent unique surgical challenges and influence the required operation. EGD can also effectively be used to treat tumor hemorrhage and avoid the need for urgent gastric operations. EUS can determine the depth of penetration through the layers of the gastric wall and potentially identify tumors that can be extirpated using endoscopic resection techniques. Only intragastric GIST that arise from the superficial circular muscular layer or muscularis mucosa can be removed with endoscopic enucleation^[20]. These procedures are technically demanding and require considerable experience and skill. Some of these cases take place in the endoscopy suites, but are often coordinated with surgical specialists to assist with the management of hemorrhage or gastric perforation. Often these resections are best performed in surgical operating rooms using laparoscopic-assisted techniques with an experienced surgeon present for the operation.

SURGICAL TREATMENT

Surgical treatment of gastric GIST is the only known cu-

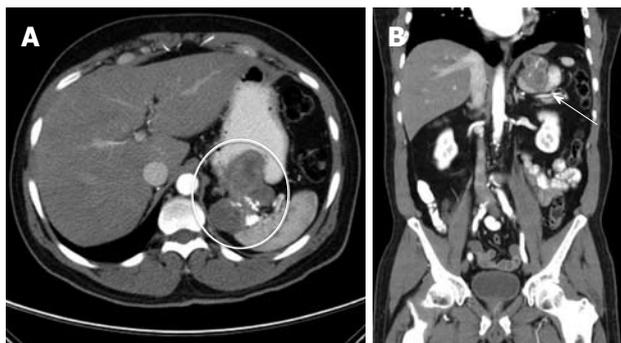


Figure 4 Locally-advanced gastric gastrointestinal stromal tumors. A: Representative contrast-enhanced computed tomography images show a large, proximal gastric gastrointestinal stromal tumors that invades into the splenic hilum (oval); B: On the coronal images the arrow indicates a heterogeneous mass invading into the spleen with areas of viable tumor and necrotic areas represented by calcifications.

rative therapy^[1]. It is essential to completely remove the entire tumor without violating the capsule of the mass. Tumor spillage or hemorrhage is associated with high locoregional recurrence rates and/or development of peritoneal sarcomatosis^[18]. Given the rarity of lymphatic dissemination, regional lymphadenectomy is not routinely performed. Since these tumors originate from the muscular layer of the gastric wall, enucleation is an option, but may be associated with higher recurrence rates unless the intramuscular pedicle can be clearly identified. Standard operations include both “open” and minimally invasive operations. Wedge or a “full-thickness” partial gastrectomy is an effective strategy for tumors that are located along the lesser or greater curvature of the stomach^[21]. Posteriorly-based gastric GIST often require transgastric resections through an anterior longitudinal gastrotomy; the tumor is everted and its pedicle divided with a linear stapling device^[22]. Anatomic gastrectomy (i.e., subtotal or total gastrectomy) is reserved for large tumors that involve a significant portion of the stomach. Endoscopic-assisted, laparoscopic gastric resections are cutting-edge operations that combine precise intraoperative localization of these tumors with gastric-volume preservation techniques.

NCCN guidelines suggest that small (< 1 cm) gastric GIST without high-risk endoscopic ultrasonographic features (i.e., irregular borders, cystic spaces, ulceration, echogenic foci and heterogeneity) may be followed with close endoscopic surveillance at 6-12 mo intervals (<http://www.nccn.org>). In the absence of biopsy-proven metastatic disease, patients with an acceptable performance status and GIST confined to the stomach should undergo complete surgical resection. In patients with marginally resectable tumors or in cases that GIST are potentially resectable, but the need for concomitant en bloc organ resection or total gastrectomy is likely, consideration should be given to neoadjuvant treatment with imatinib mesylate to cytoreduce or “downstage” tumors so that a less morbid or less extensive operation can be considered in the future (Figure 4)^[23-25].

Multiple single institutions highlight the increased use of laparoscopic or minimally-invasive operations for gastric GIST^[12-16]. Resection techniques include: (1) laparoscopic transgastric resections; (2) laparoscopic full-thickness or “wedge” resections; (3) laparoscopic extramucosal enucleation; and (4) combined laparoscopic, endoscopic resections^[26]. The five largest published reports of laparoscopy resections for gastric GIST are summarized in Table 1^[12-16]. Most of these retrospective series include non-GIST, benign submucosal tumors (leiomyomas). Although a formal meta-analysis was not performed given the small number of patients, general trends are evident. It appears that minimally-invasive operations for gastric GIST have been successfully used to treat patients with large tumors in difficult locations (i.e., proximal stomach and gastroesophageal junction). The data also suggest reasonable operative times, acceptable complication rates, and few conversions to open operations. Since none of the series had strict criteria for postoperative discharge to home, the reported postoperative length of stay is difficult to interpret, but was shorter than historic controls for open operations. Importantly, despite nearly one-third of the patients having intermediate to high-risk lesions, nearly 100% were completely removed and did not recur after 1-4 years of follow-up^[12-16]. We urge caution in broadly extrapolating these results to all patients with gastric GIST; most series had relatively short follow-up, involved a considerable selection bias, and most operations were performed by surgeons with considerable experience with these techniques^[27]. Our institutional experience with laparoscopic resection of GIST suggest that these techniques are both feasible and effective treatment for tumors less than eight centimeters in diameter. We advocate using a multidisciplinary approach with combined surgical oncology and minimally-invasive specialists to estimate the biologic behavior and determine the optimal method of resection. Cutting-edge modifications include the use of robot-assisted laparoscopic resections^[28], natural orifice surgery^[29], gasless laparoscopic resections^[30], single-port techniques^[31,32], and novel methods of removing posteriorly based tumors^[26]. One report described an experimental transgastric technique that utilized the retractable, metal-rimmed EndoCatch bags to elevate posterior wall GIST to facilitate laparoscopic stapled transection of the tumor pedicle^[33].

OUTCOMES AFTER SURGICAL RESECTION OF GASTRIC GIST

Since GISTs are rare neoplasms that demonstrate a spectrum of biologic behavior, outcomes following surgical resection are difficult to ascertain. Recurrence free survival appears dependent on tumor size, location, and mitotic rate^[6,34]. Prior to the use of imatinib mesylate as an adjuvant treatment following complete resection of gastric GIST, several large, retrospective reports suggest

local recurrence rates as high as 40% and five-year survival rates as ranging between 40%-90%^[1,35-38]. Dematteo *et al*^[11] published a series of 200 patients with GIST in 2000; more than half of these patients had gastric GIST. In the 93 patients with primary GIST, 80 (86%) had a complete resection with a median disease-specific survival of 54%. Fujimoto *et al*^[36] reported a series of 140 patients that had curative operations for gastric GIST. The five- and ten-year overall survival rates for the 129 patients with “curative” operations were 93% and 88%, respectively. Independent predictors of poor prognosis included male patients [hazard ratio (HR) = 0.469, $P = 0.013$], tumor size greater than or equal to 10 cm (HR = 20.98, $P = 0.001$), a mitotic index of 10+ (HR = 45.95, $P < 0.001$), and epithelioid cell histologic component (HR = 5.32, $P = 0.014$). Models to estimate the risk of recurrence have been created from large, retrospective data set of patients with verified GIST^[6,34]. Size (> 10 cm) and mitotic rates greater than five per 50 high-powered fields are the most significant variables that predict malignant behavior.

Conventional chemo- and radiation therapy are historically ineffective adjuvant treatments for GIST and do not significantly improve survival in patients with recurrent, metastatic or unresectable primary tumors^[18]. The evolution of targeted therapy has dramatically altered outcomes for patients with advanced GIST. Imatinib mesylate is an orally bioavailable, selective molecular inhibitor of cellular tyrosine kinases. First used to treat Philadelphia chromosome-positive chronic myelogenous leukemia, imatinib inhibits tyrosine receptor kinases such as PDGFR and KIT^[39]. The Federal Drug Administration (FDA) approved imatinib mesylate for use in patients with metastatic GIST in 2002. The American College of Surgeons Oncology Group (ACOSOG) phase II non-randomized Z9000 trial examined the use of adjuvant imatinib for one-year following complete resection of high-risk GIST (> 10 cm tumors or ruptured GIST). Imatinib-use was associated with decreased recurrence rates (*vs* historic controls)^[40]. The ACOSOG Z9001 was a randomized, double-blind, placebo-controlled, multicenter trial that conclusively showed a statistically significant reduction in the risk of recurrence with one-year of adjuvant imatinib mesylate therapy (400 mg daily dose; HR = 0.35, range: 0.22-0.53, $P < 0.0001$)^[41]. Seven hundred and thirteen patients with completely resected c-KIT positive GIST (greater than 3 cm) were randomized in an intention to treat analysis. At a median follow-up of 19.7 mo, the study was halted when it became evident that only 30 (8%) of patients in the imatinib group and 70 (20%) in the placebo arm had recurrent disease identified. Further maturation of this data is necessary to determine whether the adjuvant treatment improves overall survival in treated patients.

The FDA approved imatinib mesylate in 2008 as adjuvant therapy following complete resection of GIST for all patients without restrictions on time (to initiate therapy) or histopathologic criteria. The European Medicines Agency approved adjuvant imatinib in 2009

for adult patients with resected c-KIT-positive GIST at significant risk of relapse of disease. At least one year of postoperative imatinib mesylate therapy (400 mg daily) is now considered the standard of care for tumors greater than 3 cm with high-risk features (> 5-10 mitoses/50 high power field) per the results of ACOSOG Z9001^[2]. Several postoperative models of risk assessment have been used to estimate the likelihood of recurrence for patients who do not meet the aforementioned criteria^[6,34]. The optimal duration of imatinib and long-term survival benefit remains the subject of several ongoing randomized, controlled international cooperative group trials and industry-sponsored studies. Current protocols include the recently completed EORTC 62024 trial that randomized 900 patients with completed resected intermediate- and high-risk GIST to receive either two years of adjuvant imatinib mesylate *vs* observation. The primary endpoint of the EORTC trial was overall survival, so the final results will require approximately ten years for complete analysis. The Scandinavian Sarcoma Group phase II trial, (SSGXVII; one *vs* three years of adjuvant imatinib mesylate) and the non-randomized Novartis Pharmaceutical Trial (NCT00867113; five years of adjuvant imatinib) were both designed to test extended use of adjuvant imatinib mesylate following complete resection. Patients at a higher risk of recurrence may justify indefinite use of adjuvant therapy. Three recent cooperative group trials using imatinib in patients with locally-advanced, unresectable or metastatic GIST have suggested that the KIT mutation genotype may have prognostic value to estimate the duration of response and optimal dose of imatinib mesylate^[10,42,43]. Patients in these trials with exon 11-mutations had better treatment outcomes (improved tumor response, progression-free survival, and overall survival) when compared to patients with KIT exon 9-mutants and wild-type patients. At the American Society of Clinical Oncology annual meeting in 2010, it was reported that only deletions (all types) in the KIT exon 11 gene was associated with an increased risk of recurrence^[44]. Heinrich *et al*^[43] also reported that GIST with KIT exon 9-mutations had higher tumor response rates to neoadjuvant imatinib mesylate with daily doses of 800 mg (*vs* 400 mg).

POST-OPERATIVE SURVEILLANCE

NCCN guidelines suggest that following complete resection of gastric GIST; patients should be followed with comprehensive history and physical examinations every 3-6 mo for 5 years, then annually (<http://www.nccn.org>). Abdominal/pelvic contrast enhanced CT scans were recommended every 3-6 mo for at least three to five years postoperatively. Given the risk of renal insufficiency with iodinated contrast and the cumulative ionizing radiation exposure with frequent CT scans, we believe that less intensive surveillance programs should be advocated. MRI remains an acceptable alternative for suitable patients and avoids the deleterious radiation exposure that is associated with serial CT scans. It is reasonable to

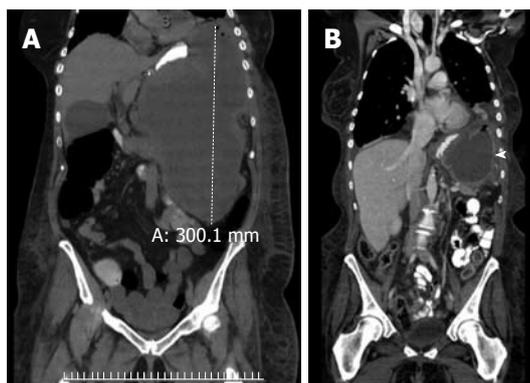


Figure 5 Neoadjuvant treatment of a locally-advanced gastrointestinal stromal tumors with imatinib mesylate. A: This woman presented with abdominal pain and fullness. A computed tomography (CT) scan identified a massive (> 30 cm), homogeneous tumor in the gastric fundus that was exophytic and extending caudally towards the pelvic inlet; B: After tissue diagnosis confirmed a gastric gastrointestinal stromal tumor (GIST), the patient was treated with six months of low-dose imatinib mesylate (400 mg/d) until a maximal response was achieved. The coronal views of this interval CT scan demonstrated a much smaller, well-encapsulated, homogenous tumor (solid white arrowhead). She had a radical resection of the gastric GIST and was free of disease until 24 mo when she developed a metastatic lesion in the left lateral segment of the liver. Following complete metastectomy, she was treated with several targeted tyrosine kinase inhibitors until she ultimately succumbed from her metastatic disease 19 mo from her second operation and 43 mo from her initial operation.

consider an EGD at one-year after resection to rule out a local or anastomotic recurrence. Less frequent surveillance programs have been suggested for small (< 2 cm), low-risk tumors. Patients on investigational adjuvant protocols routinely are scanned more frequently to determine the efficacy of treatment.

NEOADJUVANT TREATMENT OF LOCALLY-ADVANCED GASTRIC GIST

Locally-advanced “unresectable” or borderline-resectable gastric GIST are often treated with neoadjuvant imatinib mesylate therapy prior to surgical resection (Figure 5)^[45]. Theoretically, the use of preoperative imatinib may “downstage” or substantially cytoreduce GIST preoperatively and diminish the need for concomitant, en bloc organ resections. Over the past five years, there have been several small, single-institution; retrospective reports documenting outcomes following neoadjuvant treatment of borderline or locally-advanced GIST (Table 2)^[25,44,46-51]. Approximately 75% of these highly selected patients with “unresectable” GIST were subsequently treated with R0/R1 resections. The duration of neoadjuvant therapy and best method of detecting maximal treatment effect have been the subject of two recent phase II trials^[52,53]. The RTOG 0132/ACRIN 6665 cooperative group trial prospectively administered “neoadjuvant” imatinib mesylate (600 mg/d) for eight weeks to patients with both potentially resectable (*n* = 30) and recurrent/metastatic GIST (*n* = 22)^[53]. The majority of patients had disease stabilization; only 12% had a partial tumor response to therapy. Patients were

Table 2 Summary of retrospective single-institutional experience with surgical resection of metastatic gastrointestinal stromal tumor after treatment with imatinib mesylate *n* (%)

Ref.	Number of patients	R0/R1 resections
Sym <i>et al</i> ^[47]	24	15 (62)
DeMatteo <i>et al</i> ^[25]	49	39 (80)
Gronchi <i>et al</i> ^[48]	38	31 (82)
Raut <i>et al</i> ^[49]	69	57 (83)
Rutkowski <i>et al</i> ^[51]	24	22 (92)
Bonvalot <i>et al</i> ^[46]	22	15 (68)
Andtbacka <i>et al</i> ^[50]	46	22 (48)
Totals	272	201 (74)

R0/R1 resections: Complete gross removal of the gastrointestinal stromal tumor with/without negative microscopic margins.

resected with minimal morbidity and given an additional two years of adjuvant therapy. The patients without metastatic disease had estimated two-year progression-free and overall-survival rates of 83% and 93%, respectively. McAuliffe *et al*^[52] randomized 19 patients with locally-advanced GIST to receive “nanoneoadjuvant” imatinib therapy; subjects were given 600 mg/d for 3 d, 5 d or 7 d prior to surgical resection. Seventeen of 19 patients had a subsequent resection without significant morbidity and were given two years of adjuvant therapy. Approximately 30% of these patients had an objective radiologic response to imatinib (CT/PET) and 12% of the resected tumors had an increase in apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay. These studies provide the proof of principle that neoadjuvant imatinib mesylate may be a safe and effective method of treating patients with locally-advanced GIST.

CONCLUSION

Gastric GIST are rare neoplasms that have traditionally required complete surgical resection to achieve cure. Both traditional and minimally invasive gastric resections can be used to remove these tumors with minimal morbidity and excellent perioperative outcomes. The revolutionary use of specific, molecularly-targeted therapies, such as imatinib mesylate, reduces the frequency of disease recurrence when used as an adjuvant following complete resection. Neoadjuvant treatment with these agents appears to stabilize disease in the majority of patients and may reduce the extent of surgical resection required for subsequent complete tumor removal. Importantly, tyrosine kinase inhibitors likely extend the progression-free survival of most patients with GIST. The optimal sequencing of therapies and incorporation of predictive genomic data highlight future challenges in this disease.

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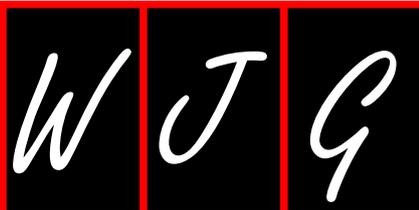
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Adult to adult living related liver transplantation: Where do we currently stand?

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Abstract

Adult to adult living donor liver transplantation (AALDLT) was first performed in the United States in 1997. The procedure was rapidly integrated into clinical practice, but in 2002, possibly due to the first widely publicized donor death, the number of living liver donors plummeted. The number of donors has since reached a steady plateau far below its initial peak. In this review we evaluate the current climate of AALDLT. Specifically, we focus on several issues key to the success of AALDLT: determining the optimal indications for AALDLT, balancing graft size and donor safety, assuring adequate outflow, minimizing biliary complications, and maintaining ethical practices. We conclude by offering suggestions for the future of AALDLT in United States transplantation centers.

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Key words: Adult to adult living donor liver transplantation; Outflow; Graft size; Liver failure; Ethics; Biliary complications

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INTRODUCTION

At the time of its initial introduction into clinical practice, many believed that adult to adult living donor liver transplantation (AALDLT) would be a panacea for the severe shortage of cadaveric donors that resulted in extensive times on the waiting list and high patient mortality. This belief was illustrated by multiple studies from the late 1990s that suggested that AALDLT was safe and claimed that it would significantly decrease mortality on the transplantation waiting list^[1,2]. The first United States AALDLT was performed in 1997, and over the next 3-5 years AALDLT was vigorously embraced by many United States transplantation surgeons. This enthusiasm was obvious in the documented increase from one to 38 United States AALDLT centers and from one to 266 United States AALDLT procedures between 1997 and 2000^[3]. However, despite rapid integration of this novel procedure into clinical practice, multiple questions remained unanswered. Little was known regarding the proper indications for AALDLT. Further, there was relatively limited data on how graft size impacted recipient and donor safety. Additionally, techniques to assure adequate outflow and minimize biliary complications

were only in their infancy. Finally, the multitude of ethical issues surrounding AALDLT had not been rigorously addressed. In 2002, possibly due to the first widely publicized death of a living donor, the number of living liver donors plummeted. The current number of annual living liver donors has now reached a relatively static plateau at about 250 donors per year, which is far below the initial peak of 524 donors in 2001 (Figure 1)^[4].

While there is a current climate of concern in the United States regarding the safety and appropriateness of AALDLT, multiple other countries (mainly Asian countries, Turkey and Egypt) have experienced a continued rise in AALDLT. Presumably, this contrast is the result of societal norms and logistic difficulties that impede cadaveric organ donation in these areas. Review of data from current United States and Asian transplantation centers along with critical review of the literature from United States surgeons who enthusiastically embraced AALDLT in its early years and then altered their practice as increasing risk became apparent, should be undertaken to help us determine how and if AALDLT can safely be reinstated as a more widely utilized procedure in United States transplantation centers. In this review, we draw upon these studies to address the current status of AALDLT. Specifically, we explore issues related to both the technical/scientific aspects of AALDLT as well as the ethical issues that surround AALDLT. We then offer suggestions for the future of AALDLT in the United States.

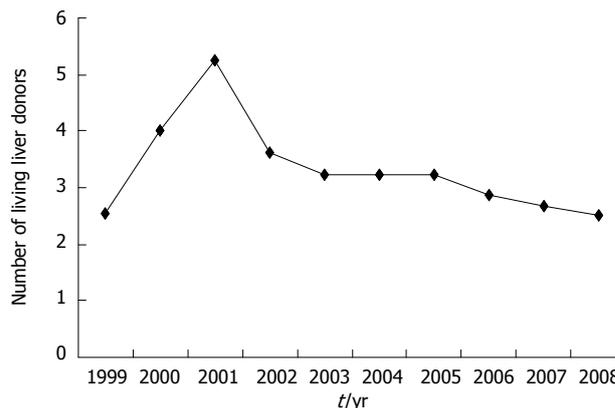


Figure 1 Number of United States living liver donors per year^[4].

managed in large-volume, Western liver transplantation centers, the transplantation rate ranges from 41%-72%, and the median waiting time for a deceased donor graft is 3.5-5 d^[6]. These statistics clearly indicate the impressive need for more readily available liver grafts.

The issue of AALDLT for ALF also sparks an ethical debate among the transplant community regarding issues such as how to assure adequate informed donor consent in a relatively pressured setting or whether it is appropriate to ask donors to give an organ to a recipient who may not have a successful outcome. Based on our recent analysis of the current ethical issues with AALDLT for ALF, we feel that with adherence to rigorous informed consent efforts mediated by a donor advocate and recognition that most donors rate the experience of donating positively even if the recipient has a poor outcome, AALDLT can be offered to patients with ALF in a way that maintains the highest ethical standards for both the donor and the recipient^[5]. Thus, given the extreme need for more liver grafts and the precedent of successful AALDLT outcomes in many Asian centers, United States centers should consider broadening the indication for AALDLT to include ALF.

In addition to efforts to determine whether AALDLT is appropriate for ALF, extensive efforts have also been directed toward determining if the MELD score can help predict the suitability of AALDLT for a given patient. The MELD score, which is based upon creatinine, total bilirubin, and INR, was originally developed by Malinchoc *et al*^[7] to predict 3 mo survival in patients undergoing elective transjugular intrahepatic portosystemic shunt procedures. The MELD score has since been shown to be a valuable predictor of pre-transplantation survival, and in 2002 it became a critical tool in assigning priority to patients on the United Network for Organ Sharing (UNOS) transplantation waiting list. At the inception of this policy, little data regarding the predictive value of the MELD score in AALDLT existed thus promoting general concern among transplantation physicians that a higher MELD score (i.e. a sicker patient) may correlate with poor outcomes after AALDLT.

OPTIMAL INDICATIONS FOR ADULT TO ADULT LIVING DONOR LIVER TRANSPLANTATION HAVE NOT BEEN ESTABLISHED

Extensive efforts have been dedicated to determining the optimal indications for AALDLT. Review of the recent literature identifies two central issues: the appropriateness of AALDLT for patients with acute liver failure (ALF) and whether the Model for End-stage Liver Disease (MELD) score can be utilized to determine the suitability of AALDLT for a given patient. Here, we briefly highlight recent literature regarding these controversial topics.

In many Asian centers where socio-cultural norms drastically limit utilization of cadaveric liver donation, AALDLT is being increasingly relied upon to offer expedient transplantation to patients with ALF (Table 1)^[5]. Despite relatively widespread use of AALDLT for ALF in many Asian countries, issues regarding graft size and donor safety have prevented widespread acceptance of AALDLT for ALF in the United States. This reluctance to utilize AALDLT in the setting of ALF presumably increases the risk of death due to failure to quickly receive a graft for these critically ill patients. For example, we have found that even when patients with ALF are

Table 1 Literature review of adult to adult living donor liver transplantation for acute liver failure^[5]

Ref.	Year	Location	n	Recipient survival (%)	Lobe utilized	Donor complications (%)	Donor survival (%)
Liu <i>et al</i> ^[38]	2002	China	16	88	Right	25	100
Nishizaki <i>et al</i> ^[39]	2002	Japan	15	80	Left	No comment	100
Wu <i>et al</i> ^[40]	2004	Taiwan, China	8	100	Right	0	100
Lee <i>et al</i> ^[41]	2007	South Korea	57	82	Both	2	100
Kilic <i>et al</i> ^[41]	2007	Turkey	6	83	Right	25	100
Campsen <i>et al</i> ^[42]	2008	United States	10	70	Right	50	100
Ikegami <i>et al</i> ^[43]	2008	Japan	44	80	Both	34	100
Park <i>et al</i> ^[44]	2010	South Korea	40	85	Both	24	100

This concern was based primarily upon the relatively poor early outcomes of AALDLT for critically ill patients. This issue was addressed in work by Testa *et al*^[8] that investigated the appropriateness of AALDLT for patients with decompensated end-stage liver disease. The authors highlighted several early studies of AALDLT that described a 1 year survival of about 50% for critically ill patients which was far inferior to the 77%-80% 1 year survival reported for patients with a more favorable clinical status. The authors also cited their own 1 year survival for AALDLT in patients with a MELD score > 30 as 43%, which although relatively low, was considered successful given the high likelihood of patient death before a cadaveric donor graft could be allocated to them^[8]. The relatively low survival rates reported in these studies led to a general consensus that critically ill patients should not be candidates for AALDLT. However, Testa *et al* advocated that rather than abandoning the procedure for these patients, the outcomes of AALDLT in the critically ill could be improved by gaining further experience in appropriate donor selection and working to overcome the technical difficulties of the operation^[8]. Recent data from various transplantation centers, especially those in Korea, that heeded this recommendation and continued working to overcome the technical issues that initially contributed to poor outcomes now demonstrate survival in critically ill patients that closely parallels survival of patients who are less ill. Additionally, Hwang *et al*^[9] and Bhangui *et al*^[10] have recently demonstrated the success of AALDLT for patients with hepatocellular carcinoma. These improvements in outcome across a variety of disease etiologies and recipient clinical status suggest that AALDLT is an appropriate procedure for even the most critically ill patients.

To further understand which critically ill patients would benefit most from AALDLT, several studies have since emerged to specifically investigate the predictive validity of the MELD score in AALDLT. For example, in a retrospective review of 62 AALDLT recipients by Hayashi *et al*^[11], MELD score failed to predict 1-year patient or graft survival. To further analyze the predictive capacity the MELD score and 23 other preoperative factors Morioka *et al*^[12] conducted a retrospective review of 335 cases of AALDLT in Japan between 1994 and 2003. The authors concluded that lack of pre-transplant encephalopathy, MELD score ≤ 30 (including points

for persistent ascites and low serum sodium), and donor age < 50 were the key factors for obtaining successful outcomes with AALDLT. This work was contrasted by Durand *et al*^[13] who conducted a retrospective review of 331 DDLT and 128 AALDLT cases to develop statistical models to determine the most efficacious means of organ allocation. This group determined that AALDLT is most advantageous when performed in patients at high risk of death. More simply, these authors demonstrated that the most critically ill patients will derive the most robust statistical benefit from AALDLT^[13]. The studies reviewed here offer slightly conflicting suggestions regarding which patients will benefit most from AALDLT. Further work must be performed to more clearly delineate the indications for the procedure and to establish a meaningful selection criteria for potential recipients.

However, it is our belief that current data generally suggests that the indications for AALDLT should be broadened. Reflection on the drastic improvements in survival for critically ill patients (including those with ALF) in many Asian centers illustrates how diligent refinement of surgical technique and increased procedural experience can result in drastically improved outcomes of AALDLT for even the most critically ill patients. Perhaps United States transplantation centers can call upon the wealth of knowledge generated by these centers to successfully incorporate AALDLT into the routine treatment of United States patients.

DELICATE BALANCE BETWEEN GRAFT SIZE AND DONOR SAFETY

In addition to determining the optimal indications for AALDLT, the transplantation community must advise on the delicate balance of assuring adequate graft size while maintaining donor safety. It is well accepted that AALDLT must be performed in careful balance between recipient needs and donor safety. For example, if acquiring adequate graft volume for the recipient equates to unacceptable risk for the donor, the transplantation must not be performed. Currently, a graft weight to body weight ratio of > 0.8% and a graft size of at least 40% of the standard liver volume is accepted as a minimum requirement for donation^[14]. It has been advocated by some that use of a right lobe graft as compared to a

left lateral lobe as is used in pediatric living donor liver transplantation (LDLT) may allow for larger graft size while still maintaining reasonable donor risk. While it is presumed that the increased graft size places donors at a higher risk following a right hepatectomy, no controlled studies substantiate this concern. Further, presumption that donor morbidity or mortality is directly and solely related to the extent of the liver resection is not reasonable given the numerous other factors that may contribute to poor donor outcomes. The Korean experience demonstrates that with increased operative experience, strict donor selection, and an institutional focus on AALDLT, complications and mishaps can be drastically minimized^[15].

Numerous studies have also been conducted in an effort to determine the safest selection criteria for donors. Authors at a prominent United States transplantation center reviewed the screening of 66 potential donors for 15 eventual AALDLT procedures^[16]. The group relied upon 3D helical imaging including hepatic lobe volume renderings, vascular anatomy, virtual resection planes, preoperative arteriography, and medical/psychological examination, and found that even with robust preoperative donor screening in an experienced hepatobiliary center, morbidity occurred in 67% of donors^[16]. A slightly more recent study of 893 AALDLT cases between 1994 and 2005 in Korea demonstrates how modification of graft size and careful donor selection can result in marked reduction in donor morbidity^[15]. Specifically, until 2001, this group reported an AALDLT donor complication rate of 6.7% which was predominantly due to complications in right lobe liver donors. In 2002, authors changed their donor selection procedure such that liver resection exceeding 65% of total liver volume was avoided except in young donors with no evidence of hepatic steatosis. This change resulted in a reduction of donor morbidity to 1.3% and prompted authors to conclude that a majority of major living donor complications are avoidable through strict selection of living donor/graft type (with cautious selection of the donor right liver if it appears to be larger than 65% total liver volume), intensive postoperative surveillance, and prompt feedback regarding surgical technique. Perhaps most interestingly the authors commented that the experience that they gained from implementation of AALDLT has actually optimized all hepatobiliary surgery practices at their institution^[15]. There is also an interesting body of work evaluating the accuracy of preoperative assessment of graft volume *via* 3D-CT volumetry in AALDLT. Both Hiroshige *et al*^[17] and Kayashima *et al*^[18] have demonstrated that 3D-CT volumetry may overestimate graft volume by as much as 13%, especially in donors under 30 yr old. Authors suggest that this may be due to graft dehydration secondary to University of Wisconsin solution. Studies such as these illustrate the difficulty in assuring accurate pre-operative assessment of graft volume. Given the obvious importance of assuring adequate graft

size, future work to further optimize pre-operative assessment of graft volume is certainly warranted. Clearly, the transplantation community will benefit from further study regarding how to safely balance donor safety and graft size; however, studies such as those discussed here suggest that we are beginning to develop a more robust understanding of how to address this critical issue. We suggest that implementation of strict policies regarding acceptable graft size is the best method for avoiding complications related to small for size grafts or resection of an unsafe donor graft volume.

However, surgical technique alone, even that delivered by expert surgeons, cannot substitute for the institutional organization at all levels of patient care that is mandatory to minimize complications. Any institution willing to offer AALDLT to its patients must invest in AALDLT. Specifically, in order to replicate the results of the best transplantation centers in the world, United States centers should focus on how the entirety of the operative experience, ranging from pre-operative donor evaluation to the number of capable transplant surgeons to the coordinated management of post-operative care, can be structured to provide the highest levels of success.

ISSUE OF OUTFLOW

The right lobe of the liver is increasingly being utilized to assure adequate graft volume in AALDLT. However, use of the right lobe brings with it a heightened risk of impaired graft outflow. Specifically, the right lobe graft carries an increased risk of early post-operative congestion of the paramedian segments 5 and 8 due to interruption of the venous drainage of the middle hepatic vein (MHV). Post-operative congestion has been shown to lead to congestive necrosis which has been reported to incite early post-operative graft failure and recipient death^[19-21]. Harvest of an extended right lobe graft with inclusion of the entire donor MHV is an effective means of preventing outflow obstruction: however, the larger graft size may increase the donor's risk of morbidity and mortality. The surgeon's decision to resect or not resect the donor MHV is thus a complicated one that rests upon several key factors. Donor residual liver volume, the importance of MHV in right lobe drainage, the ratio of graft weight to recipient body weight, and the MELD score must all be carefully evaluated prior to determining whether resection of the MHV is appropriate. Generally, it seems prudent to suggest that if the donor residual liver volume is marginal (ratio < 0.6) or the rest volume is < 30%, the MHV should remain with the donor and segments 8 and 5 should be re-anastomosed.

Several procedural modifications have been proposed to generate improved outflow in cases where the MVH must be interrupted. For example, in a review of 74 AALDLT patients, Malago *et al*^[22] observed that rapid regeneration of the graft in the first 10 post-operative days

resulted in medial displacement of the graft such that kinking, torsion, and compression/occlusion of the outflow tract resulted. By using a cadaveric iliac vein graft to create an interposition conduit that allowed drainage of all intrahepatic veins (diameter > 5 mm) draining segments 5 and 8 into the MHV, the authors devised a modification of the outflow tract that enlarged the caval orifice and assured better outflow^[22]. Successful use of this cadaveric vein outflow reconstruction procedure in patients undergoing AALDLT was also demonstrated by Dong *et al*^[19]. Continued efforts to develop procedural modifications that assure successful outcomes in recipients of AALDLT while maximizing donor safety are imperative. Additionally, it is good practice to remember that every donor-recipient pair presents a unique set of anatomical challenges, however, these challenges should not be considered separately. Favorable outcomes are a result of the safest and most feasible combination of donor and recipient need. Any compromise at the expense of either may result in serious complications and poorer outcomes. Procedural flexibility is ultimately the best policy to protect the donor and assure prompt, successful graft functioning in the recipient.

BILIARY COMPLICATIONS

In addition to issues of impaired outflow, reliance on right lobe grafts for AALDLT has brought with it an increased rate of biliary complications. Overall, since the introduction of AALDLT with right lobe grafts, biliary complications have been the leading cause of post operative complication and re-operation^[23-27]. The reported incidence of biliary complication in AALDLT ranges from 15%-60%^[25,28-31], which is substantially greater than that of cadaveric full-liver transplantation 5%-15%^[29,32,33], and pediatric LDLT with the left lateral lobe of 4%-6%^[34]. Presumably, anatomic differences between the left and right hepatic biliary systems are responsible for this differential complication rate. Specifically, reliance upon the left hepatic duct usually allows a relatively easy dissection due to straightforward ductal anatomy. However, use of the right lobe hepatic system is markedly complicated by the numerous anatomic variations of the right duct. Multiple technical modifications for biliary reconstruction have been published over the past decade (use of recipients right and left hepatic ducts, end-side reconstruction of donor ducts to native common hepatic bile duct, donor ductoplasty, stents, T-tubes, *etc.*), however none has provided a standardized, replicable method that consistently decreases the incidence of biliary complications^[24,30].

Although, biliary complications result in high recipient morbidity and occasionally mortality, early detection and prompt treatment offers a favorable rate of recovery in these patients. Perhaps most importantly, a high rate of success in non-operative management of biliary complications has been consistently demonstrated in

the literature. For example, in a review of 429 patients that underwent liver transplantation, the success rates for treatment of biliary complications by endoscopic retrograde cholangiopancreatography and percutaneous transhepatic radiologic procedures were 100% and 78% respectively^[35]. Given the consistently high rate of biliary complications despite over a decade of efforts to perfect the biliary anastomosis, assurance of robust non-operative treatment modalities is imperative. Thus while continued refinement of technique is critical, we have limited expectation of an easy fix for biliary complications in AALDLT. It is generally clear that while most of the other technical obstacles to AALDLT have found favorable solutions, we are still far from having developed a biliary anastomosis technique that will provide consistently positive results. While relying on continued advancement in minimally invasive procedures to remedy these frequent complications is currently an acceptable compromise, it is imperative that we continue significant efforts to construct a biliary anastomosis with equivalent or lower complication rates than that of deceased donor liver transplant.

ETHICAL ISSUES

The ethical issues surrounding AALDLT are complex and warrant thoughtful discussion prior to widespread implementation of the procedure. Overall, the majority of regulations on liver transplantation are guided by a simple reality: liver grafts are provided by a public supply, and the supply is insufficient to meet current societal demands. The introduction of AALDLT presumably alters this climate by increasing the available supply of grafts. This increase in supply offers the opportunity of transplantation to patients who previously had relatively minimal chances of obtaining a graft given that their clinical status was not critical enough to assure high priority listing on the transplantation waiting list or because the etiology of their disease precluded them from being a transplant candidate (hepatocellular carcinoma, polycystic liver disease, *etc.*). While introduction of AALDLT clearly expands the opportunity for many patients awaiting transplantation it carries with it multiple ethical questions.

Overall, the issue of whether AALDLT is ethically appropriate requires careful assessment of the risks and benefits to the individual donor-recipient pair. The benefits to the potential recipient are relatively obvious. AALDLT offers potential recipients decreased time on the waiting list or, in some cases, the opportunity to completely bypass the waiting list. Further, with AALDLT potential recipients are able to undergo transplantation as an elective rather than urgent surgery thereby allowing increased control of variables such as graft ischemic time. However, given the healthy status of the donor, the age old vow of physicians, “*primum non nocere*” (first do no harm), mandates that the risks and

benefits to the donor be the primary focus of a discussion of transplantation ethics. In our brief review of the literature, it is evident that donors experience a relatively high risk of morbidity and a realistic risk of mortality in even the most well-qualified transplantation centers with the most experienced transplantation surgeons. The risk of short and long-term medical morbidity is compounded by the financial burdens that may result due to ongoing medical bills and time lost from work. Currently, no system is in place to assure that donors are not excessively burdened by such issues. However, one must remember that while this risk may be of high concern for United States donors, it may be less of an issue in European countries where public health insurance typically covers all donor expenses. While substantial health and financial risk is obvious for the donor, great benefits may also be incurred by consenting to living liver donation. The psychological benefit from this altruistic decision is one of the primary benefits to donors. A vast body of literature exists that consistently supports the finding that even in cases of poor recipient outcome, undergoing donation helps donors feel as if they have done everything possible to help save the life of their loved one^[36]. Donation may also bring with it a decreased caregiver burden and increased participation of the recipient in subsequent household matters.

Several other issues are central to a thorough discussion of the ethics of AALDLT. First, there is great concern among the transplantation community that critical recipient status (especially in the setting of ALF) or familial pressure may limit the ability of potential donors to fully engage in an informed consent discussion. Many worry that donors may feel pressured to donate, and they encourage a robust donor evaluation process with inclusion of a donor advocate who assures donors the opportunity to make their decision with minimal pressure from family or members of the health care team. Additionally, many worry that performing an AALDLT in a setting in which recipients may have a poor outcome (e.g., ALF) may not be appropriate. However, multiple authors have determined that even in settings in which recipients die or suffer significant morbidity, most donors rate the donation experience positively as they feel as if it has allowed them to do all they can to help save the life of their loved one^[36]. Clearly, the ethical issues involved with AALDLT are complex and deserving of further discussion to assure the highest ethical standards are maintained when caring for the donor-recipient pair.

HAVE WE IMPROVED SINCE 1997?

Critical review of the success and failure of AALDLT over the past decade is imperative in assessing our current status. Improved transparency in outcomes reporting, development of uniform regulations, an increasing number of National Institutes of Health funded studies, and increasing procedural experience all suggest that our ability to safely and successfully perform AALDLT has

improved since the initial introduction of the procedure in 1997.

MOVING FORWARD

Critical review of AALDLT suggests that the Western world both embraced and dismissed AALDLT too quickly^[37]. A new framework for integration of AALDLT into United States medical practice that is rooted in clinical expertise, genuine need, and accurate reporting should be developed to create a new starting point for AALDLT in the United States. Focus upon the key factors of determining the appropriate indications, balancing graft size with donor safety, assuring adequate outflow, minimizing biliary complications, and mandating ethical rigor is imperative. Additionally, we offer several suggestions that we believe will assure thoughtful integration of AALDLT into United States practice. First, all AALDLT activity should be concentrated in centers with dedicated AALDLT staff and adequate field strength (capacity of surgical team to meet the technical demands of a relatively innovative procedure, proven success with all facets of hepatobiliary surgery, *etc.*). Next, we believe that proper training programs for staff along with validated criteria to demonstrate clinical competency should be developed. Further, incorporating knowledge from centers that are already world leaders in AALDLT to establish patient and graft survival that is superior to deceased donor liver transplantation must be the ultimate goal. Finally, working to improve the societal image of AALDLT is critical for assuring integration into current United States medical practice. It is our belief that careful attention to these key factors will help redefine the role of AALDLT in United States transplantation centers. And while this may not provide the panacea for long wait times and high recipient mortality that was originally assumed, we believe that robust integration of AALDLT into United States practice will offer significant improvements for patients awaiting liver transplantation.

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Multidisciplinary approach for patients with esophageal cancer

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Abstract

Patients with esophageal cancer have a poor prognosis because they often have no symptoms until their disease is advanced. There are no screening recommendations for patients unless they have Barrett's esophagitis or a significant family history of this disease. Often, esophageal cancer is not diagnosed until patients present with dysphagia, odynophagia, anemia or weight loss. When symptoms occur, the stage is often stage III or greater. Treatment of patients with very early stage disease is fairly straight forward using only local treatment with surgical resection or endoscopic mucosal resection. The treatment of patients who have locally advanced esophageal cancer is more complex and controversial. Despite multiple trials, treatment recommendations are still unclear due to conflicting data. Sadly, much of our data is difficult to interpret due to many of the trials done have included very heterogeneous groups of patients both histologically as well as anatomically. Additionally, studies have been

underpowered or stopped early due to poor accrual. In the United States, concurrent chemoradiotherapy prior to surgical resection has been accepted by many as standard of care in the locally advanced patient. Patients who have metastatic disease are treated palliatively. The aim of this article is to describe the multidisciplinary approach used by an established team at a single high volume center for esophageal cancer, and to review the literature which guides our treatment recommendations.

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Key words: Esophageal Cancer; Multimodality therapy; Multidisciplinary therapy; Chemoradiotherapy; Esophageal resection; Esophagectomy

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INTRODUCTION

Esophageal cancer is a growing epidemic with approximately 460 000 new diagnosis and 380 000 deaths annually worldwide^[1,2]. Adenocarcinoma has increased in incidence while the incidence of squamous cell esophageal carcinoma has decreased in the Western world. This seems to be linked to gastroesophageal (GE) reflux disease and Barrett's esophagus^[3-7]. The prognosis for these patients is generally poor because of the advanced stage at the time of presentation. The increase in use of pro-

ton-pump inhibitors over-the-counter has also decreased the impetus to seek physician assistance for reflux symptoms. Hence most of these patients will be diagnosed at a late stage, with approximately 50 percent of patients have advanced unresectable or metastatic cancer^[7]. Most patients are not considered curable at diagnosis and are treated with chemotherapy and radiation, mostly with palliative intent. In patients who are fortunate enough to have potentially resectable disease, the data are not clear as to the best approach. Patients with very early disease may only require endoscopic mucosal resection or surgical resection. Others are treated with a combination of chemotherapy plus radiation (chemoradiation) plus surgery if they are deemed resectable. Ideally, we would like to have large randomized trials that were powered properly to support our treatment plans. As these studies do not exist in the esophageal cancer world, we are left to rely mainly on meta-analysis, small randomized trials, and historical reports to make decisions for our patients. These treatments require specialists from surgery, medical oncology, radiation oncology, and gastroenterology. It is imperative that these individuals work in a multidisciplinary fashion in order to deliver comprehensive care. The goal of this paper is to discuss the approach of an established multidisciplinary team in the treatment of patients with locoregionally advanced disease.

EVALUATION OF THE PATIENT

To obtain an adequate volume of tissue for diagnosis, a minimum of 7 core/pinch biopsy specimens in addition to brushings are recommended at the time of endoscopy. This approach improves the accuracy of diagnosis to 98%-100%^[7]. In addition, it provides tissue for molecular marker analysis, as cancer therapy is beginning to focus on targeted therapies which may require tumor marker analysis. Staging studies should include a computed tomography of the chest and abdomen, a positron emission tomography (PET) scan and endoscopic ultrasound by a specialized gastroenterologist trained and proficient in this technique. Biopsies should be obtained from suspicious lymph nodes if accessible. An esophagram is also helpful in determining the degree of esophageal stricture. With the seventh edition American Joint Committee on Cancer Staging System, it is imperative to determine the histology of the tumor and number of lymph nodes involved. In patients who have respiratory symptoms, a bronchoscopy should be done to evaluate for tracheoesophageal fistula formation. In patients with other pulmonary or abdominal findings on imaging studies, one may wish to pursue thoracoscopy or laparoscopy. In addition, it is important to assess the performance status, the nutritional status and the patient's comorbidities of prior to determining an appropriate treatment plan.

TREATMENT APPROACHES

Treatment of cT1-2N0 disease

Surgery alone remains the standard of care for patients

with local disease (cT1N0 and some cT2N0 tumors). At our institution and other high volume institutions, patients with T1aN0 tumors are treated with minimally invasive techniques such as endoscopic mucosal resection. There is limited experience with the use of radiation or chemoradiotherapy in the curative setting for patients with cT1N0 disease^[8]. Thirty-four patients with either medically inoperable disease or refused surgery were treated with external beam alone (64 Gy) or external beam (52 Gy) plus 8 to 12 Gy with brachytherapy. The median follow-up was 61 mo, 5-year survival was 59%, 68% local relapse-free survival, and 80% cause-specific survival^[8].

For most cT2N0 tumors, surgery alone may not be sufficient since approximately 50% of patients may have lymph node metastasis^[9-12]. However, if the nodes are negative (pT2N0m0) there is no role for postoperative adjuvant chemotherapy or chemoradiation.

Treatment of cT3-4 and/or N positive disease

There remains much controversy in what is considered the current standard of care for patients with locally advanced esophageal cancer (cT3-4 and/or N positive)^[13-15]. Initially, surgical resection was the main modality for esophageal cancer treatment. Since the 1980's, studies have evaluated the utility for perioperative chemotherapy, postoperative and more commonly preoperative chemoradiation to improve outcome. These studies have been criticized for a variety of insufficiencies including inadequate power, the type of chemotherapy regimen, the dosing of chemotherapy, the radiation dose and fraction size, radiation delivery schedules, number of patients enrolled, initial staging, multiple organ sites and histologic subtype. At our institution, we advocate the use of neoadjuvant chemoradiotherapy based on the following data.

DO PATIENTS BENEFIT FROM NEOADJUVANT RADIATION?

There have been five phase III trials which evaluated neoadjuvant radiation in esophageal cancer. None of the studies have demonstrated an increase in overall survival or resectability of esophageal cancer patients treated with radiation alone^[16-20]. Nygaard *et al*^[19] reported a 3-year overall survival benefit only after adding patients who also received chemotherapy to the statistical analysis. A meta-analysis of neoadjuvant radiation revealed a trend toward improved 5-year overall survival but failed to show a statistically significant survival advantage^[21]. Data do not support the use of radiation as a single modality in the neoadjuvant treatment of esophageal cancer (Table 1). The role of radiation alone should be limited to palliation.

DO PATIENTS BENEFIT FROM PERIOPERATIVE CHEMOTHERAPY?

Neoadjuvant or perioperative chemotherapy has also

Table 1 Randomized trials of neoadjuvant radiation in esophageal cancer

Ref.	Histology	n	Rad dose (Gy)	2-yr survival, %	5-yr survival, %
Launois <i>et al</i> ^[18]	SCC	57	-	11.5	NR
		67	39-45	9/5	NR
Gignoux <i>et al</i> ^[17]	SCC	106	-	10	9
		102	33/12	16	10
Wang <i>et al</i> ^[20]	NR	102	-	33	30
		104	40	37	35
Nygaard <i>et al</i> ^[19]	SCC	50	-	NR	9 (3 yr)
		58	35 (4 wk)	NR	21 (3 yr)
Arnott <i>et al</i> ^[16]	SCC	86	-	NR	17
		90	20 (10 d)	NR	9

SCC: Squamous cell carcinoma; NR: Not reported; AC: Adenocarcinoma.

been evaluated in patients with locally advanced gastric and GE cancer. At least five phase III trials have compared cisplatin-based regimens to surgery alone in esophageal cancer and three studies showed a survival advantage (Table 2)^[22-26]. The Medical Research Council trial is the largest of these studies as it randomized 802 patients with esophageal adenocarcinoma or squamous cell carcinoma. There was a 5-year overall survival advantage of approximately 6%^[24]. The Magic trial randomized 503 patients, predominately gastric cancer patients, and demonstrates a 5-year overall survival advantage of 13%^[22]. The French Cooperative Group study randomized 224 predominately gastric cancers with a survival advantage of 14% at 5 years^[26]. All of these studies noted no evidence of increased morbidity or mortality in patients who received neoadjuvant chemotherapy. Many studies that evaluated perioperative chemotherapy have shown some overall survival benefit. This is evident in GebSKI's meta-analysis that evaluated 1724 patients who received chemotherapy and surgery versus surgery alone in 8 trials. Of note was a 7% absolute benefit in 2 years survival ($P = 0.014$) in adenocarcinoma patients only^[27]. These data are complicated because only 2 of the studies evaluate only esophageal cancer^[23,24]. While we feel there is some benefit to perioperative chemotherapy, we do not advocate its use as the data suggest neoadjuvant chemoradiotherapy to be superior in esophageal cancer.

DO PATIENTS BENEFIT FROM CONCURRENT CHEMORADIO THERAPY?

Chemotherapy combined with radiation enhances the effects of radiation by synergistically damaging the DNA following cell cycle synchronization^[28,29]. Chemotherapy theoretically also reduces the risk of distant metastatic disease by eradication of micrometastases^[30]. Chemoradiation is useful in both the neoadjuvant setting for all esophageal cancer patients or in the adjuvant setting for patients with GE junction tumors. Additionally, in patients who are not surgical candidates chemoradiation may be used as definitive treatment^[31]. Ideally, concurrent

chemoradiation should be done by a multidisciplinary group proficient in these procedures as many situations may result in a less favorable outcome. Situations which may occur include unnecessarily missed chemotherapy or radiation doses for complications which could be managed by groups more experienced in this technique. Additionally, the use of unconventional chemotherapy or radiation regimens or erroneous staging studies may also be problematic.

Initially, concurrent chemoradiation was evaluated as definitive treatment for patients who were not surgical candidates in the Radiation Therapy Oncology Group (RTOG) 85-01 trial^[31]. In this study 134 patients were randomized to cisplatin combined with infusional fluorouracil and concurrent radiation or to radiation alone. The patients predominately had esophageal squamous cell carcinoma. Interim analysis revealed that a statistically significant survival advantage favoring concurrent chemoradiotherapy hence changing the treatment paradigm in inoperable locally advanced esophageal cancer. The 5-year overall survival was 27% *vs* 0% with radiation alone^[31]. Despite the reduction in the risk of persistent disease or local recurrence with concurrent chemoradiotherapy compared to radiation alone, the incidence of locoregional failure was a dismal 47%^[31]. Hence, in an effort to reduce locoregional failure, radiation dose was then addressed by the INT 0123 trial^[32]. A total of 236 patients were randomized to high (68.4 Gy) or low (50.4 Gy) dose radiation all given with concurrent cisplatin and infusional fluorouracil per the RTOG 85-01 regimen. An interim analysis failed to reveal a local control or survival benefit with high dose radiation hence, 50.4 Gy has become standard of care for both neoadjuvant and definitive radiotherapy^[32].

Patients with esophageal cancer have unacceptably high locoregional failure rates of approximately 50% with chemoradiation and a dismal prognosis of 20%-25% at 5 years with surgery alone^[23,33-35]. Based on the limited success of these two approaches, a number of studies evaluating the combination of chemoradiation and surgery were developed.

DO PATIENTS BENEFIT FROM SURGERY?

Surgery has been considered an essential part of the treatment of patients with esophageal carcinoma^[36]. Past experiences showed that a nonsurgical approach was associated with mediocre survival results^[37]. However, the better survival achieved with surgical therapy may have a high price. In 1980, Earlam *et al*^[38] reviewed the literature and reported 29% mortality for esophagectomy. Today, some still quote these numbers as a justification for nonsurgical approach to esophageal cancer, stating if a patient with esophageal cancer may either die by the tumor or die by the knife. However, it is hard to believe that these numbers remain true after significant improvements in antibiotics, intensive care, surgical equipment, and technique. Additionally, developments in chemotherapy and radiotherapy have occurred as well.

What is the best surgical technique?

There are 3 different basic approaches for esophagectomy: (1) transhiatal; (2) transthoracic; and (3) *en bloc* or radical. The transhiatal approach has a theoretical advantage of a decreased morbidity and mortality due to the avoidance of a thoracotomy (and thus, a decreased operative time and pain). Even though concern has been raised about lesser oncologic radicality, several studies compared the outcomes for transhiatal versus transthoracic esophagectomy. Two meta-analysis in 2 different decades (2001 and 2011) showed no differences in survival comparing these two approaches^[39,40]. The transthoracic group; however, had significantly more respiratory complications, wound infections, and early postoperative mortality, whereas anastomotic leak, anastomotic stricture, and recurrent laryngeal nerve palsy rates were significantly higher in the transhiatal group. Population studies reached the same conclusions. Chang *et al*^[41] evaluated a pool of 868 patients from the American Surveillance, Epidemiology, and End Results-Medicare linked database (1992 to 2002) with similar results. Additionally, Connors *et al*^[42] consulted the registries of 17 395 patients from the American Nationwide Inpatient Sample database and found similar outcomes for both procedures.

The need for lymphadenectomy (radical esophagectomy) is an ongoing debate in esophageal surgery. Also, it is unclear if the patients that may benefit from these procedures are the ones with early cancer or locally advanced tumors. Moreover, the extent of the lymphadenectomy (1 field-thoracic, 2 fields- thoracic and abdominal, 3 fields-thoracic, abdominal and cervical) is a controversial topic. The lack of randomized trials addressing this issue increases the controversy. Although some studies showed better survival after *en bloc* esophagectomy others showed results similar to a transhiatal esophagectomy^[43,44]. Morbidity and mortality for this procedure is not always reported; however, it seems to be high, especially in 3-field^[45].

Minimally invasive surgery has the advantages of better cosmetic results, reduced operative stress, postoperative immobility, and pain. As far as we are aware, no randomized controlled trials have compared minimally invasive and open esophagectomy to date. Available data, including 3 recent meta-analysis suggests that minimally invasive esophagectomy is similar to conventional esophagectomy in terms of complications, oncologic radicality and survival^[46-49].

Modern outcomes for mortality and survival

Different studies from experienced centers show a rate of mortality close to 0 in patients with non-advanced or even advanced tumors^[50-53]. The application of standardized protocols with a multidisciplinary team improved significantly the outcomes of esophagectomy. It is not easy to access the survival related to esophagectomy only since most series of surgery alone are related to initial cancer and most surgeons refer patients to neoadjuvant

or adjuvant chemotherapy^[54]. However, patients with potentially resectable tumor not referred for surgery have a lower survival rate^[55].

Relationship between volume and outcomes

Different papers repeatedly reported better outcomes for esophagectomy in high volume centers^[42,56,57]. This better results may be attributable to surgeons' experience, since a decrease in more than 50% in the index of complications following esophagectomy is observed when the operation is performed by surgeons experienced in more than 100 esophagectomies^[58]. However, hospital volume is also important, since the preparedness of the multidisciplinary team and hospital services to attend esophagectomy patients is crucial to better outcomes. Even low volume hospitals with high nurse ratios, lung transplantation services, complex medical oncology services, bariatric surgery services, and positron emission tomography scanners have lower mortality rates compared with low-volume hospitals with none of these characteristics^[59]. Very interestingly, survival was not linked to volume^[60].

DO PATIENTS WHO UNDERGO SURGERY BENEFIT FROM NEOADJUVANT CHEMORADIATION?

To date, there have been eleven randomized trials performed evaluating the utility of neoadjuvant chemoradiotherapy added to surgery (Table 3). These trials have incorporated a variety of chemotherapy regimens, doses and fraction sizes of radiation and timing of both chemotherapy and radiation. Of these studies only 3 have shown a benefit with concurrent chemoradiotherapy. The CALGB 9781 study randomly assigned patients to cisplatin, infusional fluorouracil with concurrent radiation and surgery or to surgery alone. The study was unable to adequately accrue due to patient and investigator bias favoring the neoadjuvant arm. Despite the lack of accrual in this study there was an impressive five-year overall survival of 39% with multimodality treatment and 16% with surgery alone ($P = 0.002$)^[61]. The study performed by Walsh *et al*^[62] randomized 113 patients with adenocarcinoma to cisplatin, infusional fluorouracil and radiotherapy followed by surgery or to surgery alone. The median overall survival was 16 mo with multimodality treatment and 11 mo with surgery alone ($P = 0.01$). Three-year survival of 32% with multimodality treatment and 6% with surgery alone ($P = 0.01$). This study has been criticized for inadequate radiation dose, inadequate fluorouracil dose, survival in the control arm lower than historical controls, and lack of adequate staging prior to chemoradiotherapy^[62]. The CROSS trial has been reported in abstract form by van der Gaast *et al*^[63]. A total of 363 patients with adenocarcinoma or squamous cell carcinoma were randomized to preoperative paclitaxel/carboplatin plus 41.4 Gy *vs* surgery alone.

Table 2 Randomized trials of peri-operative chemotherapy in gastric and esophageal cancer

Ref.	Histology	Regimen	n	Resection	pCR	5-yr survival %	P value	Median survival (mo)
Kelson <i>et al</i> ^[23]	SCC (45%)	S	227	59%	NA	23 (3 yr)	NS	16.1
	AC (55%)	CF→S→CF	213	62%	2.50%	26 (3 yr)		14.9
Cunningham <i>et al</i> ^[22]	AC - Gastric	S	253	66%	NA	23	0.009	20
	25% GEJ	ECF→S→ECF	250	69%	0%	36		24
MRC <i>et al</i> ^[24]	SCC (35%)	S	402	54%	NA	17	0.004	13.3
	AC (65%)	CF→S	400	62%	NA	23		16.8
Roth <i>et al</i> ^[25]	AC/SCC	S	20	NR	NR	5	NS	9
		BVC→S	19			25		9
Ychou <i>et al</i> ^[26]	AC						0.02	
	75% GEJ	S	111	111	NA	24		NR
	25% Gastric	CF→S→(CF)	113	113	NR	38		NR

CF: Cisplatin and 5-fluorouracil; SCC: Squamous cell carcinoma; ECF: Epirubicin, cisplatin and 5-fluorouracil; AC: Adenocarcinoma; NA: Not Applicable; GEF: Gastroesophageal junction/distal esophagus; NR: Not recorded; NS: Not significant; S: Surgery; BVC: Bleomycin, vindesine, cisplatin; pCR: Protein catabolic rate; GEJ: Gastroesophageal junction.

Table 3 Randomized trials of neoadjuvant combined modality therapy for esophageal cancer

Ref.	Cell type	n	Total dose (Gy)	5-yr survival, %	Median survival (mo)	P value	Criticism
Nygaard <i>et al</i> ^[19]	SCC	47	BP + 35 + surg	11.5 (3 yr)	8	NS	Unconventional chemotherapy and low dose RT
			Surgery	9.5 (3 yr)	7		
Bosset <i>et al</i> ^[34]	SCC	143	P + 37 + surg		19	NS	Split course RT and unconventional chemo schedule
			Surgery	9	19		
Tepper <i>et al</i> ^[61]	SCC (25%)	30	PF + 50.4 + surg	39	54	< 0.001	Only 56 of 475 planned patients entered
	AC (75%)	26	Surgery	16	21		
Walsh <i>et al</i> ^[62]	AC (100%)	58	PF + 40 + surg	32 (3 yr)	16	< 0.05	Only 6% 5 yr survival benefit with surgery alone
			Surgery	6 (3 yr)	11		
Gaast <i>et al</i> ^[63]	SCC (25%)	175	Carbo/tax + 41 + surg	59 (3 yr)	49	< 0.001	Only 41 Gy RT
	AC (75%)	188	Surgery	48 (3 yr)	26		
Le Prise <i>et al</i> ^[85]	SCC	41	PF + 20 (split) + surg	19 (3 yr)	10	NS	Only some patients received split course radiotherapy chemotherapy
			Surgery	14 (3 yr)	10		
Apinop <i>et al</i> ^[86]	SCC	35	PF + 20 + surg	24	10	NS	Low dose RT
			Surgery	10	7		
Lee <i>et al</i> ^[87]	SCC	51	PF + 45.6 (bid) + surg	49 (3 yr)	28	NS	1.2 Gy bid radiation
			Surgery	51 (3 yr)	27		
Urba <i>et al</i> ^[88]	SCC (25%)	47	PF + 45 + surg	30 (3 yr)	17	NS	15% survival benefit but not statistically significant
	AC (75%)	50	Surgery	16 (3 yr)	18		
Burmeister <i>et al</i> ^[89]	SCC (37%)	128	PF + 35 + surg	17	22	NS	Only 35 by radiation delivered
	AC (62%)	128	Surgery	13	19		
Mariette <i>et al</i> ^[90]		97	PF + 45 + surg	NR	32	0.66	T1-2 only, Hi postoperative mortality c CRT
			Surgery		44		

P: Cisplatin; BP: Bleomycin and cisplatin; NS: Not significant; PF: Cisplatin and 5-fluorouracil; AC: Adenocarcinoma; SCC: Squamous cell carcinoma; NR: Not reported; NS: Not significant; RT: Radiation; CRT: Chemoradiotherapy.

With a median follow-up of 32 mo patients who received chemoradiation had a significant benefit in 3-year survival (59% *vs* 48%, *P* = 0.011). There was also an increase in (RO) resection rates 67% *vs* 92%, 0.002 favoring chemoradiotherapy^[63].

Given the contradictory and inconclusive results in many of the trials evaluating neoadjuvant multimodality treatment based on disparate study populations, differing histology, differing chemotherapy and radiotherapy doses and regimens, and small numbers of patients, data have been pooled in an effort to synthesize the data into larger numbers to discover if a survival benefit exists^[64]. The first meta-analysis published by Urschel *et al*^[65], included nine randomized controlled trials and 1116

patients. A trend toward 3-year survival improvement favoring neoadjuvant chemoradiotherapy was noted with the most pronounced effect with concurrent chemoradiotherapy as compared to a sequential approach. There was a decreased risk of local-regional recurrence but concerning trend toward increased treatment mortality with multimodality treatment. There was no difference in the risk of distant recurrence. Fiorica *et al*^[66] noted an improvement in patients who received neoadjuvant chemoradiotherapy. GebSKI *et al*^[27] also evaluated neoadjuvant chemotherapy and chemoradiotherapy compared to surgery alone in a meta-analysis. This recent meta-analysis evaluated 1209 patients with both adenocarcinoma and squamous cell carcinoma of the esophagus in

ten trials. A statistically significant benefit was noted with neoadjuvant chemoradiotherapy compared to surgery alone with a 19% decrease in the risk of death corresponding to a 13% absolute difference in 2 year survival. An absolute survival benefit of 7% was noted for neoadjuvant chemotherapy as compared to surgery alone. The Preoperative Chemotherapy or Radiochemotherapy in Esophagogastric Adenocarcinoma Trial (POET) attempted to determine in a prospective, randomized fashion if neoadjuvant concurrent chemoradiotherapy is more beneficial than perioperative chemotherapy^[67]. There was a trend toward improved pathologic complete response with neoadjuvant chemoradiotherapy but the study was closed early due to lack of accrual^[67]. Given these data, we plan neoadjuvant chemoradiotherapy in our eligible patients.

DO PATIENTS WHO UNDERGO CHEMORADIATION BENEFIT FROM SURGERY?

Two randomized trials examine whether surgery is necessary after chemoradiation. In the FFCO 9102 trial, 445 patients with clinically resectable T3-4N0-1M0 squamous cell carcinoma of the esophagus received initial chemoradiation^[68]. Patients initially received 2 cycles of 5-fluorouracil (5-FU), cisplatin, and concurrent radiation (either 46 Gy at 2 Gy/d or split course 15 Gy weeks 1 and 3^[68]). The 259 patients who had at least a partial response were then randomized to surgery *vs* additional chemoradiation which included 3 cycles of 5-FU, cisplatin, and concurrent radiation (either 20 Gy at 2 Gy/d or split course 15 Gy). There was no significant difference in 2-year survival (34% *vs* 40%, $P = 0.56$) or median survival (18 mo *vs* 19 mo) in patients who underwent surgery *vs* additional chemoradiation. These data suggest that for patients who initially respond to chemoradiation, they should complete chemoradiation rather than stop and undergo surgery. The German Oesophageal Cancer Study Group compared preoperative chemoradiation followed by surgery *vs* chemoradiation alone^[69]. In this trial, 172 patients < 70 years old with uT3-4N0-1M0 squamous cell cancers of the esophagus were randomized to preoperative therapy (3 cycles of 5-FU, leucovorin, etoposide, and cisplatin, followed by concurrent etoposide, cisplatin, plus 40 Gy) followed by surgery *vs* chemoradiation alone (the same chemotherapy but the radiation dose was increased to 60-65 Gy +/- brachytherapy). In patients who underwent surgical resection, 35% had complete pathologic response and 33% had no evidence of lymph node involvement following neoadjuvant therapy^[69]. Despite a decrease in 2-year local failure (36% *vs* 58%, $P = 0.003$) there was no statistically significant difference in 3-year survival (31% *vs* 24%) for those who were randomized to preoperative chemoradiation followed by surgery *vs* chemoradiation alone. The practice at our institution is to closely observe patients with

esophageal squamous cell cancer who have a complete clinical response. Patients with adenocarcinoma continue to require surgical resection as these studies only evaluate patients with squamous cell cancer and studies to address adenocarcinoma have not been done.

DO PATIENTS WHO HAVE SURGERY BENEFIT FROM ADJUVANT TREATMENT?

In an effort to address locally advanced gastric and GE junction cancers adjuvant chemoradiotherapy was evaluated (MacDonald)^[70]. This trial (INT 0116) enrolled 556 patients with gastric and GE junction (approximately 20%) adenocarcinoma. Patients were randomized to surgery alone or surgery followed by adjuvant leucovorin-modulated fluorouracil with concurrent radiation (45 Gy) in cycle 2 of 4 total cycles. There was an improvement in median overall survival with adjuvant therapy 36 mo *vs* 27 mo in the observation group ($P = 0.005$)^[70]. Treatment related toxicity prevented completion of the treatment in 17% of patients^[70]. There are no data evaluating the utility of adjuvant therapy in patients with more proximal esophageal tumors. At our institution, we plan neoadjuvant chemoradiation in locally advanced esophageal cancer patients. If following surgery, the pathology upstages the cancer, we plan for adjuvant treatment only in cancers of the GE junction.

WHAT IS OUR PRACTICE AND RATIONALE?

In summary, although there is good rationale for its use, it is not clear that the combination of surgery and chemoradiation regardless of the sequence, improves the survival results of either treatment alone. The survival benefit is likely to be 5%-10% with multimodality therapy. Currently, the standard of care in treatment of locally advanced tumors at our institution is to place patients on neoadjuvant chemoradiotherapy provided that it is feasible. We take into account the tumor location, size of the radiation field, comorbidities, and performance status in determining what the best multimodality approach is. There are many institutions, especially in Europe, who use neoadjuvant chemotherapy only. It is our practice to use neoadjuvant chemoradiotherapy because of the findings of the CALGB 9781, Walsh study, multiple meta-analysis and more recently the POET study^[27,61,62,67]. The GebSKI meta-analysis quoted a 13% absolute benefit in 2 year survival with neoadjuvant chemoradiotherapy and a 7% absolute benefit with neoadjuvant chemotherapy^[27]. This is almost a doubling of the benefit conferred with perioperative chemotherapy alone. Additionally, the POET study, the only study which compares neoadjuvant chemotherapy to neoadjuvant chemoradiotherapy demonstrates a trend toward increased pathologic complete response at resection and survival with neoadjuvant chemoradiotherapy^[67]. This study was performed in patients with GE junction

tumors and was closed early due to poor recruitment.

Additionally, we feel surgery may not be required in patients with esophageal squamous cell carcinoma provided they have a complete response to neoadjuvant therapy. Given that squamous cell carcinomas often recur locally, observation alone may be acceptable in this small group of patients. For those patients who have adenocarcinoma, we feel resection is still standard of care.

WHAT IS BEING EVALUATED CURRENTLY IN ESOPHAGEAL CANCER?

Despite improvements noted with multimodality treatment in esophageal cancer, cure rates are consistently dismal^[27]. With new interest in targeted agents in cancer demonstrating benefit in malignancies of the head and neck, breast, lung, colon and pancreas have generated evaluation in the esophagus^[71-73]. Multiple molecular pathways have been evaluated at the molecular level with potential targets in esophageal cancer including cyclin dependent kinases, nuclear factor κ , matrix metalloproteinases, inhibition of cyclooxygenase-2, c-MET (a protooncogene that encodes a protein known as hepatocyte growth factor), epidermal growth factor receptor (EGFR) and vascular endothelial growth factor^[72].

Over-expression of EGFR proteins may occur in 30%-70% of both adenocarcinoma and squamous cell carcinoma of the esophagus. Over-expression is associated with increased aggressiveness of the malignancy and poor prognosis^[74-76]. Clinical trials have been initiated trying to take advantage of this protein. The Southwest Oncology Group initially targeted this protein by using single agent cetuximab as a second-line therapy with discouraging results^[77]. More recent studies have evaluated cetuximab or other monoclonal EGFR antibodies with chemotherapy appear to be more promising^[78-80]. Recently, EGFR-2 (Her-2-neu) has also been evaluated in gastric and esophageal cancers over expressing human EGFR-2 HER2 (ToGA trial) with promising results^[81]. These targeted agents are currently undergoing evaluation in a multimodality setting with chemoradiotherapy. Safran *et al*^[82] have evaluated 57 patients with esophageal cancer with weekly carboplatin, paclitaxel, cetuximab, and concurrent radiation (50.4 Gy). Complete clinical response was achieved in 70% of patients. Forty-nine of patients went on to surgery with a pathologic complete response rate of 27%. The RTOG 0436 is an ongoing phase III trial evaluating weekly carboplatin, paclitaxel, and concurrent radiation with or without cetuximab in locally advanced inoperable patients. Additionally, given the results on the ToGA trial trastuzumab is currently under investigation with cisplatin, paclitaxel, and concurrent radiation for locally-advanced, HER2 overexpressing adenocarcinoma of the esophagus^[83].

Survival benefits of neoadjuvant therapy appear small, but it should be noted this is similar to other treatments for other lethal malignancies^[84]. The need to treat approximately 8 patients with a toxic regimen to cure

one additional patient is not ideal, yet these odds must be discussed with a patient who is felt to be medically fit to withstand an esophagectomy^[64]. Additionally, patients will often question about adjuvant therapy. While appropriate, in patients who have GE junction tumors only this is poorly tolerated post surgically, involves a larger radiation field, and radiation doses are lower^[70]. Hence, our recommendations in the locally advanced resectable patient remain neoadjuvant chemoradiotherapy followed by esophagectomy. How targeted therapies will affect our approach in locally advanced esophageal carcinoma and is currently under investigation^[73,76]. Additionally, studies arrived at neoadjuvant concurrent chemoradiation regimens based on PET response to induction chemotherapy are underway.

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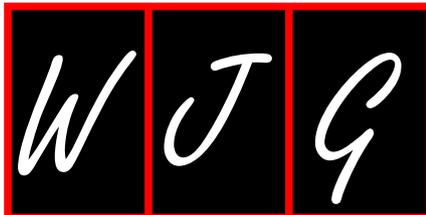
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Laparoscopic rectal cancer surgery: Where do we stand?

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Abstract

Large comparative studies and multiple prospective randomized control trials (RCTs) have reported equivalence in short and long-term outcomes between the open and laparoscopic approaches for the surgical treatment of colon cancer which has heralded widespread acceptance for laparoscopic resection of colon cancer. In contrast, laparoscopic total mesorectal excision (TME) for the treatment of rectal cancer has been welcomed with significantly less enthusiasm. While it is likely that patients with rectal cancer will experience the same benefits of early recovery and decreased postoperative pain from the laparoscopic approach, whether the same oncologic clearance, specifically an adequate TME can be obtained is of concern. The aim of the current study is to review the current level of evidence in the literature on laparoscopic rectal cancer surgery with regard to short-term and long-term oncologic outcomes. The data from 8 RCTs, 3 meta-analyses, and 2 Cochrane Database of Systematic Reviews was reviewed. Current data suggests that laparoscopic rectal cancer resection may benefit patients with reduced blood loss, earlier return of bowel function, and shorter hospital length of stay. Concerns that laparoscopic rectal cancer surgery compromises short-term oncologic outcomes including number of lymph nodes retrieved and circumferential resection margin

and jeopardizes long-term oncologic outcomes has not conclusively been refuted by the available literature. Laparoscopic rectal cancer resection is feasible but whether or not it compromises short-term or long-term results still needs to be further studied.

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Key words: Rectal cancer; Laparoscopy; Total mesorectal excision; Anterior resection; Abdominoperineal resection

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INTRODUCTION

Laparoscopic colon resection was introduced in 1991^[1,2]. Concern for port site metastasis and inadequate oncologic clearance initially hampered its adoption in the treatment of colon and rectal malignancy^[3-6]. However, recently large comparative studies and multiple prospective randomized control trials (RCTs) have reported equivalence in resection margin, lymph node collection, tumor recurrence, postoperative complications, and long-term outcomes between open and laparoscopic

resection for colon cancer^[7-12]. In addition, these studies demonstrated earlier recovery of bowel function, less postoperative pain, and decreased hospital stay with the laparoscopic approach which has heralded widespread acceptance for laparoscopic resection of colon cancer^[8,9,13-16]. In contrast, laparoscopic total mesorectal excision (TME) for the treatment of rectal cancer has been welcomed with significantly less enthusiasm.

While it is likely that patients with rectal cancer will experience the same benefits of early recovery and decreased postoperative pain from the laparoscopic approach, whether the same oncologic clearance, specifically an adequate TME can be obtained is of concern^[17-23]. Involvement of the circumferential resection margin (CRM) after TME is a prognostic factor for local recurrence^[24-28]. Marijnen *et al.*^[29] found that in the Dutch Rectal Cancer Trial, 13.1% of patients with a positive CRM developed a local recurrence within 2 years of follow-up, whereas patients with a margin > 2 mm had a local recurrence rate of 3.3% at 2 years ($P < 0.0001$). Postoperative radiation did not lead to a reduction in the local recurrence rate (17.3% *vs* 15.7% local recurrence in patients with CRM < 1 mm with and without adjuvant radiotherapy respectively, $P = 0.98$)^[29]. In addition, preoperative radiotherapy had no significant effect on the prevention of local recurrence in patients with positive CRM (9.3% in the irradiated group *vs* 16.4% in the surgery alone group, $P = 0.08$) highlighting the importance of adequate surgery. In conventional open resection of rectal cancer, considerable variation between surgeons in oncologic outcomes has been demonstrated^[30]. Differences in local recurrence and disease-free survival may be amplified by the technical challenges of laparoscopic proctectomy. While, the laparoscopic approach provides a magnified view compared to open surgery, TME and autonomic nerve preservation which are prerequisites for satisfactory oncologic and functional results require significant laparoscopic expertise^[31]. A number of studies have reported on the safety and feasibility of laparoscopic low anterior resection (LAR) and abdominoperineal resection (APR) with TME but there is no level one evidence supporting laparoscopic TME in terms of oncologic outcomes^[19,20,32-36]. The aim of this study is to provide a systematic review of the short-term and long-term oncologic outcomes of laparoscopic rectal cancer resection.

DATA SOURCE

Peer-reviewed papers published on laparoscopic rectal cancer resection were found by searching the following terms in the Ovid Medline, PubMed, and Cochrane Database of Systematic Reviews from 1993 to 2010: "laparoscopy", "laparoscopic surgery", and "rectal cancer". Review articles found using the search terms "colon cancer" or "rectal cancer" and "laparoscopy" were also reviewed to find pertinent articles. All relevant articles were assessed and inclusion and exclusion criteria applied.

Study designs included prospective RCTs, meta-analyses, and Cochrane Database of Systematic Reviews. Studies were included if short-term outcomes, morbidity and mortality, or oncologic data specifically, recurrence rates, number of lymph nodes retrieved, margin status, and overall survival for patients undergoing curative laparoscopic rectal cancer resection were reported. When more than one trial containing overlapping patient inclusion periods and data was reported from the same institution, the most recent publication was included. Studies were excluded if they (1) reported both colonic and rectal outcomes, but did not analyze rectal cancer outcomes individually; (2) were non-randomized comparative trials, descriptive trials, or case reports; (3) were not published in the English language; and (4) reported on patients undergoing palliative treatment (non-curative surgical intent).

The majority of data on laparoscopic resection for rectal cancer come from non-randomized comparative and descriptive studies. The literature review yielded a total of 79 studies published in the English language from 1993 to 2010. Sixty-five studies were excluded because they were non-randomized comparative trials or descriptive trials. One meta-analysis was excluded because individual studies were not analyzed according to the site of disease or the type of resection. The remaining 2 Cochrane reviews, 3 meta-analyses, and 8 RCTs comparing laparoscopic *vs* open TME for rectal cancer form the basis of this review. When assessing the data on laparoscopic resection of rectal cancer it is important to remember that results may vary greatly based on level of the tumor, APR *vs* LAR, use of neoadjuvant chemotherapy, and completeness of TME.

OUTCOMES OF INTEREST

Intraoperative outcomes include: duration of operation, blood loss, length of incision, and conversion rate. Short-term parameters of interest include: early postoperative complications (hemorrhage, anastomotic leak, wound complications, chest infection, prolonged ileus, incidence of pulmonary embolism or deep vein thrombosis, and urinary infection/retention), and mortality. Oncologic outcomes reviewed include: number of lymph nodes retrieved, margin status, completeness of TME, local recurrence, and overall survival.

Intraoperative results

The proven benefits of laparoscopy noted in colon cancer surgery including decreased intraoperative blood loss, smaller length of incision, less postoperative pain, faster recovery of intestinal function, and shorter length of hospital stay likely also apply to rectal cancer surgery^[37]. In RCTs (Table 1) the mean operative time for open surgical resection of rectal cancer ranged from 106 to 284 min compared to 120 to 245 min for laparoscopic resection (Table 2). As expected, duration of operation was significantly longer in the laparoscopic group com-

Table 1 Patient characteristics from randomized control trials

Ref.	Patients			M/F		BMI		Age (yr)		% Pre-op ChemoRT	
	Total	Open	Lap	Open	Lap	Open	Lap	Open	Lap	Open	Lap
Kang <i>et al</i> ^[40]	340	170	170	110/60	110/60	24.1 (3.2)	24.1 (3.2)	59.1 (9.9)	57.8 (11.1)	100	100
Ng <i>et al</i> ^[45]	153	77	76	48/29	37/39	NA	NA	65.7 (12)	66.5 (11.9)	NA	NA
Lujan <i>et al</i> ^[31]	204	103	101	64/39	62/39	NA	NA	66.0 (9.9)	67.8 (12.9)	74.8	72.3
Ng <i>et al</i> ^[39]	99	48	51	30/18	31/20	NA	NA	63.5 (12.6)	63.7 (11.8)	0	0
Guillou <i>et al</i> ^[7] /Jayne <i>et al</i> ^[12]	343	113	230	NA	NA	26 (4)	25 (4)	69 (12)	69 (11)	NA	NA
Braga <i>et al</i> ^[38]	168	85	83	64/21	55/28	NA	NA	65.3 (10.3)	62.8 (12.6)	14.1	16.9
Zhou <i>et al</i> ^[35]	171	89	82	43/46	46/36	NA	NA	45 (30-81)	44 (26-85)	NA	NA
Araujo <i>et al</i> ^[34]	28	15	13	10/5	9/4	25.6 (17.1-38.5)	23.5 (21.7-24.6)	56.4 (24-78)	59.1 (31-75)	15 (100%)	13 (100%)

BMI: Body mass index; ChemoRT: Chemoradiation.

Table 2 Intraoperative characteristics of patients from randomized control trials

Ref.	Number of patients (%)							Conv %	Op time (min)		Blood loss (mL)		Length of incision (cm)	
	Total	Open			Lap				Open	Lap	Open	Lap	Open	Lap
		Total	LAR	APR	Total	LAR	APR							
Kang <i>et al</i> ^[40]	340	170	146	24	170	151	19	1.2	197.0	244.9	217.5	200.0	20.0	5.0
			(75.9)	(14.1)		(88.8)	(11.2)		(62.9)	(75.4) ^a	(150.0-400.0)	(100.0-300.0) ^a	(18.0-23.0)	(4.5-6.0)
Ng <i>et al</i> ^[45]	153	77	77	0	76	76	0	30.3	154.0	213.1	337.3	280.0	NA	NS
			(100)	(0)		(100)	(0)		(70.3)	(59.3) ^a	(0-2542)	(0-3000)		
Lujan <i>et al</i> ^[31]	204	103	81	22	101	77	24	7.9	172.9	193.7	234.2	127.8	NA	NA
			(78.6)	(21.4)		(76.2)	(23.8)		(59.4)	(45.1) ^a	(± 174.3)	(± 113.3) ^a		
Ng <i>et al</i> ^[39]	99	48	0	48	51	0	51	9.8	163.7	213.5	555.6	321.7		
			(0)	(100)		(0)	(100)		(43.4)	(46.2) ^a	(0-4720)	(0-3000)		
Guillou <i>et al</i> ^[7] /Jayne <i>et al</i> ^[12]	343	113	79	34	230	167	63	34	180	135	NA	NA	22	10
			(69.9)	(30.1)		(72.6)	(27.4)		(135-220)	(100-180)			(18-29)	(6-17)
Braga <i>et al</i> ^[38]	168	85	74	11	83	76	7	7.2	209	262	396	213	19.1	5.8
			(87.1)	(12.9)		(92)	(84)		(72)	(72) ^a	(50-1600)	(50-1600) ^a	(± 3.1)	(± 0.8) ^a
Zhou <i>et al</i> ^[35]	171	89	89	0	82	82	0	NA	106	120	92	20	NA	NA
			(100)	(0)		(100)	(0)		(80-230)	(110-220) ^a	(50-200)	(5-120) ^a		
Araujo <i>et al</i> ^[34]	28	15	0	15	13	0	13	0	284	228 ^a	NA	NA	NA	NA
			(0)	(100)		(0)	(100)							

Conv: Conversion rate; LAR: Low anterior resection; APR: Abdominoperineal resection; NA: Not available. ^aP < 0.05 vs Open.

pared to the open group in 6 of the 8 RCTs^[7,22,31,38-40]. Similar results were reported in RCTs of open vs laparoscopic resection for colon cancer. Zhou *et al*^[35] reported both shorter open and laparoscopic operative times compared to other trials with no significant difference between the two operative approaches (120 min vs 106 min for laparoscopic vs open resection respectively, P = 0.051). However, no details were provided on tumor stage, conversion rate, or whether the analysis was performed on an intent-to-treat basis. Araujo *et al*^[34] was the only RCT to demonstrate significantly shorter operative times with laparoscopic compared to open resection (228 min vs 284 min respectively, P = 0.04). However, they attributed these results to fact that the surgical team performing laparoscopic APR was the same whereas open APR was often performed by different surgical teams. In addition, extraction of the specimen from the perineum likely decreased operative time because there was not an abdominal incision to close.

Two meta-analyses included operative time as an outcome of interest. Aziz *et al*^[41] included 22 studies compar-

ing laparoscopic vs open rectal cancer resection in 2071 patients and found that operative time was significantly increased with the laparoscopic group as compared to the open group with a weighted mean difference (WMD) of 40.18 (95% CI, 26.46-56.13). Gao *et al*^[42] performed a meta-analysis of short-term outcomes after laparoscopic resection for rectal cancer based on 11 studies and included 643 patients which reported no difference in operating time between open and laparoscopic approaches with a WMD of 1.59 (1.2-1.98).

Intraoperative blood loss was significantly less for the laparoscopic group compared to the open group in 4 of 6 RCTs and ranged from 20 mL to 321.7 mL and from 92 mL to 555.6 mL in the laparoscopic and open groups respectively (Table 2)^[31,35,38,40]. Araujo *et al*^[34] did not specifically report on the amount of intraoperative blood loss but there was no statistically significant difference in the need for blood transfusions between the two groups which was attributed to the fact that in an APR the majority of blood loss occurs during the perineal portion of the case which is the same regardless of surgical access.

Table 3 Short-term oncologic outcomes of patients from randomized control trials

Ref.	LN		Positive margin (CRM/distal) (%)	
	Open	Lap	Open	Lap
Kang <i>et al</i> ^[40]	18 (13-24)	17 (12-22)	7 (4.1)/NA	5 (2.9)/NA
Ng <i>et al</i> ^[45]	12 (7)	11.5 (7.9)	1 (1.3)/NA	2 (2.6)/NA
Lujan <i>et al</i> ^[31]	11.57 (5.10)	13.63 (6.26) ^a	3 (2.9)/0	4 (4.0)/0
Ng <i>et al</i> ^[39]	13.0 (7)	12.4 (6.7)	2 (4.2)/NA	3 (5.9)/NA
Guillou <i>et al</i> ^[7] / Jayne <i>et al</i> ^[12]	NA	NA	(14)/NA	(16)/NA
Braga <i>et al</i> ^[38]	13.6 (6.9)	12.7 (7.3)	2 (2.4)/0	1 (1.2)/0
Zhou <i>et al</i> ^[35]	NA	NA	NA	NA
Araujo <i>et al</i> ^[34]	11.9	5.5 ^a	NA	NA

LN: Lymph nodes; CRM: Circumferential resection margin; NA: Not available. ^a*P* < 0.05 vs Open.

A recent Cochrane review by Breukink *et al*^[43] evaluating the safety and efficacy of elective laparoscopic TME for the resection of rectal cancer found that in the majority of studies blood loss was reduced with the laparoscopic approach although this did not translate to fewer blood transfusions. Length of incision was measured in 3 of 8 RCTs and ranged from an average of 5 cm to 10 cm with the laparoscopic approach compared to an average of 19.1 cm to 22 cm with the open approach (Table 2)^[7,38,40].

Seven of the 8 trials reported a conversion rate which ranged from 0%-34% (Table 2)^[7,12,22,31,34,38-40]. Conversion to the open approach was commonly defined as length of incision greater than the size needed for tumor extraction or premature abdominal incision to allow improved mobilization. In the majority of studies conversion to open surgery was required because of local tumor invasion or difficult dissection in a narrow pelvis although bulky tumor, dilated small bowel, dense adhesions, bleeding, rectal perforation, difficulty mobilizing the splenic flexure, failure to identify or injury to the ureter, ischemia of the descending colon, and anastomotic failure were also cited. Breukink *et al*^[44] reported that 36 of 48 studies assessed conversion and showed a highly variable rate ranging from 0% to 33%. However, they report that the lack of consensus in the definition made results difficult to interpret. In addition, surgeon experience and patient selection criteria were often not mentioned.

Two trials reported particularly high rates of conversion. Ng *et al*^[45] had a conversion rate of 30.3% but they did not routinely perform preoperative staging with computed tomography scans and therefore frequently converted after diagnostic laparoscopy. Twelve of the 23 patients randomized to laparoscopic surgery were converted to open due to local tumor invasion, bulky tumor, or dilated small bowel which may have been recognized by preoperative imaging. In the CLASICC trial the conversion rate for laparoscopic resection of rectal cancer was reported at 34% and attributed to excessive tumor fixation and uncertainty of tumor clearance^[7]. Surgeon learning curve may account for this high rate of con-

version as evidenced by the fact that the overall rate of conversion dropped by year of study from 38% in year one to 16% in year six. However, consistent with several non-randomized reports, in the CLASICC trial patients converted to open resection had a higher operative mortality compared to patients in the laparoscopic or open groups (9% vs 1% vs 5% respectively)^[7]. Conversion was also associated with worse oncologic outcomes in non-randomized comparative and descriptive studies^[46].

Short-term oncologic outcomes

While the number of lymph nodes retrieved can vary based on age, gender, tumor site, use of pre-operative radiation, and tumor grade, the extent and quality of surgical resection can also have an impact on the number of nodes collected and is therefore often considered a surrogate marker of the oncologic completeness of the resection^[47-53]. The American Joint Committee on Cancer recommends that at least 12 lymph nodes be examined in patients with rectal cancer to confirm the absence of nodal involvement by the tumor^[54]. In addition, a number of studies have reported that the number of lymph nodes examined may be associated with patient outcome^[55,56]. Six of the 8 RCTs reported the mean number of lymph nodes retrieved with a range of 5.5 to 17 nodes in the laparoscopic group compared to 11.6 to 18 nodes in the open group (Table 3)^[22,31,34,38-40]. In 4 of the 6 trials the number of lymph nodes isolated was not significantly different based on surgical approach. Araujo *et al*^[34] reported a significantly lower yield of lymph nodes with laparoscopic rectal resection compared to open resection (5.5 vs 11.9 respectively, *P* = 0.04). However, the number of lymph nodes obtained in the study by Lujan *et al*^[31] was higher in the laparoscopic group (13.63 vs 11.57 in the laparoscopic vs open approach respectively, *P* = 0.026). They suggested that laparoscopy offered better dissection and accuracy due to better visualization and exposure of structures with less manipulation of the mesorectum especially in a narrow pelvis. Four of the 8 RCTs reported the use of pre-operative chemo-radiation. In these trials, the mean number of lymph nodes retrieved ranged from 5.5 to 17 nodes in the laparoscopic group and from 11.6 to 18 nodes in the open group^[31,34,38,40]. Anderson *et al*^[57] found that in the 17 trials that reported the number of lymph nodes retrieved, the mean number of nodes was 10 for the laparoscopic group and 12 for the open group (*P* = 0.001) with the majority of trials reporting a median of 11 or fewer nodes obtained. In 9 of these 17 trials, both groups were treated with preoperative radiation therapy and reported a mean of 10 lymph nodes harvested in the laparoscopic group and 11 in the open group.

One of the greatest concerns of laparoscopic TME is that obtaining a complete oncologic resection will be more difficult. Involvement of the circumferential or distal margin is one of the most important prognostic factors in rectal resection with TME and can lead to an increase in local recurrence and a reduction in survival. Radial margins of less than 2 mm are associated with

Table 4 Short-term outcomes of patients in randomized control trial

Ref.	Length of stay (d)		Anastomotic leak (%)		Wound infection (%)		Ileus (%)		Pain/PCA use (mg) or (number of shots)		Mortality	
	Open	Lap	Open	Lap	Open	Lap	Open	Lap	Open	Lap	Open	Lap
Kang <i>et al</i> ^[40]	9 (8-12)	8 (7-12)	0	2 (1.2)	11 (6.5)	2 (1.2) ^a	22 (12.9)	17 (10)	156.9 (117.0-185.2)	107.2 (80.0-150.0)	0	0
Ng <i>et al</i> ^[45]	10.0 (3-39)	8.4 (2-32) ^a	4 (5.2)	1 (1.3)	9 (11.7)	5 (6.6)	2 (2.6)	1 (1.3)	8.3 (0-49)	4.9 (0-23) ^a	3 (3.9)	2 (2.6)
Lujan <i>et al</i> ^[31]	9.9 (6.8)	8.2 (7.3)	10 (12)	5 (6)	2 (1.9)	0 (0)	8 (7.8)	6 (5.9)	NA	NA	3 (2.9)	2 (1.9)
Ng <i>et al</i> ^[39]	11.5 (5-38)	10.8 (5-27)	NA	NA	4 (8.3)	0 (0)	2 (4.2)	1 (2.0)	11.4 (0-49)	6.0 (0-47) ^a	1 (2.8)	1 (2.5)
Guillou <i>et al</i> ^[7]	13 (9-18)	11 (9-15)	9 (7)	26 (10)	15 (12)	33 (13)	NA	NA	NA	NA	NA	NA
Jayne <i>et al</i> ^[12]												
Braga <i>et al</i> ^[38]	13.6 (6-80)	10 (6-27) ^a	9 (10.6)	8 (9.6)	13 (15.3)	6 (7.2)	2 (2.3)	2 (2.4)	NA	NA	1 (1.2)	1 (1.2)
Zhou <i>et al</i> ^[35]	13.3 (3.4)	8.1 (3.1) ^a	3 (3.4)	1 (1.2)	NA	NA	NA	NA	NA	NA	0 (0)	0 (0)
Araujo <i>et al</i> ^[34]	< 10.5	10.5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

PCA: Patient controlled analgesia; NA: Not available. ^a*P* < 0.05 vs Open.

a local recurrence rate of 16% compared to a significantly reduced local recurrence rate of 6% with margins greater than 2 mm^[27]. Six of the 8 RCTs reported the involvement of the CRM and no difference was found by surgical approach (Table 3)^[7,31,38-40,45]. In the majority of trials the rate of CRM involvement was less than 5%. Patients with positive radial margins often had tumor invading the pelvic side wall or adjacent structure and were frequently converted from a laparoscopic to an open procedure^[39]. In the CLASICC study, the only multi-center trial, a positive CRM was identified in 14 of 97 (14%) patients with open surgery and in 30 of 193 (16%) patients with laparoscopic rectal resection (*P* = 0.8)^[7]. Of patients undergoing anterior resection, the CRM was positive in 16 of 129 (12%) individuals in the laparoscopic group and in 4 of 64 (6%) individuals in the open group (*P* = 0.19). While there is a non-significant higher positivity of the CRM in the laparoscopic anterior resection group, this is once again likely due to the fact that the learning curve was not completed before the start of this study. Two RCTs reported on distal margin status and the incidence of distal margin positivity was not significantly different between the two surgical approaches and in fact was 0%^[31,38]. All 3 meta-analyses and the Cochrane review by Breukink *et al*^[44] found no difference in positive margins based on surgical access.

Postoperative course: Less postoperative pain, faster recovery of intestinal function, and shorter length of stay are important benefits of laparoscopic colorectal surgery. Only 3 of 8 RCTs compared the exact amount of post-operative pain medication and 2 of these studies reported a significant reduction in analgesic use in the laparoscopic group (Table 4)^[39,40,45]. Zhou *et al*^[35] did not quantify the exact usage of pain medication, but found no significant difference in the number of days parental analgesics were necessary (4.1 vs 3.9 in the open and laparoscopic groups respectively, *P* = 0.225).

Resumption of bowel function was usually reported on post-operative days 3 to 5 and ability to tolerate a solid food diet was reported on post-operative days 3 to 6^[7,31,35,39,40,45]. In the majority of RCTs earlier bowel

movements and diet advancement was reported with the laparoscopic approach. The return of bowel function and reduction in wound pain was thought to contribute to earlier discharge after laparoscopic surgery. While in a majority of trials, the length of stay was not significantly different between surgical approaches, there was a trend toward decreased length of stay with laparoscopic rectal surgery. Breukink *et al*^[58] found that laparoscopic TME resulted in earlier return of normal diet, less pain, less narcotic use and a shorter hospital stay.

Complications: Rectal cancer surgery is associated with a high rate of morbidity and mortality. Post-operative mortality in RCTs ranged from 1%-4% and demonstrated no statistically significant difference based on surgical approach (Table 4). The rate of post-operative complications ranged from 6% to 69% and with the exception of Zhou *et al*^[35] did not differ significantly between laparoscopic and open groups. Wound infection and urinary tract infection accounted for the majority of perioperative complications in both groups. There was a higher incidence of wound infection with the open approach however this did not reach statistical significance. Breukink *et al*^[58] found no difference in morbidity between the laparoscopic and open groups although there was a trend toward lower morbidity with laparoscopic TME. Aziz *et al*^[41] found no difference in perioperative morbidity between the 2 groups while Gao *et al*^[42] found that the overall morbidity rate of the laparoscopic group was significantly lower than that of the open group.

Anastomotic leak is the most serious complication after sphincter sparing rectal cancer resection especially with neoadjuvant chemoradiation. In addition, development of an anastomotic leak is reported to be associated with decreased long-term survival and higher rates of local recurrence after curative resection for colorectal cancer^[59-63]. Operative expertise and selective diversion in high risk patients has resulted in an anastomotic leak rate of 1%-17% in most published series studying laparoscopic resection for rectal cancer^[46,64,65]. Consistent with reports from non-randomized comparative trials, RCTs demonstrated no significant difference in the incidence

Table 5 Long-term oncologic outcomes of patients in randomized control trials

Ref.	Mean F/U (mo)		LR (%)		DFS (%)		OS (%)	
	Open	Lap	Open	Lap	Open	Lap	Open	Lap
Kang <i>et al</i> ^[40]	NA	NA	NA	NA	NA	NA	NA	NA
Ng <i>et al</i> ^[45]	108.8 (69.8-168.7)	112.5 (71.1-168.3)	7.1	4.9	80.4 (5.1)	82.9 (4.9)	55.1 (6.5)	63.9 (6.6)
Lujan <i>et al</i> ^[31]	34.1 (20)	32.8 (18.9)	5.3	4.8	81	84.8	75.3	42.1
Ng <i>et al</i> ^[39]	90.1 (27.0-145.5)	87.2 (22.8-150.0)	11.1	5	73.6 (8.1)	78.1 (6.9)	76.5 (7.3)	75.2 (7.2)
Guillou <i>et al</i> ^[7] /Jayne <i>et al</i> ^[12]	36.8 (20.0-61.5)	36.8 (20.0-61.5)	10.1	9.7	70.4/46.9	70.9/49.8	66.7/57.7	74.6/65.2
Braga <i>et al</i> ^[38]	53.6	53.6	5.2	4	NA	NA	NA	NA
Zhou <i>et al</i> ^[35]	1.0-16.0	1.0-16.0	NA	NA	NA	NA	NA	NA
Araujo <i>et al</i> ^[34]	47.2	47.2	13.3	0	NA	NA	NA	NA

F/U: Follow-up; LR: Local recurrence; DFS: Disease-free survival; OS: Overall survival; NA: Not available.

of anastomotic leak between the laparoscopic and open technique for the resection of rectal cancer (Table 4).

While the incidence of perioperative morbidity was not different based on surgical access, fewer patients had long-term complications with laparoscopic rectal cancer resection compared to the open approach. Adhesion related bowel obstruction was the most common long-term morbidity. With a median follow-up of greater than 9 years, Ng *et al*^[45] found that adhesion-related obstruction requiring hospitalization (18.9% *vs* 2.7%) and reoperation (6.8% *vs* 0%) was higher in the open group. They report a cumulative probability of adhesion-related bowel obstruction at 10 years of 20.5% in the open group and 3.9% in the laparoscopic group ($P = 0.001$)^[45]. Kuhry *et al*^[66] performed a systematic review including 12 trials (3346 patients) to evaluate the long-term outcomes of laparoscopically assisted *vs* open surgery for resectable colorectal cancer. Data on long-term complications was not separated by site of disease but the overall occurrence of incisional hernia (7.9% *vs* 10.9%, $P = 0.32$) and reoperation for adhesions (1.1% *vs* 2.5%, $P = 0.30$) was not statistically difference between laparoscopic and open resection. Long-term studies need to be done to determine if laparoscopy decreases the incidence of intra-abdominal adhesion formation by reduced surgical trauma, less tissue handling, and smaller incisions.

Long-term oncologic outcomes

A number of the clinical trials were performed to determine the safety and feasibility of the laparoscopic approach for rectal adenocarcinoma and therefore the data we have for long-term outcomes is limited (Table 5). Braga *et al*^[38] found no difference in local recurrence (4.0% in the laparoscopic group *vs* 5.2% in the open group, $P = 0.97$), overall five-year survival, or disease-free five-year survival based on surgical approach. With a median follow-up of 87.2 mo in the laparoscopic group and 90.1 mo in the open group, Ng *et al*^[39] demonstrated that after curative resection, the probability of five-year survival was 75.2% *vs* 76.5% for laparoscopic *vs* open APR respectively ($P = 0.20$). In addition, stage-by-stage comparison for the two groups showed no statistical difference. There were no port site recurrences and overall recurrence rates were not significantly different between

the two groups (laparoscopic 20% *vs* open 25%, $P = 0.60$). Despite the higher rate of circumferential margin positivity in patients undergoing laparoscopic anterior resection in the CLASICC trial, there was no difference in local recurrence, three- year overall or three-year disease free survival between the two approaches (open OS 66.7% and laparoscopic OS 74.6%, $P = 0.17$; open DFS 70.4% and laparoscopic DFS 70.9%, $P = 0.72$; open LR 7.0% and laparoscopic LR 7.98%, $P = 0.70$)^[12]. In addition, there was no significant difference in the rates of local recurrence, three-year overall survival, or three-year disease-free survival in patients undergoing laparoscopic *vs* open APR^[12]. However, the sample size is small and therefore larger studies are needed for conclusive results. Ng *et al*^[45] published results of a randomized trial of laparoscopic *vs* open anterior resection for upper rectal cancer with a median follow-up of 9 years. No difference in local recurrence, overall survival, or disease-free survival was reported. Although these studies suggest comparative oncologic outcomes between laparoscopic and open rectal cancer resection, they include small sample sizes and are almost all are single institution studies, highlighting the need for large, multi-center RCTs to provide confirmatory data.

In a meta-analysis by Anderson *et al*^[57] 18 of 24 studies reported recurrence rates. With a mean follow-up of 35 mo for both groups, overall local recurrence was not statistically different between the 2 groups (laparoscopic 7% *vs* open 8%, $P = NS$). Eleven studies provided sufficient data to compare overall survival. Overall survival was 72% for patients undergoing laparoscopic rectal cancer resection and 65% for open resection at an average of 4.4 years ($P = 0.5$). Subset analysis by Kuhry *et al*^[66] demonstrated no significant difference between laparoscopic and open rectal cancer resection in terms of local recurrence (laparoscopic 7.2% *vs* open 7.8%, $P = 0.46$), development of distant metastases (laparoscopic 13.5% *vs* open 9.1%, $P = 0.60$), or cancer-related mortality (laparoscopic 9% *vs* open 10%, $P = 0.16$). While, this data is encouraging, it is no conclusive.

CONCLUSION

The primary goal of this study was to outline and review

the short-term and long-term oncologic outcomes and complications of laparoscopic rectal cancer resection compared to the gold standard of conventional open resection currently available in the literature. Due to the heterogeneity in tumor stage, surgeon experience, and surgical technique, descriptive and non-randomized trials were not included in this review. However, because of the relatively few RCTs, information on the long-term outcomes is sparse and our conclusions are thus based on a small number of patients. A second limitation is that in a number of these trials data accrual started before the effectiveness of neoadjuvant therapy had been proven and thus the majority of patients did not receive pre-operative chemoradiation which is the current standard of care. Given these limitations, we found no difference in adequacy of oncologic resection, perioperative morbidity, recurrence rates, overall survival, or disease-free survival between open and laparoscopic rectal cancer resection.

In conclusion, RCTs have demonstrated that laparoscopy does not adversely affect cancer related survival in patients with adenocarcinoma of the colon. Concerns about the technical difficulty of TME may have contributed to the exclusion of rectal cancer patients from most of these large multicenter RCTs resulting in little data on oncologic outcomes with laparoscopic rectal cancer resection.

Laparoscopic rectal dissection is technically more demanding than open and constraints of a narrow pelvis may result in difficulty assessing and obtaining adequate surgical margins. However, there are several proposed benefits of laparoscopic rectal resection. A clear and magnified view of the pelvis provided by the improved optics of laparoscopy may aid sharp dissection for TME and assist in identification of vital pelvic structures including the ureters and autonomic nerves. In addition, pneumoperitoneum may separate the parietal and visceral fascia of the mesorectum facilitating dissection in this plane. Laparoscopic rectal cancer resection has a steep learning curve but increased experience with both open and laparoscopic TME will lead to shorter operating times and decreased morbidity^[67].

Current data suggests that laparoscopic rectal cancer resection may benefit patients because of reduced blood loss, earlier return of bowel function, and shorter hospital length of stay^[68,69]. Concerns that laparoscopic rectal cancer surgery may compromise short-term oncologic outcomes including number of lymph nodes harvested and CRM positivity do not appear to be supported by the available literature. However, there is a paucity of data concerning long-term oncologic outcomes and complications with laparoscopic rectal cancer surgery. There are two large, multicenter RCTs that are currently being conducted: the COLOR II trial in Europe and the ACOSOG-Z6051 trial in the United States^[70]. Both of these studies are comparing the laparoscopic and open approach for treatment of resectable rectal cancer. Results from these trials will provide information on the

long-term outcomes of laparoscopic rectal cancer resection and are eagerly awaited. In view of the lack of level one data on oncologic outcomes, laparoscopic TME for locally advanced, curable rectal cancer should only be performed within the confines of a RCT.

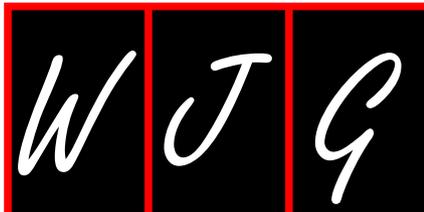
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Minimally invasive approaches for the treatment of inflammatory bowel disease

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Abstract

Despite significant improvements in medical management of inflammatory bowel disease, many of these patients still require surgery at some point in the course of their disease. Their young age and poor general conditions, worsened by the aggressive medical treatments, make minimally invasive approaches particularly enticing to this patient population. However, the typical inflammatory changes that characterize these diseases have hindered wide diffusion of laparoscopy in this setting, currently mostly pursued in high-volume referral centers, despite accumulating evidences in the literature supporting the benefits of minimally invasive surgery. The largest body of evidence currently available for terminal ileal Crohn's disease shows improved short term outcomes after laparoscopic surgery, with prolonged operative times. For Crohn's colitis, high quality evidence supporting laparoscopic surgery is lacking.

Encouraging preliminary results have been obtained with the adoption of laparoscopic restorative total proctocolectomy for the treatment of ulcerative colitis. A consensus about patients' selection and the need for staging has not been reached yet. Despite the lack of conclusive evidence, a wave of enthusiasm is pushing towards less invasive strategies, to further minimize surgical trauma, with single incision laparoscopic surgery being the most realistic future development.

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Key words: Laparoscopic surgery; Inflammatory bowel disease; Ulcerative colitis; Crohn's disease

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INTRODUCTION

The past 20 years have seen dramatic improvements in the treatment of inflammatory bowel disease (IBD)^[1]. Medical therapy, especially with the advent of biologics, has significantly increased efficacy of disease control, even if the actual reduction of the need for surgery is still debated, and concerns have been raised about po-

tential negative impact on postoperative outcomes^[2,3]. In this setting, the introduction and implementation of minimally invasive surgical techniques has substantially improved outcomes and quality of life in this particularly frail patient population^[4,5]. After the first description of laparoscopic colectomy about 20 years ago, laparoscopic surgery slowly has gained wide acceptance for the treatment of colorectal diseases, showing several advantages in short-term outcomes over open surgery in randomized trials and meta-analysis, with comparable safety and long-term results^[6-9]. However, the diffusion of laparoscopy for IBD is proceeding particularly cautiously, given the magnitude of the procedures required for the most complex cases and the difficulty in handling severely inflamed tissues, as proven by the high conversion rates observed even in the hands of surgeons with documented experience in IBD and laparoscopic surgery^[10,11]. Crohn's disease (CD) and ulcerative colitis (UC) represent real surgical challenges, due to thickened mesentery, strictures, abscesses, inflammatory masses, and enteric fistulae in CD, and intense inflammation leading to colonic distension and high risk of bleeding and accidental perforation in UC^[12,13]. The quest for further reduction of surgical trauma is ongoing, and if the natural orifice transluminal endoscopic surgery has unsolved issues related to the violation of uninvolved hollow viscera, costs and specific training, single incision laparoscopic surgery (SILS) seems to be a reasonable approach capable of minimizing the overall trauma and extent of incisions, with benefits in short term outcomes and cosmesis^[14-17].

The aim of this article is to provide a comprehensive review of the state of the art in minimally invasive approaches to IBD, highlighting the current standard of care, with a glance at the most promising future directions.

CD

Approximately 70% of patients with a diagnosis of CD will eventually require a surgical treatment, due to failure of medical therapy, septic complications, recurrent intestinal obstruction, and malnutrition^[18]. The treatment of CD has traditionally represented a challenge even in open surgery, with just two prospective randomized trials comparing laparoscopic *vs* standard approach published to date^[19,20], and with the long-term results of these studies only recently available^[21,22]. The panintestinal involvement and inflammatory complications, along with the additional risk for postoperative complication, increased by the aggressive medical management, make CD patients particularly poor laparoscopic candidates^[23]. Concerns have been raised about missing occult segments of disease and critical strictures due to the lack of tactile sensation, technical difficulty due to inflamed bowel mesentery and the presence of adhesions, fistulas, and abscesses^[24]. In order to overcome this issues, some authors have advocated the use of laparoscopic-assisted or hand-assisted laparoscopic surgery procedures, with the rationale that an incision is needed for specimen extraction, and the

handling of inflamed Crohn's tissue is easier and safer when an assisted method is used, while maintaining the advantages of a minimally invasive approach^[25]. The intrinsic difficulty of this surgery is further confirmed by a study by Hamel *et al*^[26], that showed no differences in morbidity or conversion between the earlier and the latter time periods of the experience, thus negating the effects of the learning curve. Alves *et al*^[27] looked at the risk factors of conversion in a prospective study on 69 patients undergoing primary laparoscopic ileocecal resection, observing a conversion rate of 30%, with recurrent CD, intra-abdominal abscess and fistula independent risk factors on multivariate analysis. Even if minimally invasive surgery for CD is technically complex, requiring specific training and longer operating time^[28], data in the literature confirm the safety and efficacy of this approach in terms of postoperative pain, cosmesis, return to normal activity, and, more importantly, surgical recurrence rates^[29]. Despite this evidence, in a recent study by Lesperance *et al*^[30] on 49 609 patients admitted for CD that required surgical treatment from the 2000-2004 Nationwide Inpatient Sample, only 2826 cases (6%) underwent a laparoscopic resection, demonstrating that the vast majority of CD patients are still undergoing open conventional surgery, with a minimal invasive approach mostly reserved for patients who are younger (< 35 years old), female, admitted to a teaching hospital, with ileocecal, uncomplicated disease. The increased adoption of the laparoscopic approach for the treatment of CD in teaching hospitals confirms the peculiar technical complexity of minimally invasive procedures in this setting, requiring more skilled colorectal surgeons, as can be found in referral centers where specific laparoscopic training programs are implemented.

Terminal ileal CD

The small samples size and selection bias explain the conflicting results in the initial published series of ileocolonic CD treated by laparoscopic surgery^[28,31-34]. In our series of selected consecutive patients with elective, complex and even recurrent terminal ileal CD, laparoscopic patients had faster postoperative recovery - partially related to less postoperative pain and consequent decreased need for intravenous narcotics - and similar operating times compared to the open cohort, without increased complication and recurrence rates, with potential overall cost savings^[35]. In regards to the issue of costs associated with laparoscopy, Young-Fadok *et al*^[36], in a case match study comparing 33 cases of laparoscopic ileocolic resections with 33 open, showed significantly lower direct and indirect costs in the laparoscopic group. The strongest evidence available comes from the only two prospective randomized trials present in the literature, both conducted on small samples of highly selected patients. Although such populations might be far from the reality of a tertiary referral center, it is the only way to randomize CD patients given the panintestinal, relentless nature and often unpredictable presentation of the disease. In the trial by Maartense *et al*^[19], patients with

a fixed palpable inflammatory mass, prior median laparotomy, earlier bowel resection, or pregnancy were excluded. In this study, the laparoscopic approach showed longer median operating time, shorter hospital stay, lower 30-d post-operative morbidity, but no differences in quality of life, the primary endpoint of this study. After a median follow-up of 6.7 year, there were no differences in recurrence rate and need for reoperation between open and laparoscopic group, with a 58% relapse free rate and no patients in the laparoscopic group requiring a reoperation for incisional hernia or adhesive small bowel obstruction^[21]. Even if a minimal invasive approach did not impact the overall quality of life, body image and cosmesis scores were significantly higher after laparoscopy^[21]. These data differ from the previous observation by Thaler and colleagues, that found long-term quality of life significantly reduced in patients with CD compared to general healthy population, irrespective of the surgical approach, with recurrence identified as the only significant predictor of poor quality of life^[37]. In the other randomized trial, Milsom *et al*^[20] included only patients with isolated Crohn's disease of the terminal ileum with or without cecal involvement. The results of this study demonstrated that laparoscopy offers faster recovery of pulmonary function, fewer minor complications, and a trend towards shorter length of stay compared with conventional surgery, even if no differences in the amount of morphine equivalents, return of bowel function and length of stay were found. After a mean follow-up of 10.5 years there were no significant differences between groups with regard to use of medications to treat CD and recurrence rates, both clinical and surgical. Furthermore, two laparoscopic patients underwent lysis of adhesions while none did in the open group, with an incidence of incisional hernia repair of 4% in the laparoscopic group *vs* 14% in the open (both differences were not statistically significant)^[22]. Recently, Dasari *et al*^[38] conducted a meta-analysis of the aforementioned trials, and found that laparoscopic patients had a trend towards less wound infection and shorter hospital stay, with comparable incidence of other postoperative complications, duration of postoperative ileus, incidence of anastomotic leak and intraabdominal abscess, 30-d reoperation rate, and actuarial disease recurrence rates. To date, three meta-analysis comparing laparoscopic and open surgery for ileocolonic CD have been conducted, all demonstrating that laparoscopic surgery is associated with prolonged operative time, shorter duration of postoperative ileus, shorter hospital stay and lower incidence of early postoperative complications^[39-41]. Other significant findings from these studies also include similar intraoperative blood loss and complications^[41], with a trend toward lower overall costs with laparoscopic surgery^[39], and no differences in the rate of disease recurrence^[40]. With regard to the long-term outcomes, the study from Washington University, comparing 63 CD patients treated laparoscopically with 50 open ileocolic resections, found that the two groups had a recurrence rate of 9.5% and

24%, respectively (difference not statistically significant), with the laparoscopic group having shorter mean follow-up, thus confirming the non-inferiority of the laparoscopic approach. Interestingly, 50% of the recurrences in the laparoscopic group and 33% in the open group were able to be retreated laparoscopically^[29].

Laparoscopy in complicated/recurrent CD

In complicated CD laparoscopy is even more challenging. Seymour and Kavac analyzed their series of 17 patients managed with laparoscopic approach for complicated CD (defined as for the presence of fistulas, multiple or long-segment disease, abscesses and previous operations). In this study, conversion to open procedures was not always required, but operative time and postoperative hospital stay were longer compared to laparoscopic ileocecal resections for uncomplicated disease, with major complications occurring in 18% of patients^[42]. In the literature, surgical recurrence rates are reported as high as 70% to 90%, and multiple procedures are required in more than 30%^[12]. In a recent study from France, of 62 reoperations for CD recurrence in 57 patients, 29 were performed laparoscopically. While no differences between the two groups were observed in terms of use of a temporary stoma, mean operating time, postoperative mortality (nil in both groups), overall morbidity rate, severe complications, median hospital stay, and conversion rates, a higher number of intraoperative intestinal injuries was reported in the laparoscopic group (5 *vs* 0) ($P = 0.01$). The occurrence of fistulizing disease was a risk factor for conversion, and conversion did not seem to affect complication rate^[43]. A study from Japan looked at 16 laparoscopic procedures for CD recurrence at the anastomotic site out of 61 attempted laparoscopically by experienced surgeons in 52 patients. The result of this study showed that while the operating time was significantly longer in the recurrent group, there were no differences in the rates of postoperative complications and hospital stay, with the repeated laparoscopic operations performed using the same small incision as that of the primary operation. The advantage of a minimally invasive primary approach are supported by the fact that the operating time was shorter and blood loss was less in patients who underwent the primary procedure laparoscopically^[44]. Finally, in the experience by Goyer *et al*^[45] on 54 complex CD (defined as recurrent or complicated by abscess and/or fistula) compared with 70 patients with uncomplicated CD, the complex group had increased operative time, conversion rates and use of temporary stoma. Conversely, no differences were noted in overall postoperative morbidity, including major surgical postoperative complications and hospital stay, leading to the conclusion that complex CD should not be considered an absolute contraindication to a laparoscopic approach in experienced hands.

Crohn's colitis

In contrast with the data available on minimally invasive

surgery for terminal ileal CD, very few series have been published on CD of the colon. The feasibility and safety of a laparoscopic approach to subtotal colectomy for CD was addressed by Hamel *et al*^[46], who observed a higher rate of intraoperative complications compared to ileocolic resection, while hospital stay and postoperative complication rate did not differ between the two groups. Contrasting results come from a recent case match study by the Cleveland Clinic group on 27 laparoscopic and 27 open cases, with a conversion rate of 26%. In this series, laparoscopic colectomies took longer with similar blood loss and postoperative complications, along with a trend towards shorter time to first bowel movement and length of stay, which became statistically significant in favor of laparoscopy when overall length of stay included 30-d readmissions^[47]. In our own personal experience on 125 patients who underwent colectomy for CD, 44% by a laparoscopic approach, the conversion rate was 10.9%, median operative time, blood loss, return of bowel function and length of post-op stay were reduced in the laparoscopic group, while postoperative complications and disease recurrence rates were similar, suggesting that a laparoscopic approach for CD of the colon is safe and feasible in the hands of experienced surgeons^[48].

Laparoscopy has a role also in creating diverting stomas for severe perianal CD, reducing the number of incisions to few trocars and the ostomy site. In a study by Liu *et al*^[49] on 80 patients who underwent laparoscopic stoma creation over a 10-year period (ileostomy 30, colostomy 49, conversion 1), the overall morbidity rate was 11% with five major complications requiring reoperation, and no further stoma complications recorded within a 1-year follow-up.

UC

Despite significant advances in the medical treatment of UC, surgery remains definitive cure for these patients after failure of medical management or diagnosis of neoplastic degeneration^[50,51]. A restorative procedure with the creation of an ileal pouch anal anastomosis (IPAA) is universally considered the standard of care. The earliest reports of a laparoscopic approach to ulcerative colitis was published in 1992 by Peters *et al*^[52], who described the technique of laparoscopic proctocolectomy for two UC patients. The same year, Wexner and colleagues reported the first case-controlled series on the outcome of laparoscopic-assisted proctocolectomy with IPAA, showing a longer operative time compared to open procedure, and comparable postoperative ileus and hospital stay, with no short-term benefits in favor of laparoscopy^[53]. Since then, numerous series have been reported both in the adult and pediatric patient populations^[54-56], but only from single institutions with short follow-ups^[5,57]. Universally, these initial studies showed that laparoscopy took longer, with the exception of the series published by Araki *et al*^[58]. In these studies only the colonic mobilization was performed laparoscopically,

with vessel transection and rectal mobilization carried out through a mini laparotomy^[53,58-61], with the exception of the series reported by Marcello *et al*^[55], where a totally laparoscopic techniques was adopted, reserving a mini laparotomy only for specimen extraction. Subsequently, in a study from the Netherlands, 60 patients were randomized for hand assisted or laparoscopic restorative proctocolectomy with IPAA. The results from this study failed to show statistically significant differences in terms of morbidity, postoperative stay, quality of life at 3 mo after surgery, and overall costs, but the operative time for laparoscopy was significantly longer^[62]. In a subsequent study, Polle *et al*^[63] observed that female patients reported higher body image and cosmesis scores compared to open group, while there were no differences in functional outcome, morbidity, and overall quality of life. Similarly, Dunker *et al*^[59] compared 16 patients who underwent restorative surgery with laparoscopic technique with 19 open patients. The authors found that laparoscopic patients showed significantly higher satisfaction with the cosmetic results and better body image, but once again functional outcome and quality of life were similar between groups. It seems evident, as it may have been expected, that laparoscopic IPAA offers significant advantages over the open conventional procedure in terms of body image and cosmesis, important factors in the acceptance of surgery in this young patient population, while conflicting results have been reported in terms of postoperative recovery. Faster return of bowel function after laparoscopy and decreased use of narcotics have been reported by some authors, not always translating into shorter hospital stay^[57,62]. On the other hand, concerns have been raised regarding the duration of surgery often noted to be longer than open surgery even by very experienced laparoscopic surgeons, often resulting in higher costs. In regards to long-term pouch function, quality of life and complications, very few studies are available with adequate follow-up^[5,57,62,63,59]. These observations were confirmed in a Cochrane review on 607 patients from 12 studies, only one randomized, which did not found any significant differences in complications, readmission, reoperation rates and mortality. However, once again, it showed that laparoscopic IPAA is associated with a significantly longer operating time, along with the inability to confirm conclusively the presumed short-term benefits of laparoscopy, with length of follow-up too short for evaluating long-term outcomes^[64]. Similar results were obtained in a subsequent meta-analysis on 16 studies, only one randomized, by Wu *et al*^[13]. Postoperative fasting time and hospital stay were shorter for laparoscopy, and overall complication rates were higher after open surgery. Once again, laparoscopy took significantly longer and no advantages were demonstrated in terms of recovery of bowel function, postoperative septic complications, anastomotic leakage, postoperative bowel obstruction, blood loss, and mortality^[13]. In our personal experience with 73 laparoscopic IPAA with a mean follow-up of 24 mo, the minimally invasive ap-

proach offered a statistically significant earlier return of flatus and resumption of diet, less intraoperative blood loss, and lower incidence of incisional hernias compared to 106 open IPAA, with no differences in overall complication rate, pouch function and quality of life^[65].

The controversy about the safety of a single-stage procedure has not been resolved yet. Since long-term functional outcomes after IPAA are threatened by the occurrence of pouch-related septic complications, every effort should be made to reduce such complications and to identify patients at risk for pouch-related sepsis^[66]. In a study by Marcello *et al*^[67] on 59 patients who underwent laparoscopic proctocolectomy for UC, where only 9 patients received a diverting stoma at the primary procedure, 9 patients, all on high dose immunosuppressors or elevated body mass index, required a secondary ileostomy for postoperative complications. Better results were reported by Ky *et al*^[68], with only one out 32 patients with an anastomotic leak requiring secondary diversion after one-stage laparoscopic restorative proctocolectomy. It is hard to analyze these data since these results can be influenced by patients' selection; pelvic sepsis is reported to occur in up to 23% of patients after IPAA for UC, especially after the introduction of biologic therapy for IBD, in most cases secondary to an anastomotic leak^[66,69-72]. In a recent study on 118 UC patients treated with a minimally invasive approach, we compared a 3-stage approach (laparoscopic abdominal colectomy followed by pouch surgery with a diverting loop ileostomy, 50 patients) with a 2-stage approach (laparoscopy colectomy with IPAA and diverting stoma at the initial operation, 68 patients). We observed a significant higher rate of septic complications in the 2-stage group (38.2% *vs* 21%, $P < 0.05$), despite 3-stage patients had been receiving a more aggressive medical therapy in the immediate preoperative period^[73].

The role of laparoscopy for the treatment of ulcerative colitis in the emergency setting has been investigated by two studies. In the study by Bella and Seymour, 18 patients underwent laparoscopic-assisted restorative proctocolectomy for fulminant colitis, reporting a postoperative complication rate of 33%, with a length of stay of 5.0 d, which was shorter compared to the 8.8 d reported for the 6 open cases analyzed in the study^[74]. The other study, by Marcello *et al*^[67], reviewed the data from 19 laparoscopic and 29 conventional total colectomies with end ileostomy and mucous fistula buried within subcutaneous tissue for acute, not fulminant, UC, demonstrating longer operative time (210 *vs* 120 min) but lower complication rates (16% *vs* 24%), earlier return of bowel function (1 *vs* 2 d) and shorter length of stay (4 *vs* 6 d) for the laparoscopic group.

SILS IN IBD

During the last few years an increasing number of reports and case series on SILS colorectal resections for both benign and malignant diseases have been reported. Few

studies have been published comparing SILS to standard laparoscopy, showing potential for improved short-term outcomes^[75-78]. Besides the obvious cosmetic advantage resulting from a reduced number and size of scars - particularly important in a young IBD patient population - limiting the incisions seems to result in less postoperative pain, less use of narcotic pain medications, with consequent faster recovery and earlier discharge, along with a lower incidence of wound-related complications^[17,76,78-80]. These data are still preliminary, with only few cases of SILS for UC published to date^[4,16,17,75-78,81-92]. We believe that particularly for total abdominal colectomy (TAC) the SILS approach is a very attractive option in this patient population, representing a true "scarless" procedure, with the only access to the abdominal cavity at the site of the future stoma. Our preliminary results with the adoption of a well-standardized SILS approach to TAC confirm the potential of this technique in improving the postoperative recovery in selected patients, without significant increases in operative time and costs^[93].

CONCLUSION

During the past three decades the evidence has been accumulating in favor of a minimally invasive approach to IBD. Crohn's disease is probably one of the most challenging diseases to treat laparoscopically for the colorectal surgeons, especially when the disease is located in the colon and involves multiple segments, thus explaining the fact that in the United States the majority of CD patients are still approached with open surgery. Laparoscopic IPAA for UC has been shown to be feasible, but to date the evidence present in the literature is still not conclusive. Current data suggest a shorter length of stay, shorter ileus, faster recovery and less postoperative pain, along with better cosmesis with minimally invasive surgery. On the other hand, significantly longer operative times with laparoscopy are universally reported. Our goal and responsibility is to explore new avenues for a true minimally invasive approach to IBD and to train the next generation of surgeons to facilitate wide spread acceptance of laparoscopy.

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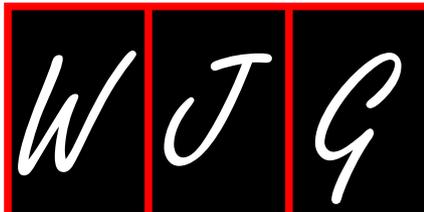
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Impact of minimally invasive surgery on the treatment of benign esophageal disorders

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Abstract

Thanks to the development of minimally invasive surgery, the last 20 years have witnessed a change in the treatment algorithm of benign esophageal disorders. Today a laparoscopic operation is the treatment of choice for esophageal achalasia and for most patients with gastroesophageal reflux disease. Because the pathogenesis of achalasia is unknown, treatment is palliative and aims to improve esophageal emptying by decreasing the functional obstruction at the level of the gastro-esophageal junction. The refinement of minimally invasive techniques accompanied by large, multiple randomized control trials with long-term outcome has allowed the laparoscopic Heller myotomy and partial fundoplication to become the treatment of choice for achalasia compared to endoscopic procedures, including endoscopic botulinum toxin injection and pneumatic dilatation. Patients with suspected gastroesophageal reflux need to undergo a thorough preoperative workup. After establishing diagnosis, treatment for gastroesophageal reflux should be individualized to patient characteristics and a decision

about an operation made jointly between surgeon and patient. The indications for surgery have changed in the last twenty years. In the past, surgery was often considered for patients who did not respond well to acid reducing medications. Today, the best candidate for surgery is the patient who has excellent control of symptoms with proton pump inhibitors. The minimally invasive approach to antireflux surgery has allowed surgeons to control reflux in a safe manner, with excellent long term outcomes. Like achalasia and gastroesophageal reflux, the treatment of patients with paraesophageal hernias has also seen a major evolution. The laparoscopic approach has been shown to be safe, and durable, with good relief of symptoms over the long-term. The most significant controversy with laparoscopic paraesophageal hernia repair is the optimal crural repair. This manuscript reviews the evolution of these techniques.

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Key words: Gastroesophageal reflux disease; Esophageal achalasia; Hiatal hernia; Laparoscopic fundoplication; Laparoscopic Heller myotomy

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INTRODUCTION

The last two decades have seen a shift in the treatment algorithm of benign esophageal disorders, largely due to the introduction and development of minimally invasive surgery. In the early 1990s, it became clear that a minimally invasive approach for esophageal achalasia, gastroesophageal reflux disease, and paraesophageal hernias, provided outcomes comparable to those achieved by open techniques but with less postoperative pain, shorter hospital stay and earlier return to work^[1-3].

The late 1990s and last 10 years saw the evolution of the laparoscopic approach thanks to better instrumentation and expertise. As more of these procedures were performed, more long-term data became available allowing improved analysis of the technique and the outcome. For example, in the early 1990s a left thoracoscopic Heller myotomy was considered the surgical procedure of choice for achalasia, yet now the standard of care is a laparoscopic Heller myotomy with partial fundoplication as this operation allows better relief of dysphagia and a lower incidence of postoperative reflux^[4-8]. In a similar fashion, both total fundoplication and partial fundoplication were initially considered equivalent for the treatment of gastroesophageal reflux disease, however, long-term follow-up indicates that a laparoscopic total fundoplication is superior^[9]. In addition, a laparoscopic approach to paraesophageal hernias offers fewer complications and shorter hospital stay than to the open approach^[10].

In this review, we focus on the impact of minimally invasive surgery on the treatment of achalasia, gastroesophageal reflux disease (GERD), and paraesophageal hernias.

ACHALASIA

Esophageal achalasia is a primary esophageal motility disorder characterized by lack of esophageal peristalsis and inability of the lower esophageal sphincter (LES) to relax properly in response to swallowing. The goal of treatment is to relieve the functional obstruction caused by the LES, therefore allowing emptying of food from the esophagus into the stomach by gravity. The treatment of this disease is based on a delicate balance between elimination of the functional obstruction caused by the LES and the need to prevent gastroesophageal reflux into an aperistaltic esophagus, with risk of developing complications such as strictures, Barrett's esophagus, and even adenocarcinoma^[11-14].

Open era

In 1913, Heller first described the simultaneous performance of two myotomies, on the anterior and posterior sides of the esophagus, to treat patients with achalasia. A decade later, Zaaijer would restrict the procedure to a single anterior myotomy. For decades, Heller myotomy was the standard treatment for esophageal achalasia. In the 1970s and 1980s, pneumatic dilatation became the primary form of treatment as it was believed that the results were equivalent to those of a myotomy but this

approach avoided the postoperative pain and the long recovery time which followed surgery. Very few Heller myotomies were performed during this era, and they were mostly reserved for patients whose dysphagia did not improve with balloon dilatation or whose esophagus was perforated during a dilatation^[15].

During this era, the myotomy was performed by either a left thoracotomy or a laparotomy. Ellis described his results using a left transthoracic approach to perform a Heller myotomy^[6]. His myotomy extended for only 5 mm onto the gastric wall, the rationale being that a short myotomy relieved dysphagia but avoided reflux, therefore obviating the need for a fundoplication. His results showed relief of dysphagia in about 90% of patients and symptomatic reflux in 5%^[12]. However, when gastroesophageal reflux was objectively measured postoperatively by manometry and pH monitoring, abnormal esophageal acid exposure was found in 29% of patients^[16]. These findings underscored the importance of objective measurement of reflux in achalasia patients.

A trans-abdominal approach was used mostly in Europe and South America. Bonavina *et al*^[17] from Italy reported the results of trans-abdominal Heller myotomy and Dor fundoplication in 206 patients operated between 1976 and 1989. The operation yielded "excellent" or "good" results in 94% of patients. Abnormal postoperative gastroesophageal reflux, as measured by pH monitoring, was present in 8.6% of patients only.

Minimally invasive era

A shift in the treatment algorithm of achalasia has slowly occurred in the last decade due to the excellent outcome of minimally invasive techniques. In 1992, we reported our initial experience with a thoracoscopic approach^[1], following the technique first described by Shimi *et al*^[18]. Using the guidance provided by intraoperative endoscopy, we performed a left thoracoscopic myotomy which extended for only 5 mm onto the gastric wall. Like Ellis^[12], the rationale for a shorter myotomy was to relieve dysphagia while trying to avoid postoperative reflux. The hospital stay was short, postoperative discomfort was minimal, and the recovery was fast^[1]. Long-term follow-up showed relief of dysphagia in almost 90% of patients^[19].

Despite these good results, some shortcomings of the thoracoscopic technique soon became apparent. This approach required a double lumen tube for one lung ventilation and a chest tube. In addition, the exposure of the gastroesophageal junction was limited by the diaphragm. Most striking though was that a thoracoscopic myotomy was associated with reflux in up to 60% of patients when studied by postoperative pH monitoring^[19]. A significant amount of postoperative reflux after thoracoscopic Heller myotomy was also demonstrated by others^[20,21].

These limitations of the thoracoscopic approach were the key reasons for the switch to a laparoscopic approach.

Is a fundoplication necessary?

A laparoscopic Heller myotomy alone is also associated with a high incidence of postoperative reflux, with the

risk of severe esophageal damage. In several retrospective studies, objective evidence of reflux was demonstrated in about 60% of patients postoperatively^[22,23].

Subsequent randomized control trials confirmed this observation and suggested that a fundoplication decreases the incidence of this problem. In 2003, Falkenback *et al*^[24] reported the results of a prospective and randomized trial comparing myotomy alone *vs* myotomy with Nissen fundoplication. Using postoperative pH monitoring, they found abnormal reflux in 25% of patients who had a myotomy and fundoplication as compared to 100% of patients who had a myotomy alone. Twenty percent of patients in the myotomy alone group subsequently developed Barrett's esophagus^[24].

Another randomized trial compared Heller myotomy alone *vs* Heller myotomy and Dor fundoplication. Pathologic gastroesophageal reflux was demonstrated by pH monitoring in 48% of patients after Heller myotomy alone but in 9.5% only when a Dor fundoplication was added^[25]. Relief of dysphagia was similar in the 2 groups. Both the total (Nissen) and partial (Dor) fundoplications prevent reflux in the majority of patients. The above trials provide Level 1 evidence that a fundoplication should be performed at the time of a laparoscopic Heller myotomy.

Partial vs total fundoplication

In patients with gastroesophageal reflux, a laparoscopic total fundoplication is the procedure of choice, even when esophageal peristalsis is weak^[9]. In contrast, because of the absence of peristalsis in achalasia, a total fundoplication may cause too much resistance at the level of the gastroesophageal junction, therefore impeding the emptying of food from the esophagus into the stomach. This can eventually cause persistent or recurrent dysphagia.

There have been long-term studies demonstrating that postoperative dysphagia is initially significantly decreased after a Heller myotomy with a total fundoplication^[24,26,27]. Yet, most surgeons have abandoned the use of a total fundoplication and switched to a partial fundoplication based on other long-term data showing that dysphagia eventually recurs in most patients that have a total fundoplication. For example, Duranceau *et al*^[28] initially reported excellent results with a Heller myotomy and total fundoplication, as dysphagia improved in most patients and there was no symptomatic reflux. Ten years later, however, they noted that symptoms had recurred in 82% of patients probably due to complete decompensation during the follow-up period^[29].

A prospective, randomized trial comparing long-term results of laparoscopic Heller myotomy and Dor fundoplication *vs* laparoscopic Heller myotomy and floppy-Nissen fundoplication for achalasia recently demonstrated that there was a statistically significant difference in dysphagia rates (2.8% *vs* 15%, $P < 0.0001$). Although both techniques achieved long-term reflux control, the recurrence rate of dysphagia was significantly higher among patients who underwent a Nissen fundoplication^[30].

These data provide Level 1 evidence supporting the use of a partial fundoplication with a Heller myotomy as the preferred choice of fundoplication for achalasia.

Partial fundoplication: Anterior vs posterior

Currently, deciding between which partial fundoplication is best, either a posterior (Toupet) or an anterior fundoplication (Dor) after Heller myotomy, is controversial. Some groups favor a posterior fundoplication as it might be more effective in preventing reflux and because it might keep the edges of the myotomy separate^[4,5,31-33]. One retrospective study demonstrated a lower rate of recurrent dysphagia in the Toupet fundoplication group compared to the Dor fundoplication group (3% *vs* 17%). However, conclusions from this study are limited since the comparison groups were not equivalent: the follow-up period for the Dor fundoplication group was longer and the length of the myotomy was longer in the Toupet fundoplication group^[33].

Our preference is for a Dor fundoplication because it is simpler to perform, in that there is no need for a posterior dissection. In addition, it adds the advantage of covering the esophageal mucosa. It certainly is the procedure of choice if there is any suspicion of injury to the mucosa or if a perforation has occurred. Many studies have demonstrated that a laparoscopic Heller myotomy with anterior fundoplication significantly relieves the symptoms of achalasia in about 90%, while limiting gastroesophageal reflux^[6-8,32,34-37].

Recently presented at the Society of American Gastrointestinal and Endoscopic Surgeons annual meeting in 2011 were the findings of a prospective, randomized, and multicenter study comparing laparoscopic Heller myotomy and Dor fundoplication and laparoscopic Heller myotomy and Toupet fundoplication. Follow-up monitoring by pH monitoring at six months showed no statistical significance between a Dor fundoplication and Toupet fundoplication^[38].

The last two decades have witnessed a shift in the treatment algorithm for esophageal achalasia. The refinement of minimally invasive techniques accompanied by large, multiple randomized control trials with long-term outcome has allowed the laparoscopic Heller myotomy and partial fundoplication to become the treatment of choice for achalasia.

GERD

The pathophysiology of GERD is multifactorial and complex. It is well established that the lower LES plays a key role in the antireflux mechanism. This is evidence that the striated muscles of the crus fail to exert its synergistic action with the LES when a hiatal hernia is present, thus, causing reflux^[39]. In addition, esophageal dysmotility can also play a crucial role: 40% to 50% of patients with GERD have low amplitude of peristalsis or an abnormal propagation of peristaltic waves^[40]. As a consequence, acid clearance is impaired, potentially caus-

ing more mucosal injury. Furthermore, it has been demonstrated that a mixed reflux of both gastric and duodenal juices plays a role in the pathogenesis of GERD, particularly in patients with Barrett's esophagus. This has a profound impact on treatment considering that proton pump inhibitors only control acid secretion and not duodenal reflux^[41]. The pH of the refluxate is changed, yet the underlying problem has not been addressed: the competence of the lower esophageal sphincter. The laparoscopic 360° fundoplication addresses this very issue, which can block either type of refluxate.

Open era

Nissen^[42] first described an antireflux procedure with a 360° wrap in 1956. Reflux symptoms improved, yet there were problems with dysphagia and inability to belch. Multiple modifications and different types of partial fundoplications were then introduced over the years. In 1986, DeMeester *et al*^[43] described several modifications to operative technique that would prove to be valuable. Increasing the bougie size seemed to reduce the incidence of postoperative dysphagia. Similar results were obtained by shortening the length of the fundoplication. Finally, a more extensive mobilization of the fundus increased the incidence of complete distal esophageal sphincter relaxation.

Laparoscopic 360° fundoplication

The laparoscopic 360° fundoplication (total) was initially described by Dallemagne *et al*^[44] in 1991. Despite some surgeons favored partial fundoplication in some settings, it was shown that laparoscopic partial fundoplication was not as effective in controlling reflux as a laparoscopic total fundoplication^[9].

A 360° fundoplication using minimally invasive techniques is associated with a low morbidity, a short hospital stay, and excellent outcomes^[45,46]. The operation controls reflux by improving esophageal motility, both in terms of LES competence and quality of esophageal peristalsis^[9,47]. Long-term studies have shown that fundoplication controls symptoms in 93% of patients after 5 years and 89% after 10 years^[48]. Postoperative dysphagia has been described in approximately 8% of patients. However, this usually resolves in most patients within a few months and rarely requires any intervention^[9].

Clarifying indications

The indications for surgery have changed in the last twenty years. Surgery was often considered for patients who did not respond well to acid reducing medications. However, the paradigm has changed. Today, the best candidate for surgery is the patient who has excellent control of symptoms with proton pump inhibitors^[49].

We feel that laparoscopic 360° fundoplication is indicated in the following circumstances: (1) when heartburn and regurgitation are not completely controlled by medication; (2) when it is thought that cough is induced by reflux. For instance, Mainie *et al*^[50] demonstrated that

patients resistant to proton pump inhibitors (PPIs) with non-acid or acid reflux demonstrated by multichannel intraluminal impedance-pH monitoring and with a positive symptom index, can be treated successfully by a laparoscopic 360° fundoplication); (3) poor patient compliance with medical treatment; (4) cost of medical therapy is prohibitive (most insurance companies in the United States will pay for one PPI pill per day only, with many patients prescribed more frequent dosing); (5) postmenopausal women with osteoporosis. It has been shown that PPIs and H2 blockers may increase the risk of hip and femur fractures because of decreased calcium absorption^[51]; and (6) young and very symptomatic patients in whom life-long medical treatment is not advisable.

Finally, in a recently published meta-analysis of medical *vs* surgical management for GERD, Wileman *et al*^[52] have shown that, in adults, laparoscopic fundoplication is more effective than medical management for the treatment of GERD in the short to medium term. Surgery, however, carries some risks and its application should be individualized as the decision to undergo fundoplication should be based on patient and surgeon preference.

The minimally invasive approach to antireflux surgery has allowed surgeons to control reflux in a safe manner without troublesome side effects. Long term outcomes are excellent and risk is minimal. Nonetheless, patients with suspected reflux need to undergo a thorough preoperative evaluation. After establishing diagnosis, treatment for gastroesophageal reflux should be individualized to patient characteristics and a decision about an operation made jointly between surgeon and patient.

PARAESOPHAGEAL HERNIA

Paraesophageal hernias are rare and only make up a small fraction of hiatal hernias. Yet because of their association with serious morbidity and potential mortality, knowing how to treat these patients is essential.

Open era

Like achalasia and gastroesophageal reflux, the treatment of patients with paraesophageal hernias has also seen a major evolution. Thanks to the advent of minimally invasive techniques, a laparoscopic repair has slowly replaced an open approach *via* a laparotomy or left thoracotomy.

In the 1980s, an open technique was standard of care and was shown to be effective. Ellis *et al*^[53] demonstrated an 88% benefit from an open paraesophageal hiatal hernia repair *via* laparotomy. Their technique commonly included an antireflux procedure and a Stamm gastrotomy. Average length of stay was 9.5 d and the complication rate was 24%.

Minimally invasive era

The last two decades have seen the emergence of laparoscopy for treatment of paraesophageal hernias. Though it is a technically challenging operation, the laparoscopic approach has been shown to be safe, durable, with good

relief of symptoms over the long-term, decreased post-operative pain, and a rapid return to normal activities. The majority of patients also undergo an antireflux procedure as well^[54,55]. The basic principles of the operation include excising the hernia sac, mobilizing the esophagus extensively in the posterior mediastinum, closing the hiatus, and a fundoplication.

Current status

Despite popularization of the laparoscopic approach, many controversies remain. The majority of surgeons advocate complete excision of the hernia sac for several reasons. A sac remnant could potentially act as a potential lead point for a recurrence or interfere with the crural repair or fundoplication. Others argue that removing the sac can potentially cause injury to the mediastinum and pleura and that it is not necessary. Aly *et al*^[56] showed that sac excision was not routinely performed and had no significant effect on quality of life measures and post-stop barium swallow radiographs.

The most significant controversy with laparoscopic paraesophageal hernia repair though is recurrence rate. In several large volume studies, hiatal hernia recurrence rate is reported as high as 28%-44% for the laparoscopic repair^[56-59]. These rates are significantly higher than for open repair. However, prior open series rarely measured outcomes objectively as they routinely do now.

When repairing paraesophageal hernias, there can be a significant amount of tension with a primary repair, which ultimately may lead to recurrence. Due to the significant use of synthetic mesh for tension free repairs inguinal hernias, these were soon applied to paraesophageal hernias repairs. One randomized control trial showed a significant reduction in recurrence rates at the one year follow-up period^[60]. However, there was a significantly higher rate of dysphagia and potential for erosion into the esophagus or stomach^[60,61]. Synthetic mesh is rarely used now.

In 2006, Oelschlager *et al*^[62] published a multicenter, prospective randomized trial assessing biologic prosthesis for paraesophageal hernias as a buttress with a primary repair. They used small intestinal submucosa (SIS), an acellular xenograft consisting primarily of porcine collagen. Their findings at six month follow up were promising. The primary outcome measure was a greater than 2 cm recurrent hernia on upper gastrointestinal (UGI) series. At six months, patients who had the biologic prosthesis buttress repair had a 9% recurrence rate compared to 24% in the group who had a primary repair alone of the hiatus.

The long-term data from that study were recently presented at the 2010 American College of Surgeons Annual Clinical Congress. Unlike the short-term data, there was no difference in recurrence in long-term follow up. With a median follow-up of 58 mo, there was no statistical difference in symptom control, quality of life measures or UGI recurrence rates between the primary repair alone *vs* primary repair with a SIS buttress.

They concluded that both types of repair gave long and durable relief, but the benefit of reducing hiatal hernia recurrence in the short term had diminished by the five year mark^[63]. There have been new biologic mesh materials and redesigning of existing materials that will need to be studied in the future.

CONCLUSION

Despite controversies for laparoscopic paraesophageal hernia repairs, there are clear advantages when compared to open repair. Hospital stays are usually around two days and much shorter than before, pain is improved, and patients have less complications. More long-term data will be needed to assess the optimal crural repair.

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Hepatic expression and cellular distribution of the glucose transporter family

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Abstract

Glucose and other carbohydrates are transported into cells using members of a family of integral membrane glucose transporter (GLUT) molecules. To date 14 members of this family, also called the solute carrier 2A proteins have been identified which are divided on the basis of transport characteristics and sequence similarities into several families (Classes 1 to 3). The expression of these different receptor subtypes varies between different species, tissues and cellular subtypes and each has differential sensitivities to stimuli such as insulin. The liver is a contributor to metabolic carbohydrate homeostasis and is a major site for synthesis, storage and redistribution of carbohydrates. Situations in which the balance of glucose homeostasis is upset such as diabetes or the metabolic syndrome can lead metabolic disturbances that drive chronic organ damage and failure, confirming the importance of understanding the molecular regulation of hepatic glucose homeostasis. There is a considerable literature describing the expression and function of receptors that regulate glucose uptake and release by hepatocytes, the most important cells in glucose regulation and

glycogen storage. However there is less appreciation of the roles of GLUTs expressed by non parenchymal cell types within the liver, all of which require carbohydrate to function. A better understanding of the detailed cellular distribution of GLUTs in human liver tissue may shed light on mechanisms underlying disease pathogenesis. This review summarises the available literature on hepatocellular expression of GLUTs in health and disease and highlights areas where further investigation is required.

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Key words: Hepatic; Liver; Glucose transporters; Glucose; Transport; Hepatocyte

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INTRODUCTION

Provision of a regular supply of glucose and other carbohydrates for fuel is vital for human survival and these are transported into cells using members of a family of integral membrane glucose transporter (GLUT) molecules^[1]. To date 14 members of this family, also called the solute carrier 2A (SLC2A) proteins have been identified which can be divided on the basis of transport characteristics (intrinsic or inducible, specificities) and sequence similarities^[2] into several families (Classes 1 to 3)^[3,4]. The expression of these different receptor subtypes varies between different species, tissues and cellular

subtypes and each has differential sensitivities to stimuli such as insulin.

The liver is a contributor to metabolic carbohydrate homeostasis and is a major site for synthesis, storage and redistribution of carbohydrates. At its simplest, after a meal hepatocyte GLUTs take up glucose from the portal bloodstream and it is converted to glycogen for storage. In a glucose-depleted state, this glycogen can then be converted back to glucose for fuel with up to 70% of total hepatic glucose production arising *via* this route^[5]. Situations in which the balance of glucose homeostasis is upset such as diabetes or the metabolic syndrome can lead metabolic disturbances that drive chronic organ damage and failure, which confirms the importance of understanding the molecular regulation of glucose homeostasis. The liver is the major store of glycogen, regulates the availability of glucose and acute liver failure is associated with profound hypoglycemia. This has led to a large body of work investigating the expression and function of receptors that regulate glucose uptake and release by hepatocytes, the most import cells in glucose regulation and glycogen storage but there is less appreciation of the roles of GLUTs expressed by other cell types within the liver. Thus to date expression of GLUT-1, GLUT-2^[6,7], GLUT-9^[8] and GLUT-10^[9] has been documented on hepatocytes but little is known about their expression or function on other cell types. However all cells require carbohydrate to function and there is evidence that non-parenchymal cells may contribute to glucose disposal. For example sinusoidal endothelial cells bind insulin with high affinity, and endothelial insulin-responses may be rate-limiting for glucose uptake^[10]. Thus a better understanding of the detailed cellular distribution of GLUTs in human liver tissue may shed light on mechanisms underlying disease pathogenesis. We begin by discussing the extrahepatic expression and functions of these proteins.

EXTRAHEPATIC EXPRESSION AND FUNCTION OF CLASS I GLUCOSE TRANSPORTERS

This family contains the proteins GLUTs 1 to 4 and 14 (SLC2A1-4, 14). The gene for GLUT-1 (*SLC2A1*) the most ubiquitous transporter is located on chromosome 1p35-p31.3 and generates a 54 Kd protein in humans and rodents^[11]. It has a high K_m for glucose ($K_m = 1-2$ mmol/L) and is mainly responsible for basal glucose and uptake^[12], but can also transport other hexose carbohydrates including mannose, galactose, glucosamine, 3-O-methylglucose and 2-deoxy-d-glucose. GLUT-1 is, expressed in most cells^[13] at low levels, with highest expression reported on erythrocytes, the blood brain barrier, neuronal membranes, eye, placenta and lactating mammary glands^[14-16]. Murine embryonic expression also suggests a developmental role^[17,18]. Over expression of GLUT-1 has been documented in a variety of tumours^[19] and is associated with increased proliferation rates and

increased mortality^[20] leading to its use as a diagnostic/prognostic marker in some cancers^[21,22].

The GLUT-2 (*SLC2A2*) gene located on chromosome 3q26-1-q26.2 encodes a 524 amino acid protein. GLUT-2 can efficiently transport sugars due to its high V_{max} and K_m for glucose, and is well suited to managing large bi-directional fluxes of glucose in and out of cells^[23]. It also transports other dietary sugars such as galactose, mannose and fructose with a high affinity for glucosamine^[11,24,25]. GLUT-2 is highly expressed in the liver, pancreatic beta cells, and on the basolateral surface of kidney and small intestine epithelia^[26,27] with expression regulated by sugars and hormones^[23,28]. Glycogenesis in the rare autosomal recessive disorder Fanconi-Bickel Syndrome has been associated with mutations in GLUT-2^[29], and diabetes mellitus in patients with prolonged hepatitis C virus (HCV) infection has been linked to virally-induced reduction in hepatocyte expression of GLUT-2^[30].

GLUT-3 (SLC2A3) was initially identified from muscle cell cDNA^[31]. Expression localises to the membrane of slow twitch muscle fibres^[32] and it is implicated in muscle regeneration and cell fusion^[33]. However its major role is in neurons, supplying the high glucose demands in the brain^[11,34] and it is increased in brain tumour cells^[35]. The gene for GLUT-3 is located on chromosome 12p13.3 and encodes a 496aa protein^[12] which transports glucose with a high affinity ($K_m = 1.8$ mmol/L) and maltose, xylose, dehydroascorbic acid, mannose and galactose^[25]. It is also present in fat, kidney, heart, placenta and liver at lower levels^[36], and is vital for the supply of substrate to early post-implanted embryos^[37]. White blood cells, which need an increased supply of glucose to fuel immune functions, express several GLUTs including GLUT-3^[34] the expression of which is decreased in diabetes^[38].

GLUT-4 (SLC2A4) was cloned and sequenced by several groups in 1989^[39-41]. It is a 55kDa protein responsible for more than 50% of all body glucose uptake^[42]. In the absence of insulin it is sequestered in intracellular vesicles and rapidly translocated to the plasma membrane in response to insulin. GLUT-4 transports glucose ($K_m = 5-6$ mmol/L), dehydroascorbic acid and glucosamine^[11,24]. Highest levels of expression are detected in insulin sensitive tissues such as skeletal and cardiac muscle, brown and white adipose tissue^[11] and endothelial cells^[43]. Expression has also been documented in monocytes, and like GLUT-3 is reduced in insulin-resistant individuals^[44]. Mutations in the gene have been associated with diabetes.

GLUT-14 (SLC2A14) was identified and cloned by Wu X *et al.*^[45] in 2002. It is located on chromosome 12p13.3, has a high sequence similarity to GLUT-3 and may have arisen as a result of gene duplication. The protein contains sugar transporter signature motifs predicted to exhibit glucose transport activity^[45]. Two splice variants have been identified in the testis^[45]. Mutations of GLUT-14 and its drosophila homologue have been associated with Alzheimers disease in genome wide as-

sociation studies in patients and insect models^[46,47] and may explain the reported brain-specific dysregulation of glucose metabolism seen in this condition.

EXTRAHEPATIC EXPRESSION AND FUNCTION OF CLASS II TRANSPORTERS

This family contains the transporters GLUT-5, GLUT-7, GLUT-9 and GLUT-11. GLUT-5 (SLC2A5) mRNA is detected mainly in the small intestine where it is found at both the apical and basolateral membranes and functions to absorb dietary fructose ($K_m = 6 \text{ mmol/L}$)^[11,48,49]. It is also expressed at lower levels in the human kidney, microglial cells, adipocytes, muscle, brain, and testes^[49,50], and in common with other transporters, expression is increased in human malignant tumours^[51]. The protein exhibits no activity for glucose transport in humans or mouse^[11,52] and its localization is not regulated by insulin^[50,53]. There is a growing interest in fructose consumption and its link with the metabolic syndrome, type 11 diabetes and obesity^[49] since consuming foods and beverages which contain excessive amounts of fructose has been linked to nonalcoholic fatty liver disease (NAFLD)^[54]. The thiazolidinedione drug pioglitazone^[55], which is used to treat type II diabetes, decreases GLUT-5 mRNA (52%) and protein (40%) in muscle fibres of type II diabetic subjects.

The GLUT-7 (SLC2A7), which was originally cloned from a human intestinal cDNA library^[56], has considerable sequence similarity to GLUT-5^[57] and is involved in uptake of sugars *via* facilitative diffusion mechanisms. Like GLUT-5 it has substrate specificity for both glucose and fructose and a key Ile-314 residue confers hexose specificity and is essential for fructose transport^[58]. GLUT-7 mRNA is detected in the small and large intestines at the brush border membrane of enterocytes^[11,49]; it is also detected in the prostate and testis^[56]. Interestingly, disparities between the localisation of expression within the small intestine and glucose and fructose substrate availabilities suggest that alternate ligands may exist^[57].

GLUT-9 (SLC2A9) shares sequence homology^[59,60] and substrate specificities^[58] with GLUT-5, GLUT-7 and GLUT-11 and, together with GLUT-2, is important for glucose-sensing by pancreatic B-cells^[61]. Expression is localised to liver, kidneys, leukocytes^[62], pancreas^[61], placenta, lung^[63], testis and adrenal gland. Two alternate isoforms have been identified, termed GLUT-9a and GLUT-9b^[64,65], and alternative splicing results in differential subcellular localisation. Both isoforms have also been reported to transport urate with high affinity^[66], and polymorphisms in the *GLUT-9* gene are linked with an increased predisposition to gout^[67]. Some polymorphisms have also been associated with an increased incidence of diabetes in Chinese populations^[68]. GLUT-9a expression increases in pregestational and gestational diabetes, and GLUT-9b is increased by insulin^[59]. In mouse, three isoforms of this transporter are reported,

and similarly elevated in diabetes^[8].

Three distinct isoforms of GLUT-11 (SLC2A11) have been identified in humans, with distinct but overlapping tissue expression patterns. Thus GLUT-11-A is expressed in skeletal muscle, kidney and heart, GLUT-11-B in adipose tissue, kidney and placenta, and GLUT-11-C in pancreas, heart, adipose tissue and skeletal muscle^[69-72]. Muscle expression is localised to slow twitch fibres^[73] and appears to be involved in myeloma cell viability and proliferation^[74]. All three variants of GLUT-11 exhibit transport activity for both glucose and fructose but not galactose when expressed in *Xenopus* oocytes^[58,70].

EXTRAHEPATIC EXPRESSION AND FUNCTION OF CLASS III TRANSPORTERS

This family constitutes the evenly numbered transporters GLUT-6, GLUT-8, GLUT-10, GLUT-12 and GLUT-13. GLUT-6 (SLC2A6)^[62] is widely expressed in normal and malignant tissue. mRNA has been detected in peripheral leucocytes, brain^[72] and spleen^[11] as well as in pancreas, testis, colon^[62] and adipose tissue^[75]. Subcellular protein expression varies with plasma membrane localisation in renal collecting tubule cells and cytoplasmic localisation in germinal cells of the testis and smooth muscle. GLUT-6 has significant sequence identity with GLUT-3 and may have arisen through insertion of GLUT-3 sequence into another gene on chromosome 5^[76].

GLUT-8 (SLC2A8) is a high capacity intracellular GLUT^[77] composed of 447 amino acids containing an N-terminal dileucine motif that permits trafficking *via* adaptor proteins to different organelles^[77,78]. Expression is highest in the testis and^[79], following insulin stimulation increases in the mid-piece of mature spermatozoa and translocates to the acrosome where the spermatozoa take up glucose to drive motility and the acrosome reaction. GLUT-8 may compensate for a lack of GLUT-4 in spermatozoa^[80,81] and the preimplantation blastocyst, which demonstrates insulin stimulated glucose uptake *via* GLUT-8 translocation^[82]. GLUT-8 is also found in some insulin receptive tissues including adipose tissue, muscle, brain, adrenal glands, spleen, heart and the liver^[62,83,84], but not adipocytes^[75] or neuronal cells^[85].

GLUT-10 (SLC2A10) is a 541aa protein in humans and 513aa in zebrafish^[11,86], which transports both glucose and galactose with high affinity^[87]. It is expressed in the brain, lungs, adipose tissue^[88], heart, placenta, and skeletal muscle with highest expression in the liver and pancreas^[9,87]. The *GLUT-10* gene, located on chromosome 20q12-13.1^[89] has been linked with type II diabetes^[9,90]. However other studies do not show any association with a diabetic phenotype^[91,92]. Development of the cardiovascular system and TGF β signalling are linked to GLUT-10 function^[93] and mutations are associated with altered angiogenesis and arterial tortuosity syndrome^[93,94] as a consequence of a loss of regulation of smooth muscle mitochondrial antioxidants production in the absence of functional GLUT-10^[89].

Table 1 Summary of reported hepatic expression of glucose transporters isoforms

Class	GLUT isoform	Hepatic expression	Subcellular expression/localisation	Protein/mRNA	Ref.
Class I	GLUT1	Yes	Sinusoidal membrane of hepatocytes, protein restricted to hepatocytes proximal to the hepatic venule, also expressed on endothelial cells, kupffer cells and cholangiocytes; hepatocyte expression in HCC	Both	[110,113,115,116,120]
	GLUT2	Yes	Hepatocytes	Protein	[124-126]
	GLUT3	Yes	Hepatocytes, bile canalicular membrane more enriched than sinusoidal membrane	Protein	[115,116,132]
	GLUT4	Yes	Stellate cells	mRNA	[115,116,134]
	GLUT14	No			
Class II	GLUT5	Yes	Normal liver tissue hepatocytes (cytoplasmic)	Both	[51,115]
	GLUT7	No			[140]
	GLUT9	Yes	Majority of expression in hepatocytes of normal liver and HCC with cytoplasmic expression in pericentral areas	Protein	[51]
Class III	GLUT11	Yes		mRNA	[115]
	GLUT6	Yes		mRNA	[76]
	GLUT8	Yes	Perivenous hepatocytes	Both	[115,143]
	GLUT10	Yes		mRNA	[86,115]
	GLUT12	Yes		mRNA	[98]
	GLUT13	No			

The table summarizes the evidence presented within this review to highlight the reported hepatocellular expression of glucose transporter (GLUT) isoforms at message and protein level. Pertinent references are cited in the extreme right hand column. mRNA: Micro RNA; HCC: Hepatocellular carcinoma.

GLUT-12 (SLC2A12) was originally identified in the MCF-7 breast cancer epithelial cell line^[95]. It is expressed in insulin sensitive tissues in humans and rodents including adipose tissue, skeletal muscle (major expression in type 1 oxidative fibres) and heart^[72,88,96-98] as well as human chondrocytes^[99]. GLUT-12 is also found in placenta, small intestine, heart and tumours with a high metabolic and capacity glucose utilisation^[100-103]. In normal human muscle GLUT-12 undergoes PI3 kinase dependent translocation from an intracellular region to the plasma membrane^[104]. Its expression in insulin sensitive tissues, and evidence that overexpression of GLUT-12 in mice improves glucose clearance rate and whole body insulin sensitivity^[105] confirm that this transporter is insulin-sensitive. The *GLUT-13 (SLC2A13)* gene encodes a 629 amino acid protein, located on chromosome 12q12. It is a H⁺/myo-inositol co-transporter^[11,106,107] also known as HMIT^[108] in neuronal cells^[106,108]. Although there are no known reports of glucose activity for GLUT-13^[11], the rat gene contains motifs which are important for glucose transport activity (<http://omim.org/entry/611036>).

LIVER SPECIFIC EXPRESSION OF GLUCOSE TRANSPORTER MOLECULES

The data reviewed above reveal the widespread distribution and diverse function of extrahepatic transporter proteins but much less is known about their expression and function in the liver. Surprisingly, there are few studies documenting changes in expression and function in disease. Defining local expression of GLUTs in tissue will shed light on disease pathogenesis. For example, diabetes is associated with altered expression of GLUT-1, GLUT-2, GLUT-3 and GLUT-8 and GLUT-9 (reviewed in^[8]) and abnormal GLUT-1 expression on tumour endothelium in HCC has prognostic and diagnostic

significance^[109-111]. A good example is the finding that diabetes in HCV is a consequence of virally induced downregulation of GLUT-1 and GLUT-2 on hepatocytes^[30]. Similarly, transport of key substrates such as fructose has been linked to NAFLD^[54]. Dysregulated glucose homeostasis and insulin resistance in NAFLD is associated with chronic organ damage affecting multiple hepatic cell types. The expression levels of transporters is not only regulated by insulin and glucose levels but also by cytokines including interleukin-6, which is increased in obesity and diabetes and can amplify insulin resistance *via* effects on GLUT-4^[112]. The hexose transporters also play important roles in the function of cholangiocytes^[113], endothelial cells and stellate cells^[114]. Thus we summarise the current state of knowledge regarding hepatocellular expression of the GLUT family of proteins, in order to highlight their potential role in tissue homeostasis and disease (Table 1).

HEPATIC EXPRESSION OF CLASS I TRANSPORTERS

The widespread expression of GLUT-1 includes the liver although the precise cellular distribution remains controversial. Because hepatocytes are capable of gluconeogenesis their need for glucose uptake is modest. GLUT-1 is expressed on the sinusoidal membrane of rat and porcine^[115,116] hepatocytes, and may be expressed to a greater extent than GLUT-2 during early post-natal development^[117]. Expression of both GLUT-1 and GLUT-2 by foetal hepatocytes allows for efficient glycogenesis at low plasma glucose concentrations^[118]. In adult animals, expression is strongest in the central acinar zones^[119]. Transcription and microsomal expression of GLUT-1 is detected in periportal and perivenular hepatocytes but membrane localisation is restricted to hepatocytes

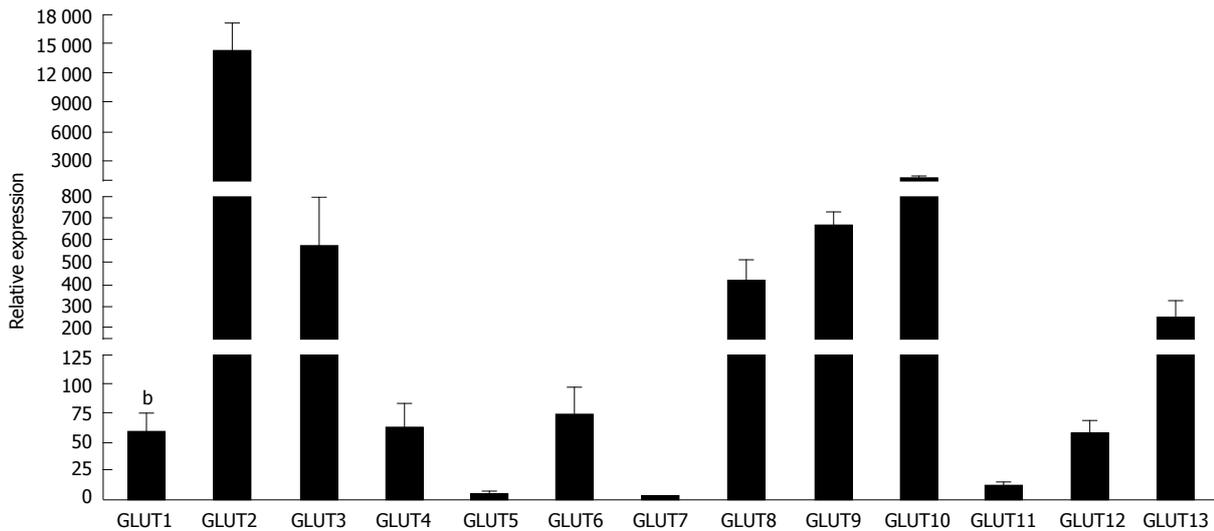


Figure 1 Expression of glucose transporter glucose transporters 1-13 micro RNA in normal human livers. This figure contains unpublished data generated by the authors. Livers were collected from patients at the Liver Unit, Queen Elizabeth Hospital, Birmingham, United Kingdom with appropriate informed written consent and local ethics committee approval. Micro RNA was extracted using standard protocols and integrity was confirmed using an Agilent 2100 Bioanalyser. Transcriptome analysis was carried out for all samples using Agilent Human Whole Genome Oligo Arrays (G4112F) in accordance with Agilent one colour microarray gene expression analysis protocol. Results are expressed as means of five normal livers \pm SE, and were run on triplicate plates. Data were normalized to pooled endogenous controls and differential expression is represented as power $2^{-\Delta\Delta CT}$. $^b P < 0.01$ vs glucose transporters (GLUT) 2.

proximal to the hepatic venule^[120] under basal conditions. Our own microarray analysis of human normal livers confirms expression of GLUT-1 and GLUT-2 in total liver mRNA (Figure 1). In hepatocellular carcinoma, variable cytoplasmic GLUT-1 is detected and is has been used to distinguish between cholangiocarcinomas and hepatocellular carcinomas (HCC)^[109] and has even been proposed as a therapeutic target for HCC^[110]. Exposure of rodents to alcohol and high fat feeding results in increased GLUT-1 and decreased GLUT-2 expression in hepatocytes which presumably reflects changes in energy metabolism in response to the dietary changes^[121].

Non-parenchymal cells, which cannot carry out gluconeogenesis, rely on glucose uptake rather than endogenous generation. GLUT-1 is the dominant receptor on both endothelial cells and Kupffer cells and levels increase in response to even brief exposure to LPS^[122]. Interestingly, acute liver failure has been associated with increased GLUT-1 expression on cerebral vasculature in response to elevated circulating ammonia levels^[123]. Cholangiocytes demonstrate basolateral expression of GLUT-1^[113] which facilitates absorption of glucose from bile.

GLUT-2 fulfils the major glucose transport role in hepatocytes^[36,124] (Figure 1). The protein localises to the sinusoidal plasma membrane of normal^[23,125] and malignant hepatocytes. Historical reports suggest a K_m for glucose transport of up to 66 mmol/L in intact rat hepatocytes^[126] although contribution from other transporters likely contributes in this study since others report lower values between 10 mmol/L and 20 mmol/L^[23]. GLUT-2 promotes rapid glucose efflux following gluconeogenesis. In the fasting state the liver produces glucose *via* glycogenesis or glycogenolysis with the conversion of glucose 6 phosphate into glucose preceding release *via*

GLUT-2^[23]. However in the fed state glucose and insulin levels rise and inhibit endogenous glucose production through effects on enzymes involved in gluconeogenesis. This is associated with removal of membrane GLUT-2 and a subsequent fall in GLUT-2 mediated release^[23]. Excess glucose is stored as glycogen or converted to lipids and hepatocyte GLUT-2 and the insulin receptor are internalised together into endosomes in response to insulin^[23,127,128]. In mice lacking GLUT-2 the rate of hepatic glucose production is not impaired indicating the presence of a facilitated diffusion-independent mechanism for glucose release^[129]. Thus the major role of hepatocyte GLUT-2 is to regulate efflux rather than uptake of glucose. However, in obesity insulin resistance drives an increase in GLUT-2 levels that may further exacerbate metabolic dysfunction in NAFLD^[130].

Much less is known about the hepatic expression and function of GLUT-3. GLUT-3 is expressed in porcine livers^[115] and localised to the plasma membrane of rat hepatocytes. Expression is focussed on the bile canalicular membrane rather than the sinusoidal membrane^[116]. Mice with GLUT-3 haploinsufficiency develop obesity and insulin resistance associated with hepatic steatosis, possibly as a consequence of foetal glucose insufficiency^[131]. GLUT-3 expression is increased on both primary and metastatic hepatic tumours, which might reflect an increased need for glucose uptake in cancer^[132]. Low levels of GLUT-3 have been reported in the human liver^[36] and are supported by our microarray analysis but detailed human studies are lacking and little is known about changes in disease.

Whilst the liver is generally considered to lack significant expression of GLUT-4^[133], a recent study reports expression of GLUT-4 mRNA in porcine liver^[115]. Al-

though there is little evidence for expression in hepatocytes, GLUT-4 has been detected in sinusoidal endothelial cells and stellate cells where it can mediate glucose uptake by semicarbazide sensitive amine oxidase mediated effects on insulin receptor signalling^[134] which explains our findings of expression at mRNA level in humans (Figure 1). Expression on stellate cells is enhanced by leptin signalling^[114] leading to HSC activation that may contribute to fibrogenesis in NAFLD. In contrast, in murine models of diet-induced obesity GLUT-4 mRNA is decreased in the liver^[135] and cirrhosis is associated with decreased extrahepatic GLUT-4 mRNA^[42]. Deletion of skeletal muscle GLUT-4 results in redirection of excess circulating glucose to the liver where it becomes fuel for conversion to lipid storage^[136-138]. Thus glucose homeostasis is maintained by a complex relationship between intra- and extra hepatic levels of GLUT-4 regulated by metabolic activity and dietary intake. To date there are no published reports concerning expression of GLUT-14 in the liver.

HEPATIC EXPRESSION OF CLASS II TRANSPORTERS

GLUT-5 protein has been detected in human hepatocytes^[51] although low to undetectable RNA levels in pigs^[115] imply species-specific differences in GLUT-5 expression. Hepatic metastases from lung and breast cancer are GLUT-5 positive^[132] but under normal conditions liver expression is minimal (Figure 1). A mechanistic link between elevations in GLUT-5 expression in small intestine and alterations in hepatic metabolism^[139] has been suggested.

GLUT-7 was initially reported as a hepatic microsomal GLUT found in the endoplasmic reticulum, which facilitated the release of glucose formed in the process of gluconeogenesis and glycogenolysis for export into the blood^[136,140]. However this has recently been challenged by studies showing that neither human nor rat livers contain GLUT-7 mRNA^[141] and our data in Figure 1, suggesting that the previous findings were due to a cloning artefact. Definitive studies need to be performed to clarify the situation.

GLUT-9 has been detected in the cytoplasm of pericentral hepatocytes in normal human liver and in HCC^[51]. The receptor appears to be functional for glucose transport because plasma membrane expression of GLUT-9 correlates with glucose influx in HepG2 cells^[142] and GLUT-9 inactivation in mouse hepatocytes leads to hyperuricosuria^[143]. Although GLUT-11 mRNA has been detected in porcine liver^[115], there are no studies documenting expression of GLUT-11 in the human liver.

HEPATIC EXPRESSION OF CLASS III TRANSPORTERS

Little is known about the hepatic expression of the

recently identified Class III transporters although our microarray data (Figure 1) is indicative of some degree of expression. Presence of mRNA for GLUT-6 has been described in hepatoma cell lines but has not been detected in normal human liver^[76]. GLUT-8 mRNA has been detected in perivenous hepatocytes in pig^[115] and mouse^[144] livers where it may regulate glycolytic flux. Mice with type I diabetes show decreased expression whereas expression increases in insulin resistance and type II diabetes suggesting that expression is regulated by insulin^[144]. Hepatic expression of GLUT-10 has been reported in pigs^[115] and zebrafish^[86] but we are unaware of any data in humans. GLUT-12 mRNA has been documented in all bovine tissues including the liver where levels are low compared to spleen and skeletal muscle^[98], but again detailed cellular expression data is currently lacking. There are no known reports of GLUT-13 expression in the liver.

CONCLUSION

Systemic carbohydrate homeostasis is maintained by a complex relationship between organs such as the pancreas, intestine, muscle and liver. Intra- and extra hepatic levels of GLUT molecules are regulated in part by metabolic activity, dietary intake, and disease state. For example, diabetes is associated with altered expression of GLUT-1, GLUT-2, GLUT-3 and GLUT-8 and GLUT-9^[8] and abnormal GLUT-1 expression on tumour endothelium in HCC^[109-111] permits efficient glucose uptake by tumour cells even at low blood glucose concentrations. Chronic fructose intake drives glucose and glycogen storage, lipogenesis and production of lipogenic intermediates as well as promoting production of very-low-density lipoproteins^[145]. This suggests that the reported hyperlipidaemic and hyperuricaemic effects of fructose, coupled with macrovesicular steatosis and lobular inflammation patterns^[146] characteristic of human NAFLD seen in rodents fed high fructose diets, may be enhanced in the context of altered expression of fructose transporters within the hepatic parenchyma and especially so for individuals with high fructose intake or pre-existing hyperlipidaemia or metabolic syndrome. New data is increasingly suggesting the merits of targeting members of the GLUT family therapeutically. Thus overexpression of GLUT-1 in tumours, particularly those with poor prognosis has been suggested as a possible means to selectively inhibit tumour cell metabolism^[147], although expression of GLUT-1 in red blood cells will likely preclude use therapeutically. Similarly targeting of GLUT-3, which is involved in neovascularisation in glioblastoma has been suggested to prevent resistance to conventional therapy^[148], and GLUT-4 is of particular interest in the context of diabetes and insulin resistance, with efforts underway to design therapeutics to enable appropriate glucose uptake independently of insulin stimulation^[149]. Alterations in hepatic expression of GLUT transporters have been described in response to

insulin resistance and hyperlipidaemia, alcohol consumption, viral infection and carcinogenesis, with diverse functions including biliary transport, fibrogenesis, urate transport and angiogenesis executed by family members in extraparenchymal cells. Combined with the central role of the liver in regulation of circulating carbohydrate therefore, future definition of the spatial, temporal and disease-specific expression of GLUTs within the liver microenvironment is key to understanding disease pathogenesis and potential hepatic complications of systemic inhibition.

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Utility of faecal calprotectin analysis in adult inflammatory bowel disease

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Abstract

The inflammatory bowel diseases (IBD), Crohn's disease and ulcerative colitis, are chronic relapsing, remitting disorders. Diagnosis, along with assessment of disease activity and prognosis present challenges to managing clinicians. Faecal biomarkers, such as faecal calprotectin, are a non-invasive method which can be used to aid these decisions. Calprotectin is a calcium and zinc binding protein found in the cytosol of human neutrophils and macrophages. It is released extracellularly in times of cell stress or damage and can be detected within faeces and thus can be used as a sensitive marker of intestinal inflammation. Faecal calprotectin has been shown to be useful in the diagnosis of IBD, correlates with mucosal disease activity and can help to predict response to treatment or relapse. With growing evidence supporting its use, over the last decade this faecal biomarker has significantly changed the way IBD is managed.

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Key words: Faecal calprotectin; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Faecal biomarker

INTRODUCTION

It is desirable to have simple diagnostic tests and disease markers for use in the assessment and follow up of chronic diseases such as inflammatory bowel disease (IBD). These markers should be easy to perform, acceptable to both patients and health workers, economical, ideally non-invasive and have a high sensitivity and specificity. Calprotectin, first described in 1980^[1], is a protein found in the cytosol of neutrophils and macrophages composed of two subunits S100A8 and S100A9. It can be detected in plasma, urine, cerebrospinal fluid, faeces, saliva, synovial fluid and colonic biopsies^[2]. It is stable in faeces for up to seven days at room temperature and has a homogenous distribution in faeces^[3], properties which lend it to testing spot faecal samples. There has been recent emphasis of the involvement of the innate immune system in the pathogenesis of IBD^[4]. Calprotectin is classed as a damage associated molecular pattern protein (DAMP) having antimicrobial protective properties. DAMPs are released by the innate immune system from damaged or activated cells, initiating and perpetuating the immune response. The extracellular release of calprotectin during times of cell stress/damage makes it an accurate marker of intestinal inflammation.

As early as 1992 it was shown that faecal calprotectin

is elevated in patients with both ulcerative colitis (UC) and Crohn's disease (CD)^[3] and this has been confirmed by subsequent studies^[5-9]. Calprotectin levels in faeces correlate with faecal excretion of 111-indium labelled leucocytes, deemed to be the gold standard for measuring intestinal inflammation ($r = 0.80$, $P < 0.0001$)^[5]. Disadvantages of this gold standard test compared with calprotectin are the exposure to radiation, cost and the three day collection of faeces. Faecal calprotectin levels have also been shown to correlate well with radiolabelled white cell scanning, another method of assessing intestinal inflammation, in adults with CD^[10]. Spot faecal samples of < 5 g have been shown to be as reliable as 24 h collection samples for measuring calprotectin levels^[3] indicating that calprotectin is evenly distributed throughout the faeces.

An elevated faecal calprotectin is not specific for IBD. Any inflammatory process within the gastrointestinal tract will result in the activation of the innate immune response and release of calprotectin. Faecal calprotectin concentration has been shown in studies to be elevated in many conditions including infection, colorectal cancer, untreated coeliac disease, microscopic colitis and diverticulitis^[11-13]. Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to cause significant increases in faecal calprotectin levels within seven days due to NSAIDs induced intestinal inflammation with endoscopic correlation^[14,15]. Proton pump inhibitors (PPIs) have been associated with significantly elevated faecal calprotectin levels, regardless of reason for PPI^[16].

Initially faecal calprotectin concentration was reported in mg/L, but more recent assays (post 2000) usually report faecal calprotectin concentration as $\mu\text{g/g}$. To compare these results, faecal calprotectin concentrations obtained using assays pre-2000 need to be multiplied by a factor of five.

USE OF FAECAL CALPROTECTIN IN DIAGNOSIS OF IBD

Diagnosis of IBD has historically been based on a combination of clinical history and examination, blood parameters, radiology and endoscopy. The addition of a faecal biomarker able to reduce the need for invasive endoscopic procedures or exposure to radiation is advantageous.

Limburg *et al.*^[12], in 2000, published a study of 110 patients attending for colonoscopy for the investigation of chronic diarrhoea showing that increased faecal calprotectin levels were significantly ($P = 0.0001$) associated with the presence of colorectal inflammation (CD, UC, microscopic colitis or diverticulitis). Within the colonic inflammation subgroup, calprotectin concentrations were highest amongst subjects with IBD. The negative predictive value of faecal calprotectin in this dataset was 93%.

IBD and irritable bowel syndromes (IBS) can present in a similar clinical fashion with symptoms such as diarrhoea and abdominal pain. Routine colonoscopy in these

patients is costly, invasive and has associated morbidity and mortality. Serum markers of inflammation such as C reactive protein (CRP) and erythrocyte sedimentation rate (ESR) in isolation are not sufficiently sensitive or specific for the diagnosis of IBD^[7]. The use of faecal calprotectin to distinguish between IBD and IBS has been analysed in several studies. In 2000 Tibble *et al.*^[7] presented results of a prospective study of 220 consecutive patients in whom the principal differential diagnosis was that of either IBS or CD. They excluded patients with UC on sigmoidoscopy and biopsy. A diagnosis of CD was made from a combination of radiological, endoscopic and histological investigations. A diagnosis of IBS was made on basis of normal investigations and a compatible history fulfilling the Rome criteria. All patients subsequently diagnosed with CD had significantly higher faecal calprotectin concentrations than those with IBS. The investigators found that using a cut-off point of 30 mg/L faecal calprotectin had a 100% sensitivity and 97% specificity in discriminating between active CD and IBS.

Schoepfer *et al.*^[17] looked at the accuracy of faecal biomarkers alone and in combination with the IBD antibodies, antineutrophil cytoplasmic antibody (ANCA) and anti-*Saccharomyces Cerevisiae* manna antibody (ASCA), in discriminating IBD from IBS. They found that the overall accuracy of faecal calprotectin for discriminating between IBD and IBS was 89% (sensitivity 83%, specificity 100%). There was only a marginal increase in overall accuracy when faecal calprotectin was combined with IBD antibodies to 91%.

Faecal calprotectin has been studied as a tool to predict abnormal small-bowel radiology^[18]. The study looked at 73 consecutive patients attending for small bowel follow through whose presenting symptoms were consistent with a possible diagnosis of IBD. The control group consisted of 25 patients with IBS, 25 normal volunteers and 25 patients with active CD. A faecal calprotectin level above 60 $\mu\text{g/g}$ predicted all abnormal barium follow through results. The negative predictive value of a single calprotectin result below 60 $\mu\text{g/g}$ of stool was 100% compared with 91% each for erythrocyte sedimentation rate cut off of 10 mm and C-reactive protein of 6 mg/L. Somewhat in contrast to this Sipponen *et al.*^[19] found that faecal calprotectin had a low utility for predicting the presence of small bowel CD on wireless capsule endoscopy, sensitivity was low at 59% with a moderate specificity of 71% using a cut-off of 50 $\mu\text{g/g}$.

A recent meta-analysis analysed 30 prospective studies comparing the diagnostic precision of faecal calprotectin against a histological diagnosis^[20]. Summary receiver operating characteristic curve analysis showed a sensitivity of 0.95 (95% CI: 0.93-0.97), specificity of 0.91 (95% CI: 0.86-0.91), and an area under the curve (AUC) of 0.95 for the diagnosis of IBD. The diagnostic precision of faecal calprotectin for IBD was higher in children than adults with better accuracy at a cut-off level of 100 $\mu\text{g/g}$ vs 50 $\mu\text{g/g}$. This meta-analysis also showed that faecal calprotectin was superior to CRP, ESR, ASCA, perinuclear anti-neutrophil cytoplasmic an-

tibodies and anti-*Escherichia coli* outer membrane porin C antibody in diagnosis of IBD.

Thus it can be stated that a normal faecal calprotectin result, in the absence of 'red flag' symptoms and in the context of positive Rome criteria, is associated with a high likelihood of subsequent non-organic diagnosis and further endoscopic or radiological evaluation may be avoided in such patients. A meta-analysis published in 2010 to assess whether the use of faecal calprotectin reduces the number of unnecessary endoscopic procedures in the investigation of suspected IBD showed that screening with faecal calprotectin would result in a 67% reduction in the number of adults requiring endoscopy. The downside of this screening strategy is delayed diagnosis in 6% of adults because of a false negative test result^[13].

Faecal calprotectin appears to better reflect disease activity in UC rather than CD^[21] but faecal calprotectin has not been found to be useful in distinguishing UC from CD. Quail *et al.*^[22] looked at faecal calprotectin concentrations in Scottish children with a diagnosis of IBD; there was no statistical difference in calprotectin concentrations between CD and non-Crohn's patients (UC or IBD type unspecified).

FAECAL CALPROTECTIN IN DISEASE ACTIVITY AND RESPONSE TO TREATMENT

In IBD, the presence of active gut inflammation is associated with migration of leucocytes, including neutrophils, to the gut mucosa^[23]. As a result the faecal stream contains increased levels of these inflammatory proteins including calprotectin. Faecal calprotectin has been shown to differentiate quiescent from active disease in both patients with CD and UC^[10,24-26]. Correlation of faecal calprotectin tends to be higher with endoscopic activity than clinical activity indices^[24,27] and indeed some studies have demonstrated no significant correlation between faecal calprotectin and clinical indices^[10,28]. In general faecal calprotectin correlates better with colonic CD rather than ileal disease^[27-31] and an inflammatory rather than a structuring/penetrating phenotype^[27,31]. Sipponen *et al.*^[24] showed that in active disease (CD endoscopic index of severity, CDEIS ≥ 3), faecal calprotectin concentrations were significantly higher in colonic than in ileal CD. Also, in limited ileal disease faecal calprotectin failed to correlate with endoscopic activity.

In UC Ricanek *et al.*^[32] showed that the median faecal calprotectin concentration was higher in patients with extensive and left sided disease distribution compared with proctitis (740 $\mu\text{g/g}$, 2106 $\mu\text{g/g}$, 86 $\mu\text{g/g}$ respectively; $P = 0.007$ and $P = 0.009$). There was no significant difference in faecal calprotectin concentration between extensive and left sided disease distribution.

There have been several studies looking at the use of faecal calprotectin to predict or monitor response to

treatment. In a study looking at 11 patients with relapsing IBD^[33] (11 CD and 27 UC) faecal calprotectin was analysed at inclusion and after 8 wk of treatment (end of study). Treatment was individualised medical therapy. A normalised faecal calprotectin concentration at 8 weeks predicted a complete response in 100% patients. There was a significant decline in faecal calprotectin levels ($P < 0.001$) in patients with UC responding to treatment defined as normalisation of clinical and endoscopic scores. Within the small subgroup of patients with CD although 81% of patients achieved a complete clinical response defined clinically as a Harvey Bradshaw Index^[34] (HBI) ≤ 5 there was no significant decline in calprotectin levels. This study was limited by small numbers and also the lack of endoscopic evidence of disease activity or remission in CD patients, it is possible that these patients had ongoing subclinical inflammation. In fact it has been shown that in patients with steroid induced clinical remission faecal calprotectin levels can remain elevated^[35,36]. This finding is in keeping with earlier studies showing incomplete mucosal healing in patients treated with corticosteroids^[37]. Sipponen *et al.*^[35] were able to show a significant decrease in faecal calprotectin ($P = 0.005$) in patients with CD who responded both clinically and endoscopically (using CDEIS) to an individualised escalation of treatment. There was no significant change in faecal calprotectin concentration in patients without endoscopic response.

Faecal calprotectin may be able to predict colectomy in patients with acute severe UC. Ho *et al.*^[38] showed that in patients with acute severe UC requiring inpatient treatment with intravenous corticosteroids faecal calprotectin was significantly higher in patients who failed to respond to medical therapy and required colectomy than those who did not ($P = 0.04$). The AUC was 0.65 ($P = 0.04$) for faecal calprotectin to predict colectomy with a maximum likelihood ratio of 9.23 at a cut-off of 1922.5 $\mu\text{g/g}$ (specificity of 97.4%). Overall in the study faecal calprotectin concentrations were high with 86% of patients having levels of $> 500 \mu\text{g/g}$ (median 1020 $\mu\text{g/g}$).

Faecal calprotectin can be used to monitor response to biological therapy. Palmon *et al.*^[39] showed that faecal calprotectin concentration decreases significantly at week 2 after an infliximab (IFX) infusion in 17 patients with CD on maintenance IFX therapy. Calprotectin levels were noted to rise back to baseline values by week 4 again despite a low median HBI. There was no endoscopic assessment of disease activity in this study. The rise in faecal calprotectin at week 4 may once again indicate a subclinical recurrence of mucosal inflammation. Sipponen *et al.*^[40] assessed the role of faecal calprotectin in monitoring clinical, using the CD activity index (CDAI) and CDEIS response to anti-tumor necrosis factor- α (TNF- α) therapy (IFX or adalimumab) in 15 patients with CD. Following 12 wk of treatment faecal calprotectin levels declined significantly from baseline level ($P = 0.001$) and changes in faecal calprotectin correlated to endoscopic appearances as scored using CDEIS (Spear-

Table 1 Correlation of faecal calprotectin with endoscopic findings, histology and clinical indices, when available

Ref.	Patient population	Endoscopic assessment score	Correlation coefficient with endoscopy (<i>r</i>)	Correlation coefficient with histology	Correlation with clinical index (<i>r</i>)
Røseth <i>et al</i> ^[6]	UC	MAYO	0.57	NA	NA
D'Inca <i>et al</i> ^[6]	CD	SES-CD	0.48	<i>r</i> = 0.12	NA
D'Inca <i>et al</i> ^[6]	UC	MAYO	0.51	<i>r</i> = 0.32	NA
Sipponen <i>et al</i> ^[24]	CD	CDEIS	0.73	NA	0.397(CDAI)
Schoepfer <i>et al</i> ^[27]	CD	SES-CD	0.75	NA	NA
Jones <i>et al</i> ^[28]	CD	SES-CD	0.45	NA	0.23 (CDAI)
Sipponen <i>et al</i> ^[31]	CD	SES-CD	0.64	0.56 (exchange ileal only disease)	0.32 (CDAI)
Sipponen <i>et al</i> ^[40]	CD	CDEIS	0.83	0.52 (exchange ileal only disease)	NA
Langhorst <i>et al</i> ^[48]	UC	MAYO	0.49	NA	NA
Langhorst <i>et al</i> ^[48]	CD	SES-CD	0.35	NA	NA
Hanai <i>et al</i> ^[63]	UC	Matts	0.81	NA	0.68 (CDAI)
Schoepfer <i>et al</i> ^[64]	UC	Rachmilewitz	0.83	NA	NA

NA: Not available; MAYO: Mayo score^[65]; SES-CD: Simplified endoscopic activity score for Crohn's disease (CD)^[66]; CDEIS: Crohn's disease endoscopic index of severity^[67]; CDAI: Crohn's disease activity index^[68]; Matts: Matts grading^[69]; UC: Ulcerative colitis.

man's rank correlation $r = 0.561$, $P = 0.03$) suggesting that faecal calprotectin is a useful non-invasive marker of mucosal response to anti-TNF- α treatment.

PREDICTING MUCOSAL HEALING

Historically clinical practice has considered interpretation of symptoms and the use of scoring systems such as the CDAI, HBI and the Rachmilewitz UC activity index^[41] to determine treatment success in IBD. These indices however tend to reflect patient well-being and quality of life rather than the degree of mucosal inflammation^[10,28]. In both CD and UC there is evidence that mucosal healing is associated with sustained remission and reduced need for surgery^[42,43] and following ileal resection the endoscopic appearance of the neoterminal ileum mucosa at 1 year post surgery has been shown to predict symptomatic relapse^[44]. Thus mucosal healing is evolving into the new goal of IBD treatment.

Røseth *et al*^[45] have demonstrated that normalisation of faecal calprotectin concentration corresponds to endoscopic mucosal healing. Seventeen patients with CD and 28 with UC clinically in remission who had faecal calprotectin concentrations of < 50 mg/L underwent endoscopic assessment of their lower GI tract and macroscopic mucosal appearances were assessed. Biopsies were also taken to assess histological inflammation. All but one of these patients with faecal calprotectin < 50 mg/L had inactive mucosal disease on colonoscopy.

Several subsequent studies have gone on to show that concentration of faecal calprotectin correlates with both histological and endoscopic disease activity in IBD and these are summarised in Table 1. Furthermore, several of the studies included in Table 1 show that correlation of faecal calprotectin with endoscopic appearances is stronger than correlation with clinical indices^[28,40].

In contrast to the evidence shown in Table 1, Denis *et al*^[46] failed to find a significant correlation between CDEIS and faecal calprotectin concentration. This was a small study of 28 patients with CD who had CDAI $>$

150 but a normal serum CRP. This lack of correlation may reflect the population studied as more than half of the patients had isolated ileal disease and overall disease activity was low (median CDEIS 3.4).

Interestingly one study in paediatric patients with IBD showed that calprotectin concentration correlated more closely with histological inflammation rather than endoscopic findings suggesting that faecal calprotectin may be more sensitive than macroscopic endoscopic appearances in evaluating disease activity status^[47].

USE OF FAECAL CALPROTECTIN TO PREDICT RELAPSE

Being able to identify patients at high risk of relapse, and those with sub-clinical intestinal inflammation, may allow adjustment of their treatment strategy thus preventing clinical relapse. A non-invasive method of identifying this would reduce cost and risk of morbidity and mortality to patients. As sensitivity and specificity of serum markers of inflammation correlate poorly with intestinal inflammation their ability to predict disease relapse is poor^[48,49]. Several studies have looked at the use of faecal calprotectin to predict relapse in patients with IBD and have demonstrated significant differences in faecal calprotectin concentration in relapsers compared with non-relapsers. A summary of these studies is shown in Table 2. Interestingly, faecal calprotectin appears less useful for predicting relapse in patients with ileal CD compared with patients with UC or colonic/ileocolonic CD^[29,30].

The major outlier in these datasets is the study by Laharie *et al*^[50] which looked at the use of faecal calprotectin to predict relapse specifically in 65 patients with CD treated with IFX induction regimen and then maintained on immunomodulator alone. There was no significant difference in faecal calprotectin concentration between those patients who relapsed by 14 wk post induction and those who did not even when the analysis was restricted to patients with pure colonic disease. No endoscopic

Table 2 The use of faecal calprotectin to predict relapse

Ref.	Patient population	Cut-off calprotectin level	Sensitivity for relapse (%)	Specificity for relapse (%)	Increased risk of relapse
Costa <i>et al</i> ^[21]	UC	150 µ/L	89	82	14 fold
	CD		87	43	
D'Incà <i>et al</i> ^[29]	UC	130 mg/kg	70	70	2.4 fold
	CD		65	62	1.7 fold
García-Sánchez <i>et al</i> ^[30]	CD + UC	120 µ/g	80	60	
	Ileal CD	223 µ/g	83	50	
Tibble <i>et al</i> ^[49]	CD + UC	50 mg/L (250 µ/g)	90	83	
Laharie <i>et al</i> ^[50]	CD post IFX		130 µ/g	61	
Kallel <i>et al</i> ^[70]	Colonic CD	250 µ/g	43	57	18.8 fold
		340 µ/g	80	90.7	
Gisbert <i>et al</i> ^[71]	CD + UC	150 µ/g	69	69	

UC: Ulcerative colitis; CD: Crohn's disease; IFX: Infliximab.

evaluation was performed after IFX induction so although the study did show a median drop in faecal calprotectin concentration of 340 µg/g following induction there may have been ongoing subclinical inflammation. Another limitation of this study is that disease relapse was defined on clinical grounds alone.

Mao *et al*^[51] performed a meta-analysis of the predictive capacity of faecal calprotectin in IBD relapse. Analysing 6 studies they found a pooled sensitivity of 78% and specificity of 73%. Capacity to predict relapse was comparable between CD and UC. Due to the small number of patients the predictive value of faecal calprotectin in ileal only CD patients was not assessed.

FAECAL CALPROTECTIN POST-SURGERY

More than 80% of CD patients require surgery within 10 years of diagnosis and by 3-5 years after surgery around a third of patients will have had a clinical relapse^[44,52]. The role of faecal calprotectin in predicting post-surgical recurrence of CD has been assessed. One study assessed 39 patients with CD undergoing bowel resection^[53]. The majority of patients (67%) had ileocolonic disease. Measurements of faecal calprotectin, CDAI and ultrasound examination were performed at 3 mo post surgery. Endoscopy was performed at 1 year regardless of patient symptoms and was considered the 'gold standard' for recurrence of disease. All patients were clinically in remission at 3 mo. Using a cut-off level of 200 mg/L, in predicting endoscopic post-surgical recurrence, faecal calprotectin has a sensitivity of 63% and specificity of 75% leading the authors to suggest that patients with elevated faecal calprotectin levels at 3 mo post surgery should be assessed endoscopically for early recurrence.

Lamb *et al*^[54] prospectively followed 13 patients for 12 mo post ileocaecal resection for symptomatic CD. Faecal calprotectin was seen to normalise after uncomplicated surgery at 2 mo and remained within normal limits in 8 patients who remained clinically in remission. In the remaining 5 patients there was an increase in faecal calprotectin concentration after initial normalisation associated with disease recurrence or post operative

intra-abdominal collections. The authors' conclusion from this small number of patients was that patients with low levels of faecal calprotectin after resection who had symptoms were unlikely to have mucosal inflammation. It should be pointed out though that there was no scheduled endoscopic evaluation of the gut mucosa during the 12 mo follow up period.

Pouchitis is an inflammatory condition with significant associated morbidity common in patients post restorative proctocolectomy^[55]. Diagnosis can be difficult histologically due to the patchy distribution of inflammation. Faecal calprotectin has been shown to reliably differentiate between inflamed and non-inflamed pouches and correlates with severity of pouchitis^[55-57] thus may reduce the need for endoscopic evaluation of the pouch in some patients.

USE OF FAECAL CALPROTECTIN TO AID DECISIONS OF WITHDRAWAL OF TREATMENT

There has been focus of late on when and in which patients it is appropriate to withdraw anti TNF-α therapy driven by costs and concerns about long term safety. Faecal calprotectin levels may assist in this decision making. Louis *et al*^[58] published results of a prospective study (STORI) of CD patients who had been in steroid free remission on IFX for at least six months. Relapse after IFX withdrawal was associated with various risk factors including a faecal calprotectin concentration of ≥ 300 µg/g. Other risk factors for relapse identified on multivariate analysis included male sex, absence of surgical resection, leucocyte counts > 6 × 10⁹/L, haemoglobin ≤ 145 g/L and hsCRP ≥ 5.0 mg/L. Patients with no more than 2 of the above risk factors had a reduced risk of relapse within 1 year (15% compared with 43.9% overall).

POINT-OF-CARE FAECAL CALPROTECTIN TESTING

Faecal calprotectin concentration is most commonly

measured using an enzyme-linked immunosorbent assay (ELISA) technique in clinical laboratories and several ELISA kits are available. Recently point-of-care or bedside faecal calprotectin tests have become available which may be advantageous in clinical circumstances such as primary care or when a more rapid result is required. These tests can be performed in less than 30 min. Correlation between faecal calprotectin concentrations measured using an ELISA technique and a point-of-care test has been shown to be good in recent initial studies^[59-62].

CONCLUSION

Faecal calprotectin is now playing a major role in the investigation and diagnosis of patients presenting to the physician with lower gastrointestinal symptoms and can obviate the need for costly invasive investigations in selected patients.

Perhaps more excitingly faecal calprotectin is dramatically changing the way IBD is managed. There is a growing body of evidence that faecal calprotectin correlates well with mucosal disease activity and this in turn makes it useful in assessing activity, monitoring response to treatment, predicting relapse and aiding in the difficult decision of whether or not to withdraw biological therapy to a degree than clinical scoring indices or other non-invasive serological markers cannot.

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Have guidelines addressing physical activity been established in nonalcoholic fatty liver disease?

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Abstract

The purpose of this review was to highlight, in relation to the currently accepted pathophysiology of non-alcoholic fatty liver disease (NAFLD), the known exercise habits of patients with NAFLD and to detail the benefits of lifestyle modification with exercise (and/or physical activity) on parameters of metabolic syndrome. More rigorous, controlled studies of longer duration and defined histopathological end-points comparing exercise alone and other treatment are needed before better, evidence-based physical activity modification guidelines can be established, since several questions remain unanswered.

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Key words: Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Physical activity; Diet

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INTRODUCTION

Compared with our ancestors, Western societies today lead a lifestyle that is much more sedentary, probably as a result of cultural changes stemming from disregarded traditional customs and modern usages. Taking into account differences in body size, our energy expenditure per kilogram of body weight has been estimated to be 40% less than that of our prehistoric ancestors^[1]. Current estimates suggest that 7 out of 10 adults are inactive or lack adequate conditioning^[2], and this lack of adequate exercise, combined with dietary indiscretion, has contributed to the worldwide epidemic of obesity and non-alcoholic fatty liver disease (NAFLD). Within the United States, data suggest that 64.5% of the adult population is now overweight or obese, with a worldwide prevalence of 40%-60%^[3-5]. Obesity, combined with host factors such as diet, sedentary lifestyle and genetic predisposition, has been directly associated with increases in the prevalence of insulin resistance, type 2 diabetes (T2D) and the metabolic syndrome. The hepatic manifestation of the metabolic syndrome, NAFLD, has also increased in prevalence, and is now considered to be around 20%-30% in Western countries. Among

morbidly obese patients undergoing bariatric surgery, approximately 90% have NAFLD and 36%-37% have the more aggressive form of fatty liver, non-alcoholic steatohepatitis (NASH)^[6,7]. In patients with NAFLD, advancing age, increasing weight, the number of features of the metabolic syndrome and the degree of insulin resistance have all been independently associated with NASH severity^[8]. Evidence-based treatment options for NAFLD are currently lacking. Recent data suggest that the thiazolidinedione class of insulin sensitizers may be efficacious, but widespread utilisation of these agents awaits further investigation^[9]. Evidence also supports a role for weight loss, achieved through exercise.

HORMONAL AND NON-HORMONAL REGULATORS OF GLUCOSE, LIPID AND ENERGY METABOLISM IN NAFLD

The majority of NAFLD patients are overweight or obese and have underlying insulin, and probably leptin, resistance that results in dysregulated energy metabolism. The regulation of glucose and lipid metabolism involves a complex interplay between adipose tissue, skeletal muscle and the liver. While our knowledge of the pathogenesis of hepatic steatosis has undoubtedly increased over the last decade, many uncertainties remain, and it remains the subject of intense investigation. Hepatic steatosis derives from several possible sources including: (1) increased free fatty acid (FFA) delivery to the liver as a result of dietary fat intake and increased lipolysis within insulin-resistant adipose tissue; (2) increased hepatic *de novo* lipogenesis (DNL); (3) decreased FFA oxidation; and (4) decreased triacylglycerol export from the liver in the form of very low-density lipoprotein. The largest contributor to hepatic steatosis in patients with NAFLD is increased FFA influx to the liver (60%), followed by DNL (26%)^[10].

Increased hepatic lipid supply

In insulin-resistant states, principally obesity and T2D, adipose tissue hormone-sensitive lipase activity is not fully suppressed by insulin, resulting in enhanced lipolysis and non-esterified fatty acid flux into the systemic circulation (Figure 1). The precise mechanism of adipocyte insulin resistance in obesity remains a subject of controversy, but an emerging body of data suggest that an altered adipocytokine milieu in visceral fat resulting from macrophage infiltration is important^[11]. This milieu is characterised by increased expression of pro-inflammatory cytokines, such as tumour necrosis factor- α and interleukin-6 (IL-6), that can directly inhibit insulin, signalling, and decreased expression of adiponectin, an anti-steatosis and insulin-sensitising adipocyte-derived cytokine (adipocytokine) in both skeletal muscle and liver^[12].

Hepatic lipid synthesis and oxidation

Energy metabolism within the liver is tightly regulated.

Two transcription factors, sterol regulatory element-binding protein (SREBP-1) and carbohydrate response element-binding protein (ChREBP), are intimately involved in hepatic glucose and lipid metabolism, and their activity is increased in animal models of NAFLD^[13,14]. The former is induced by insulin and high-fat diets, and regulates glycolytic and lipogenic gene expression resulting in increased DNL and a concomitant decrease in FFA oxidation resulting from malonyl-CoA-induced inhibition of carnitine palmitoyl transferase-1 reducing mitochondrial FFA uptake^[15]. ChREBP exerts similar effects on glycolytic and lipogenic gene expression and also increases the expression of genes involved in triglyceride synthesis. In contrast to SREBP-1, ChREBP is up-regulated by glucose, which increases its nuclear translocation and its DNA binding/transcriptional activity^[16].

Inhibition of ChREBP in *ob/ob* leptin-deficient mice reduces hepatic steatosis by decreasing lipogenesis and enhancing FFA β -oxidation, with a concomitant decrease in circulating plasma triglycerides and FFA resulting in the restoration of hepatic, skeletal muscle and adipose tissue insulin sensitivity^[14]. This study provides further evidence linking fat accumulation to insulin resistance in both hepatic and non-hepatic tissues, principally skeletal muscle^[17,18].

Insulin resistance

Skeletal muscle is the primary site for glucose disposal *via* insulin-dependent pathways, accounting for about 75% of whole-body insulin-stimulated glucose uptake^[19]. Insulin binding to the insulin receptor on the myocyte plasma membrane results in autophosphorylation of the receptor, allowing insulin receptor substrate (IRS)-1 adaptor protein to bind and undergo tyrosine phosphorylation (Figure 2A). IRS-1-associated phosphatidylinositol 3-kinase (PI3K) activity is increased, which results in downstream activation of protein kinase B, leading to enhanced glucose uptake into the cell, *via* increased glucose transporter (GLUT)-4 translocation from the cytosol to the cell membrane, and up-regulation of glycogen synthesis and glucose oxidation^[18]. In obese individuals, there is reduced insulin-stimulated glucose disposal in skeletal muscle, most probably due to increased intramyocellular lipid content (Figure 2B)^[18]. The increased concentration of FFA (or more probably their esterification product, diacylglycerol (DAG), activates the serine/threonine kinase PKC α , which phosphorylates IRS-1 on critical serine sites, thereby inhibiting its tyrosine phosphorylation and subsequently the activation of PI3K and the resulting glucose uptake, glycogen synthesis and glycolysis. Other cytokine-regulated serine/threonine kinases including I κ B kinase-b (IKKb) and JNK-1 may also be involved since the inhibition of IKKb *via* exercise^[20] or salicylates results in improved insulin sensitivity^[21]. As discussed above, hyperglycaemia resulting from skeletal muscle insulin resistance in obesity leads to increased hepatic fat synthesis, reduced fat oxidation and steatosis *via* activation of ChREBP. Moreover, the accumulation of FFA/DAG in the liver results in hepatic

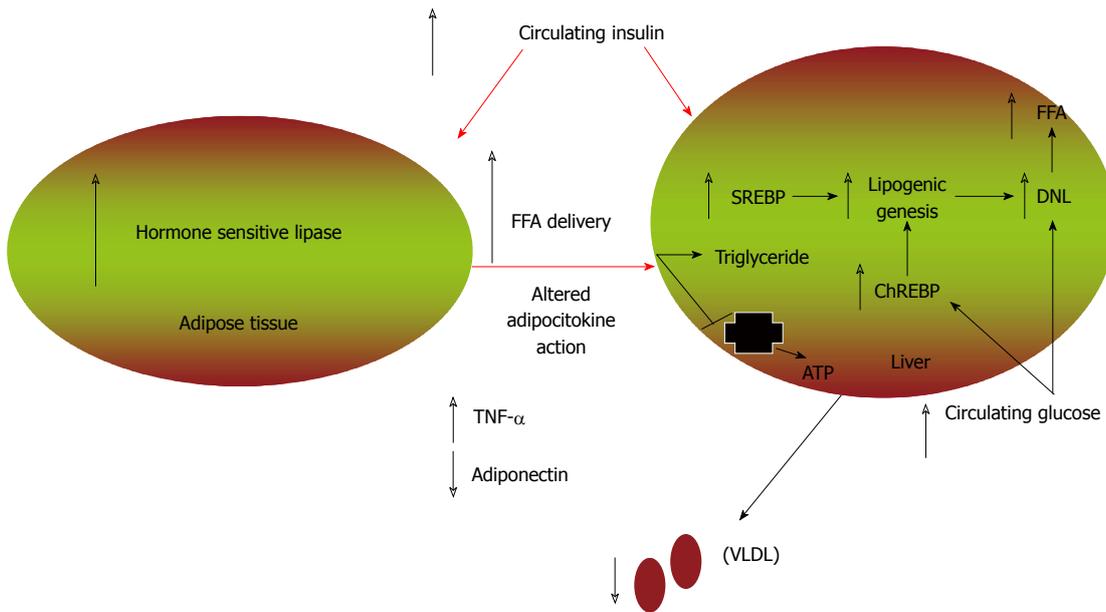


Figure 1 Mechanisms contributing to glucose and lipid dysmetabolism. In the setting of insulin resistance, there is increased adipose tissue hormone-sensitive lipase activity that results in enhanced lipolysis and increased non-esterified fatty acid (NEFA) delivery to the liver. NEFAs are preferentially esterified to triglycerides. Additionally, hyperinsulinaemia leads to increased sterol regulatory element protein (SREBP) expression, resulting in increased *de novo* lipogenesis (DNL) and decreased fatty acid oxidation. Carbohydrate response element-binding protein (ChREBP) is induced by hyperglycaemia and leads to further increases in DNL. Decreased hepatic lipid transport may also occur, in part *via* altered synthesis of apolipoprotein B, leading to decreased very low-density lipoprotein (VLDL) production. TNF- α : Tumour necrosis factor- α ; FFA: Free fatty acid.

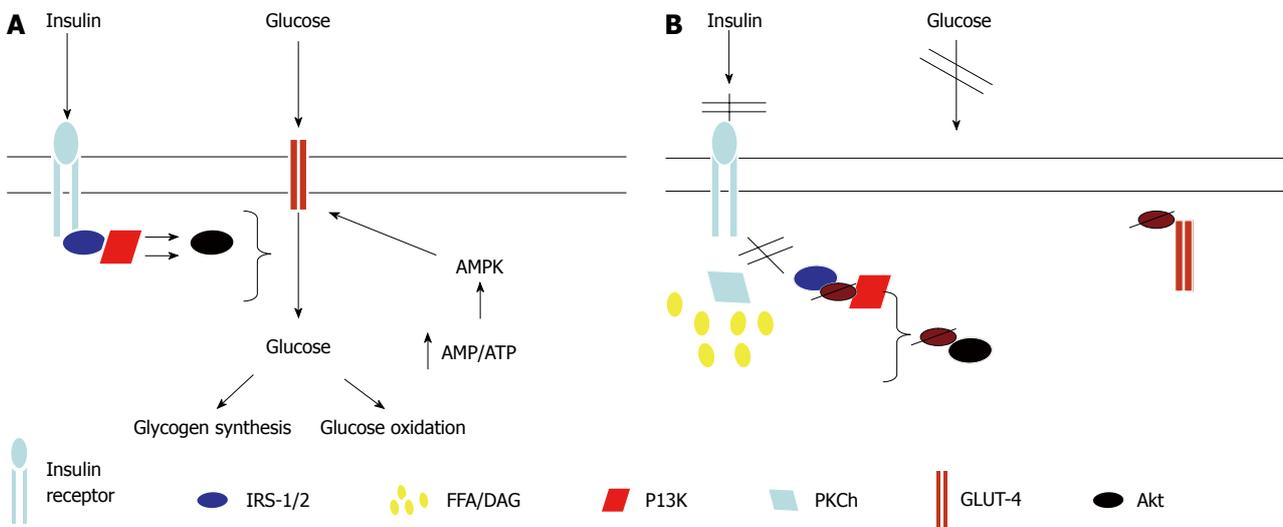


Figure 2 Mechanisms for free fatty acid-induced skeletal muscle insulin resistance. A: Normal glucose uptake into skeletal muscle occurs *via* binding of insulin to the insulin receptor, resulting in receptor autophosphorylation and subsequent binding of tyrosine phosphorylation of insulin receptor substrate (IRS)-1. Subsequently, phosphatidylinositol 3-kinase (PI3K) is activated and results in downstream activation of protein kinase B, leading to glucose transporter (GLUT)-4 translocation to the myocyte plasma membrane and glucose uptake into the cell; B: Increased intramyocellular lipid content leads to the activation of protein kinase Ch (PKCh) which results in serine phosphorylation of IRS-1, thereby inhibiting its tyrosine phosphorylation. This prevents the activation of PI3K and GLUT-4 translocation to the cell surface. Thus glucose entry into the cell is inhibited. AMPK: AMP-activated protein kinase; DAG: Diacylglycerol; FFA: Free fatty acid; Akt: Protein Kinase B.

insulin resistance, *via* PKC ϵ -induced IRS-2 serine phosphorylation, which reduces insulin's inhibitory effect on gluconeogenesis, contributing further to hyperglycaemia^[18,22].

Leptin resistance

Lack of response to the adipocytokine leptin (leptin resistance) rather than insulin resistance may be an im-

portant factor in the pathogenesis of hepatic steatosis. The evidence for leptin resistance in NAFLD, though indirect, is compelling. Leptin exerts a number of anti-steatosis effects on the liver, including inhibition of lipid synthesis *via* reduced expression of stearoyl CoA desaturase (SCD)-1^[23] and enhanced FFA oxidation, *via* up-regulation of peroxisome proliferator-activated receptor- α (PPAR- α)^[24], and yet patients with NAFLD

have increased serum leptin concentrations^[25]. Recent studies in genetically leptin resistant (ZDF) rats have dissociated the direct anti-steatotic effects of leptin from any indirect effects *via* improved hepatic insulin sensitivity^[26]. The mechanism of leptin resistance in obesity is unclear; however, it may be enhanced by the ingestion of fructose which leads to an inhibition of STAT-3, abrogating leptin-mediated PPAR- α activation resulting in decreased fatty acid oxidation, increased SREBP-1 expression and increased hepatic triglyceride content^[27]. Further inhibition of STAT-3 may occur as a result of up-regulation of suppressor of cytokine signalling-3 (SOCS-3) by adipose tissue or hepatocyte-derived inflammatory cytokines^[15,28].

AMP-activated protein kinase

Of particular relevance to modify NAFLD natural history, AMP-activated protein kinase (AMPK) has recently emerged as a key orchestrator of both hormonal and non-hormonal regulators of energy metabolism in liver, skeletal muscle and adipose tissue. Activated by an increase in the AMP/ATP ratio, AMPK activity is enhanced by physiological processes that induce metabolic stress and either decrease ATP production (ischaemia, hypoxia) or increase its consumption (exercise)^[29]. Once activated, AMPK acts to increase ATP-generating cellular events while turning off energy-consuming processes to restore energy balance. In the liver, the activation of AMPK by exercise^[30], starvation, adiponectin^[31], leptin (*via* inhibition of SCD-1), the biguanide metformin^[32] or the thiazolidinedione class of insulin sensitizers^[33] suppresses expression of SREBP-1^[34], ChREBP^[35] and acetyl-CoA carboxylase^[36] resulting in decreased DNL and increased FFA oxidation. Hepatic gluconeogenesis is also inhibited. Within skeletal muscle, exercise, leptin or drug related AMPK activation enhances glucose uptake *via* direct non-insulin-dependent GLUT-4 translocation, and increases pyruvate oxidation^[32]. In adipose tissue, hormone-sensitive lipase activation is suppressed, resulting in decreased lipolysis^[32].

ChREBP, a newly discovered transcription factor, plays an essential role in glucose-induced L-pyruvate kinase (*L-PK*)^[37] gene transcription by binding to the carbohydrate-responsive element of L-PK promoter^[38,39]. It is well known that glucose metabolism is inhibited by fatty acids, which serve as an alternative fuel source and thus conserve glucose. This phenomenon has been termed the fat sparing effect on glucose^[37,40].

Kawaguchi *et al.*^[41] investigated the mechanism by which feeding high fat diets results in decreased activity of ChREBP in the liver. It's strongly suggested that the fatty acid inhibition of glucose-induced L-PK transcription resulted from AMPK phosphorylation of ChREBP at Ser(568), which inactivated the DNA binding activity. AMPK was activated by the increased AMP that was generated by the fatty acid activation.

AMPK is a metabolic master switch mediating adaptation of the cell to variations in nutritional environ-

ment^[42]. Its activity is stimulated by increases in intracellular AMP-to-ATP ratio in response to stresses such as exercise, hypoxia, and glucose deprivation. AMPK has acute effects on energy metabolism pathways and long-term effects involving changes in gene expression.

AMPK is a major therapeutic target for the treatment of diabetes. The effect of a short-term overexpression of AMPK specifically in the liver by adenovirus-mediated transfer of a gene encoding a constitutively active form of AMPK α 2 (AMPK α 2-CA) has been investigated^[43]. The short-term overexpression of AMPK α 2-CA in the liver results in a metabolic switch from glucose to lipid metabolism. The lower plasma glucose concentrations in Ad AMPK α 2-CA-infected mice lead to an increase in hepatic lipid utilization, resulting in a decrease in white adipose mass. The concomitant accumulation of hepatic triglycerides leads to the generation of ketone bodies, which are required as alternative substrates to supply energy to peripheral tissues in conditions of low glucose availability.

Another study investigated the effects of fasting and refeeding on AMPK and ChREBP mRNA, protein and activity levels; as well as the expression of lipogenic genes involved in regulating lipid synthesis in broiler chicken (*Gallus gallus*) liver^[44]. In general, evidence was found for coordinate transcriptional regulation of lipogenic program genes in broiler chicken liver, but specific regulatory roles for AMPK and ChREBP in that process remain to be further characterized.

Moreover, thioredoxin-interacting protein (TXNIP) regulates critical biological processes including inflammation, stress and apoptosis. TXNIP is upregulated by glucose and is a critical mediator of hyperglycemia-induced beta-cell apoptosis in diabetes. In contrast, the saturated long-chain fatty acid palmitate, although toxic to the beta-cell, inhibits TXNIP expression. The mechanisms involved in the opposing effects of glucose and fatty acids on TXNIP expression are unknown. Shaked *et al.*^[45] showed that AMPK is an important regulator of Txnip transcription *via* modulation of ChREBP activity. The divergent effects of glucose and fatty acids on TXNIP expression result in part from their opposing effects on AMPK activity. In light of the important role of TXNIP in beta-cell apoptosis, its inhibition by fatty acids can be regarded as an adaptive/protective response to glucolipotoxicity. The finding that AMPK mediates nutrient regulation of TXNIP may have important implications for the pathophysiology and treatment of diabetes.

EFFECT OF EXERCISE ALONE ON OBESITY, VISCERAL FAT AND INSULIN RESISTANCE

Exercise physiology and the salutatory effects on weight loss, fat reduction and insulin sensitivity have been described in great detail. These beneficial effects are now considered to reflect, at least in part, the effect of exer-

cise on the activation of AMPK. In obese non-diabetics, exercise has been shown to reduce the risk of developing T2D by up to 46%^[33].

Physical training, consisting of 20 min cycling or running, 20 min swimming at submaximal heart rate, followed by 20 min of warm up/cool down three times per week for 4 wk, resulted in a significant reduction in body weight and percentage body fat, and this was associated with improved whole-body glucose uptake, decreased fasting insulin concentrations and increased circulating adiponectin and mRNA expression in muscle. Among patients with T2D, increasing exercise led to a reduction in fasting plasma glucose^[34].

The intensity of exercise needed to show improvement in metabolic profiles has been studied by several investigators. O'Donovan and colleagues evaluated the effects of 24 wk of moderate intensity exercise, defined as cycling three times weekly at 60% $\text{VO}_{2\text{max}}$ to burn 400 kcal, *vs* high-intensity exercise, defined as cycling three times weekly at 80% $\text{VO}_{2\text{max}}$ to burn 400 kcal, *versus* no exercise, on insulin sensitivity, triglycerides and glucose concentration^[35]. Training at 60% $\text{VO}_{2\text{max}}$ was as effective as training at 80% $\text{VO}_{2\text{max}}$ when 400 kcal were expended per session, suggesting that moderate exercise, expending 400 kcal per session, three times per week is sufficient to improve insulin sensitivity. The overall energy expenditure achieved per work-out session appears to be more important than the intensity of the exercise. This is supported by two recent studies performed in obese patients^[36,46]. Daily exercise for 12 wk, performed at not greater than 70% $\text{VO}_{2\text{max}}$ (about 80% maximum heart rate) on a treadmill to achieve 700 kcal energy expenditure (about 60 min) resulted in an 8% body weight loss and was associated with significant reductions in abdominal obesity, visceral fat, waist circumference and insulin resistance^[36]. Furthermore, this study showed that exercise without weight loss also reduced both abdominal and visceral fat. Ray *et al*^[46] also demonstrated that daily aerobic exercise for 50-60 min, starting at 60%-65% maximum heart rate and increasing to 80%-85% maximum heart rate (about 70% $\text{VO}_{2\text{max}}$) over 4 wk improved visceral fat content and this correlated with improved glucose metabolism and loss of insulin resistance. These encouraging results were seen with only a 3% weight loss over this time period.

The effects of aerobic *vs* restrictive exercise have also been debated. A Turkish study examined the effects of aerobic exercise, defined as walking briskly for 15 min and exercising on a stationary bicycle for 12-15 min three times per week the first month, exercising 20-30 min four times per week the second month, and 30-45 min five times per week the third month, in a group of 20 obese women compared with a restrictive exercise utilizing a stationary exercise unit in a similar group of women over a 3-mo period^[47]. While improvement in body mass index (BMI), fasting glucose and postprandial glucose were seen in both groups, reduced fat mass (as measured by bioelectric impedance), decreased

low-density lipoprotein and insulin resistance were seen only in the aerobic exercise group. In another study in 39 older obese men, aerobic or restrictive exercise training 3 d per week for 6 mo resulted in similar improvement in whole-body glucose disposal^[48].

Weight loss remains fundamental to the management of NAFLD, but is mistakenly perceived as the primary rationale for promoting physical activity (PA) participation. However, obesity management is not simply a function of weight loss. Outside the context of liver disease, it is well established that exercise enhances insulin sensitivity, reduces progression to T2D, and favorably modifies serum lipids independent of weight loss^[49,50]. When combined with the observation that high fitness and habitual PA are associated with improved functional capacity, quality-of-life measures, well-being, and reduced all-cause mortality^[51], the importance of incorporating PA therapy, beyond assisting weight loss, becomes apparent.

At present, there is an overall paucity of evidence concerning the benefits of PA as treatment for NAFLD, even though PA is certainly useful in NAFLD-associated diseases such as obesity, T2D and cardiovascular disease.

What is available shows a conclusive benefit of PA when coupled with energy restriction when weight loss is achieved, and it is encouraging for an independent benefit in the absence of weight loss. Although weight loss remains fundamental, patients should be counseled on the spectrum of benefits conferred by regular PA. Management should include assessment of cardiorespiratory fitness and PA levels, and the setting of lifestyle goals based on adoption of regular exercise, with a focus on the attainment of sustainable PA habits.

The dose (intensity and volume) of PA required to reduce liver fat remains unclear. Furthermore, from the present evidence, it is difficult to discern the relative importance of structured exercise and fitness *vs* less structured PA.

Although several examples of a hepatic benefit from low-dose PA therapy have been cited^[52-55], in the absence of robust data and knowledge of the long-term sustainability of such outcomes, it would seem reasonable to promote the current public health recommendations for health promotion, disease prevention, and weight management (Table 1). This recommends that individuals accumulate 20-60 min or more of moderate intensity (about 45%-70% of $\text{VO}_{2\text{max}}$) exercise on most days of the week^[51]. If weight loss is the goal, exercise confers a reduction in body weight in an apparent dose-response fashion with exercise volume, even when prescribed without associated restriction of energy intake^[56]. Greater amounts of exercise may be needed for most individuals to induce significant weight loss or prevent weight being regained in the long term. The consensus suggests that little weight loss is achieved with < 150 min of exercise per week, modest (2-3 kg) losses are attainable with > 150 min/wk (with an energy equivalent of 1200-2000 kcal/wk), and moderate weight loss (5-7.5 kg) often results from 225-420 min/wk (1800-3300 kcal) of aerobic

Table 1 Recommendations for physical activity in non-alcoholic fatty liver disease

Patients should be appropriately screened for contraindications prior to initiating exercise testing or therapy
Physical fitness assessment <i>via</i> exercise testing. Physical activity level assessment by subjective (questionnaire/ diary) or objective (e.g., accelerometer) means
Accumulate 20-60 min or more of moderate intensity rhythmic exercise using large muscle groups on at least 5 d/wk
Moderate intensity physical activity between 150 and 250 min/wk for preventing weight gain
Physical activity > 250 min/wk for clinically significant weight loss
Moderate-to-high intensity resistance training 3 d/wk for enhancing insulin sensitivity

activity^[56]. These targets can be achieved using a variety of exercise modalities, with the outcome of cardiorespiratory fitness being a reliable and easily quantifiable endpoint measure of structured aerobic exercise. Although there is currently no longitudinal evidence available concerning its benefit in NAFLD, progressive resistance training may be useful for the management of obesity-related comorbidities, particularly insulin resistance^[56]. The benefits of nonstructured leisure-time PA, including reduced sedentary time, are becoming increasingly recognized and have, in some studies, shown efficacy in improving cardiometabolic risk and promoting weight loss^[56,57]. Clear guidelines for such “lifestyle PA” are lacking, and reliable measurement, particularly of intensity, is more difficult. PA habits and adherence can be estimated by questionnaires, pedometers, and accelerometers (reviews of which can be found elsewhere)^[58], and the latter may further promote adherence to PA^[58].

Hybrid training of voluntary and electrical muscle contractions

“Hybrid exercise” is an exercise method that combines electrically stimulated and volitional contraction. This technique produces resistance against the motion of a volitionally contracting muscle by means of a force generated by an electrically stimulated antagonist^[59-62]. In particular, hybrid exercise resists utilizes electrically stimulated eccentric contractions and concentric volitional contractions with reciprocal limb movements. Both the volitionally activated agonist and the electrically stimulated antagonist contract during joint motion. The result is that both muscles are trained and that a longitudinal compressive load is placed on the bone. This technique requires minimal external stabilization as compared with conventional weight training. Matsuse *et al.*^[59] have shown that such hybrid exercise increased the extension torque of the elbow joint by about 30% and the cross-sectional areas (CSA) of the proximal upper extremity muscles by about 15% over a 12-wk period. In addition, Iwasaki *et al.*^[60] demonstrated that 6 wk of hybrid exercise effectively increased the extension of the knee joint by 19-33%. Takano *et al.*^[61] demonstrated that 12 wk of treatment increased extension torque by 39% and the CSA of quadriceps muscle by 9% in elderly subjects. However, the mechanism by which hybrid exercise achieves these increases in muscular strength and bulk is still unknown.

Muscle atrophy occurs as a consequence of denervation, injury, joint immobilization, bed rest, glucocorticoid treatment, sepsis, cancer and aging^[63]. Unfortunately,

there is no effective treatment for muscle atrophy. The maintenance of muscle mass is controlled by a balance between protein synthesis and protein degradation pathways, which is thought to shift toward protein degradation during atrophy^[63]. Recently, a signaling pathway that increases protein synthesis was shown to promote muscle hypertrophy, thereby overcoming muscle atrophy^[64,65].

The “Hybrid Training” (HYBT) method utilizing combined electrical stimulation and voluntary muscle contraction has been developed as a muscle training method^[66]. It has already been shown that the method is technically sound and clinically effective in healthy young subjects. The HYBT method increases muscle strength and mass and is as effective as the weight machine training (WMT), an effective method for improving muscle strength and hypertrophy in elderly people^[67,68].

However, a critical problem is that the equipment for WMT is large in size and takes space. In addition, unlike the WMT, the HYBT device, which is portable and not large in size, is so easy to handle that it can be placed at the bedside. Therefore, the HYBT may become a safe, effective method of muscle training for elderly people^[66].

Although skeletal muscle regulates glucose metabolism, partly by releasing IL-6, the effects of hybrid training on glucose metabolism remain unclear. Kawaguchi *et al.*^[69] showed the effects of hybrid training on glucose metabolism and serum IL-6 levels in elderly people. This study showed the safety and good adherence of hybrid training for lower extremities in elderly people. Furthermore, hybrid training decreased fasting blood glucose and serum IL-6 levels in elderly people.

Kawaguchi *et al.*^[70] investigated the therapeutic efficacy of hybrid training in patients with NAFLD. Physical inactivity is a risk factor for the development of NAFLD. HYBT of voluntary and electrical muscle contractions improved hepatic steatosis and reduced insulin resistance and serum IL-6 levels in NAFLD patients who are resistant to lifestyle counseling.

Possible role of myokine in patients with NAFLD

Skeletal muscle has recently been identified as an organ that produces and releases cytokines, which have been named “myokines”. Given that skeletal muscle is the largest organ in the human body, our discovery that contracting skeletal muscle secretes proteins sets a novel paradigm: skeletal muscle is an endocrine organ producing and releasing myokines in response to contraction which can influence metabolism in other tissues and organs. With the discovery that exercise provokes an in-

crease in a number of cytokines, a possible link between skeletal muscle contractile activity and immune changes was established.

For most of the last century, researchers sought a link between muscle contraction and humoral changes in the form of an “exercise factor”, which could be released from skeletal muscle during contraction and mediate some of the exercise-induced metabolic changes in other organs such as the liver and adipose tissue. It has been suggested that cytokines or other peptides that are produced, expressed, and released by muscle fibers and exert either paracrine or endocrine effects should be classified as “myokines”^[71]. The nervous, endocrine, and immune systems all contribute to the maintenance of homeostasis. Interestingly, although these individual systems operate independently to a certain degree, each with their own collection of highly specific cells and regulatory factors, they also depend on each other for normal development and function.

It appears that skeletal muscle has the capacity to express several myokines. To date the list includes IL-6, IL-8 and IL-15^[71]. Contractile activity plays a role in regulating the expression of many of these cytokines in skeletal muscle^[71]. The discovery that IL-6 is released from contracting skeletal muscle has generated much interest among the scientific community because this finding is somewhat paradoxical. On one hand, IL-6 is markedly produced and released in the postexercise period when insulin action is enhanced, but on the other hand, IL-6 has been associated with obesity and reduced insulin action. Given the controversy, this review focuses on the metabolic roles of IL-6.

Despite the fact that acute IL-6 treatment may enhance glucose uptake and fat oxidation in skeletal muscle, there are, nonetheless, a number of studies both *in vitro*^[72-75] and in rodents *in vivo*^[76-78] that demonstrate that IL-6 is capable of inducing insulin resistance. It appears that most, if not all, *in vivo* studies seem to suggest that IL-6 induces insulin resistance *via* adverse effects on the liver. Subjecting lean mice to chronically elevated IL-6 for 5 days causes hepatic insulin resistance^[75], while treating either *ob/ob* (leptin-deficient) mice^[77] or liver-inducible kappa kinase transgenic mice that display hepatic insulin resistance^[79] with IL-6 neutralizing antibodies improves hepatic insulin resistance. The IL-6-induced insulin resistance appears due to increased SOCS proteins (SOCS-3) expression^[75], since it is thought that SOCS-3 may directly inhibit the insulin receptor^[80]. However, even the negative effect of SOCS-3 on insulin action has recently been brought into question. Liver specific activator of transcription 3 (STAT3) knockout mice that express low levels of hepatic SOCS-3 protein, paradoxically are unable to suppress hepatic glucose production after intracerebral ventricular insulin infusion^[81]. Moreover, the prevention of IL-6 signaling either by neutralizing antibodies or by genetic deletion of IL-6 markedly reduces insulin-induced phosphorylation of hepatic STAT3^[81]. These results suggest that the local production of IL-6

is important for the phosphorylation of hepatic STAT3 induced by the brain insulin action. In a separate study, liver specific SOCS-3 knockout mice exhibited obesity and systemic insulin resistance with age^[82]. Furthermore, in this recent study, insulin signaling was reduced in skeletal muscle^[82], suggesting that deletion of the *SOCS-3* gene in the liver modulates insulin sensitivity in other organs. Possibly, the most convincing data to suggest that IL-6 may be antiobesogenic is the observation that IL-6 knockout mice develop mature onset obesity and glucose intolerance^[83]; however, even this observation is unclear^[84]. Whether IL-6 has positive effects on obesity and insulin action is clearly unresolved and requires further work. However, IL-6 unquestionably has a poor prognosis for certain inflammatory diseases^[85], and due to the immunoreactive nature of IL-6, it is clear that rhIL-6 treatment may not be a wise therapeutic treatment strategy in human disease. This is most likely due to the previously described trans-signaling of IL-6. The soluble IL-6 receptor controls the transition from the acute to the chronic phase in many proinflammatory diseases such as peritonitis^[86], a transition that can be inhibited by treatment with a soluble gp130 receptor fragment that neutralizes the trans-signaling process^[86]. Therefore, other cytokines that signal through the gp130 receptor, but which do not activate trans-signaling of IL-6, such as ciliary neurotrophic factor, show some therapeutic promise as an antiobesity therapy^[87].

NAFLD and its subsequent complications create a significant health burden, and currently there is no effective treatment strategy. The biochemical mechanisms that underlie NAFLD are unclear at this time, but there is evidence that insulin resistance is a major contributing factor. In addition, circulating concentrations of inflammatory cytokines - myokines (e.g., IL-6) as well as decreased antiinflammatory factors (e.g., adiponectin, IL-10) are not only implicated in the development of insulin resistance and T2D, but are also related to NAFLD. Such inflammatory mechanisms are fundamental in the progression of NAFLD toward higher risk cirrhotic states. Regular exercise can reverse insulin resistance, suppress low-grade systemic inflammation, and attenuate inflammatory markers associated with NAFLD. Thus, exercise has the potential to become an effective treatment and prevention modality for NAFLD and NASH.

CONCLUSION

Our knowledge of the pathological consequences of lack of adequate exercise on adipose tissue, skeletal muscle and the liver is improving, and this will help establish more specific guidelines for the proper exercise regimens that will improve underlying metabolic pathways and ultimately decrease the incidence and severity of NAFLD.

Moderate exercise, preferably a combination of aerobic and restrictive, performed 3-4 times per week, expending about 400 calories each time seems adequate to augment improvement in the metabolic profiles of patients with

NAFLD. PA has long been considered a cornerstone of a healthy lifestyle. Although its protective role in cardiovascular and metabolic diseases is well established^[88], its place and importance in NAFLD still requires scientific support and clarification. In a study, an inverse association was found between cardio-respiratory fitness categories and the prevalence of NAFLD. Whereas fitness and BMI were independent of each other in their associations with the prevalence of NAFLD, the addition of waist circumference to the regression model attenuated the association^[89]. This is in line with the fact that abdominal obesity has been shown to be a major risk factor for NAFLD, of greater importance than BMI^[90,91], and is consistent with previous studies demonstrating that exercise-induced weight loss is associated with a preferential reduction in abdominal fat^[92,93], and that, at any given weight, individuals who exercise more have less visceral fat than those who are sedentary^[94]. The suggested effect of PA on NAFLD may stem from other mechanisms as well. Exercise alone, in the absence of any change in body weight or composition, may enhance insulin sensitivity and glucose homeostasis^[95,96]. PA appears to result in insulin-receptor up-regulation in muscle tissue and hence increased delivery of glucose and insulin to the muscles^[97]. Exercise also has a beneficial effect on FFA metabolism by enhancing whole-body lipid oxidation^[98]. Hepatic triglyceride accumulation was shown to decrease with exercise intervention^[99] and hepatic FFA uptake was lower in trained compared with untrained subjects^[100]. From the perspective of NAFLD patients, weekly or daily performance of walking, swimming, or cycling might seem as simple as jumping of the cliff. Thus, it seems that among NAFLD patients, even small increments in regular PA can improve liver enzymes; encouraging information that can be provided to patients. Time spent sedentary, measured objectively by individually calibrated heart rate monitoring, predicted higher levels of fasting insulin, independent of the amount of time spent at moderate- and vigorous-intensity activity levels. This highlights the importance of reducing sedentary time in order to improve metabolic status, in addition to the benefits associated with a physically active lifestyle^[101].

Environmental factors that discourage PA include an environment that encourages automobile use rather than walking (like lack of sidewalks), and that has few cues to promote activity and numerous cues that discourage activity (television, computers, *etc.*)^[102,103]. It should be emphasized that associations between air pollution and a multitude of health effects are now well established. Given ubiquitous exposure to some level of air pollution, the attributable health burden can be high, particularly for susceptible populations affected by cardio-respiratory problems when walking, running at a slow or leisurely pace, or cycling^[104]. More rigorous, controlled studies, of longer duration and defined histopathological end-points comparing exercise alone and other treatment are needed before better, evidence-based PA modification guidelines can be established, since several questions remain unanswered. Does PA modification work equally

well in men *vs* women? Do younger patients respond better than older patients to PA modification? Is there a diversity of response among various ethnic groups or in patients with fatty liver alone compared with patients with more progressive disease? Finally, are there different lifestyle modification approaches, *i.e.*, diet alone *vs* diet and aerobic exercise, that work better for different patient populations?

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Ileocecal valve dysfunction in small intestinal bacterial overgrowth: A pilot study

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Abstract

AIM: To explore whether patients with a defective ileocecal valve (ICV)/cecal distension reflex have small intestinal bacterial overgrowth.

METHODS: Using a colonoscope, under conscious sedation, the ICV was intubated and the colonoscope was placed within the terminal ileum (TI). A manometry catheter with 4 pressure channels, spaced 1 cm apart, was passed through the biopsy channel of the colonoscope into the TI. The colonoscope was slowly withdrawn from the TI while the manometry catheter was advanced. The catheter was placed across the ICV so

that at least one pressure port was within the TI, ICV and the cecum respectively. Pressures were continuously measured during air insufflation into the cecum, under direct endoscopic visualization, in 19 volunteers. Air was insufflated to a maximum of 40 mmHg to prevent barotrauma. All subjects underwent lactulose breath testing one month after the colonoscopy. The results of the breath tests were compared with the results of the pressures within the ICV during air insufflation.

RESULTS: Nineteen subjects underwent colonoscopy with measurements of the ICV pressures after intubation of the ICV with a colonoscope. Initial baseline readings showed no statistical difference in the pressures of the TI and ICV, between subjects with positive lactulose breath tests and normal lactulose breath tests. The average peak ICV pressure during air insufflation into the cecum in subjects with normal lactulose breath tests was significantly higher than cecal pressures during air insufflation (49.33 ± 7.99 mmHg *vs* 16.40 ± 2.14 mmHg, $P = 0.0011$). The average percentage difference of the area under the pressure curve of the ICV from the cecum during air insufflations in subjects with normal lactulose breath tests was significantly higher ($280.72\% \pm 43.29\%$ *vs* $100\% \pm 0\%$, $P = 0.0006$). The average peak ICV pressure during air insufflation into the cecum in subjects with positive lactulose breath tests was not significantly different than cecal pressures during air insufflation 21.23 ± 3.52 mmHg *vs* 16.10 ± 3.39 mmHg. The average percentage difference of the area under the pressure curve of the ICV from the cecum during air insufflation was not significantly different $101.08\% \pm 7.96\%$ *vs* $100\% \pm 0\%$. The total symptom score for subjects with normal lactulose breath tests and subjects with positive lactulose breath tests was not statistically different (13.30 ± 4.09 *vs* 24.14 ± 6.58). The ICV peak pressures during air insufflations were significantly higher in subjects with normal lactulose breath tests than in subjects with positive lactulose breath tests ($P = 0.005$). The aver-

age percent difference of the area under the pressure curve in the ICV from cecum was significantly higher in subjects with normal lactulose breath tests than in subjects with positive lactulose breath tests ($P = 0.0012$). Individuals with positive lactulose breath tests demonstrated symptom scores which were significantly higher for the following symptoms: not able to finish normal sized meal, feeling excessively full after meals, loss of appetite and bloating.

CONCLUSION: Compared to normal, subjects with a positive lactulose breath test have a defective ICV cecal distension reflex. These subjects also more commonly have higher symptom scores.

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Key words: Ileocecal valve; Ileocecal sphincter; Cecum; Reflex; Lactulose breath test; Small bowel bacterial overgrowth

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INTRODUCTION

The literature regarding the ileocecal valve (ICV) and its relationship to small intestinal bacterial overgrowth (SIBO) is limited^[1-3]. Surgical section of the ileocecal ligament in dog, a procedure that suppresses the ileocolonic angle and reduces sphincter competence, increases the amount of cecoileal reflux^[4,5]. Surgical removal of the ICV maximizes reflux ultimately leading to bacterial overgrowth^[6].

Dinning *et al.*^[1] using temporary, side diverting, defunctioning ileostomies, recorded ICV pressures in a configuration close to that surgically produced by Quigley *et al.*^[7] in dogs, hence allowing precise positioning of the manometric assembly across the ICV for prolonged periods. In these patients, a sustained pressure of about 10 mmHg is observed either using a pull-through or sleeve recording over a 4.8 cm distance.

In fasted humans, phasic activity of the ICV, unrelated to motor activity of more oral or aboral zones, is observed for 35% of the recording time^[1-3]. During these waves that occur at about four to eight waves per minute, the basal tone is doubled. On the contrary, ICV tone is reduced in humans and in dogs while prolonged propagated contractions are observed on the distal ileum^[1,8]. In dogs and to a lesser extent in humans, ileal motor events propagate across the ICV into the colon. For instance,

Quigley *et al.*^[8] show that 50% of ileal discrete clustered contractions and 76% of ileal prolonged propagated contractions continued propagating in the proximal canine colon. Colonic distension is followed consistently by contraction of the ICV in dogs and in humans. This enhanced motility of the ICV comprises simultaneously an increase in tone together with larger amplitude ICV phasic pressure waves^[1-3] while the ileal contractions are unaffected^[7].

Using a technique in which radioactive tracer is instilled in the cecum it was found that backward flow from the cecum to the ileum is episodic. In dog, the volume of the refluxate is low accounting for about 7% of the total radioactivity injected to the cecum^[9]. This minimal reflux rate can be explained by the competence of the canine ICV towards reflux. Surgical section of the ileocecal ligament in dog, a procedure that suppresses the ileocolonic angle and reduces sphincter competence^[4,5], increases the amount of cecoileal reflux to 44% of the total radioactivity^[9].

In the current study, we aimed to explore whether patients with a positive lactulose breath test (indicative of SIBO) may have an incompetent ICV leading to reflux of colonic contents into the small intestine^[10]. To test this hypothesis we measured pressures within the ICV during cecal distension and compared these pressures with the results of lactulose breath tests.

MATERIALS AND METHODS

Study design and conduct

This research study was approved by the Temple University Institutional review board on 06/27/2007. This study is registered with clinicaltrials.gov ID: NCT01413945. Written Informed consent was given by all the participants prior to their inclusion in this study. All subjects filled out a symptom questionnaire on a scale of 0 to 5 with 0 being no symptom and 5 being very severe symptoms.

Eligibility requirements

Subjects undergoing screening colonoscopy were included in the study. Subjects who were currently on medications such as prokinetics, antibiotics and anticholinergics were excluded from the study. Subjects with history of gastroparesis, Crohn's disease, ulcerative colitis, diseases causing diarrhea, and long standing uncontrolled diabetes were also excluded from the study.

Assessments and outcome measurements

All subjects were sedated with Midazolam and Fentanyl. Colonic preparation was used in all subjects (polyethylene glycol preparation). A custom make water perfused manometry catheter (Mui Scientific, Mississauga, Canada) with 4 pressure measurement ports spaced one cm apart was passed through the biopsy channel of the colonoscope and placed across the ICV with at least one port in the terminal ileum and one port in the cecum (Figure 1). The simultaneous video endoscopy and pressure readings (30 Hz) were continuously recorded on a Medical

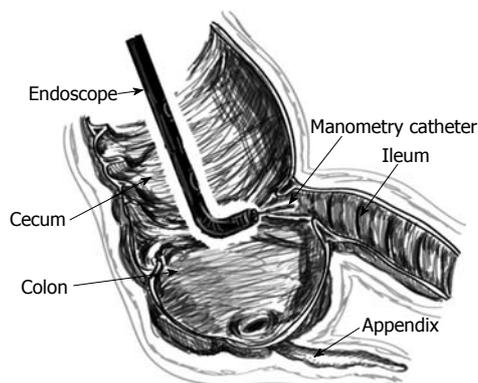


Figure 1 Schematic of the terminal ileum, ileocecal valve and colon. It shows the colonoscope and manometry catheter passing through the biopsy channel of the colonoscope. The 4 channel manometry catheter is placed across the ileocecal valve so that at least 1 channel is in the cecum and 1 channel in the ileum.

Measurement Solar System (MMS, Dover, NH) (Figure 2). Video endoscopy was used to make sure that the catheter remained in the correct position during the entire study. If the catheter position changed during the study, the catheter was repositioned according to markings on the catheter. All the air was removed from the cecum by suction through the biopsy channel of the colonoscope and baseline measurements were taken. After the baseline measurements, air was slowly and continuously instilled into the cecum through the air/water channel of the colonoscope with the air insufflation setting, set at low on endoscopy processor (Evis, Exera II, CLV-180, Olympus America Inc, Center Valley, PA). The peak pressures were measured in all the channels at a time when the pressure in ICV reached its peak between the start of air insufflation and the end of air insufflation. Only studies in which the video playback showed the catheter to be in the correct position were used for analysis. A threshold cecal pressure of 40 mmHg was used to avoid barotrauma. If the pressure exceeded 40 mmHg the air flow into the cecum was stopped. *In vivo* and cadaveric animal experiments have yielded data regarding intraluminal pressure that can lead to rupture of the colon^[11-16]. An adult human cadaveric cecum exposed to less than 40 mmHg of intraluminal pressure generally does not rupture, but cecum exposed to more than 150 mmHg of pressure always ruptures^[11-16]. The pressures needed for perforation of the right colon and cecum are lower than those needed for perforation of the sigmoid and descending colon^[12,13]. It is estimated that the upper limit of safe intraluminal human colonic pressure is 80 mmHg, because perforation can occur at pressures greater than 140 mmHg^[17]. The area under the pressure curve was measured within all the channels, from the time of the beginning of the upslope of the pressures within the cecum, after the start of air insufflation, to the time when the ICV reached its peak pressure, before the end of air insufflation.

All patients returned one month after the colonoscopy for a lactulose breath test using “Breath Tracker Digital Microlyzer”, QuinTron Instrument Company,

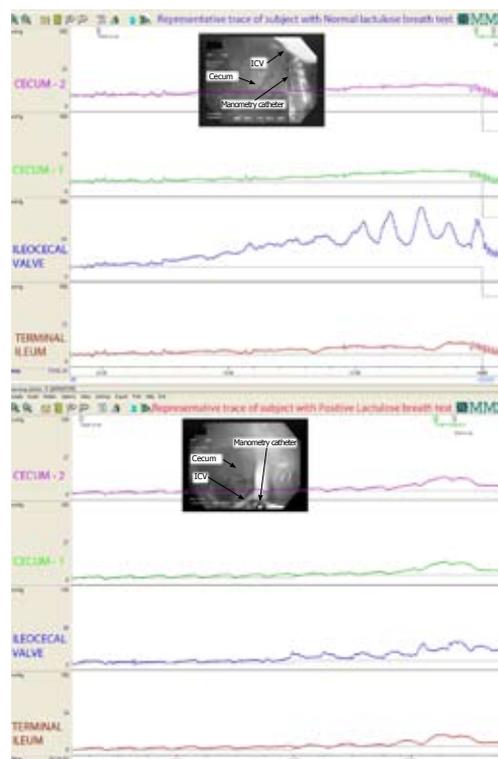


Figure 2 Representative trace of a simultaneous 4 channel manometry and video endoscopy. A: The response of the ileocecal valve during cecal distention by air insufflation in a subject with a normal lactulose breath test. Note the increase in pressure within the ileocecal valve greater than in the cecum and ileum during distension of the cecum; B: The response of the ileocecal valve during cecal distention by air insufflation in a subject with positive lactulose breath test. Note the similar pressures within all the manometry ports (a common cavity effect). ICV: ileocecal valve.

Milwaukee, WI, United States. All subjects presented for the lactulose breath test after an overnight fast. The subjects were given 10 g of lactulose in 120 mL of water to drink and instructed to breathe into a collection bag every 15 min for 3 h after a sample of their breath was collected at baseline. All breath samples were end-expiratory and analyzed immediately. The concentrations of breath hydrogen and methane were measured in parts per million (ppm). The measurements were then graphed and analyzed^[18-21].

Graphs of methane and hydrogen concentration were plotted against time. A positive lactulose breath test was defined as a double peak of hydrogen or a combination of both hydrogen and methane above 20 ppm within the first 2 h.

All subjects were given a gastrointestinal questionnaire which evaluated the following habitual symptoms: nausea, vomiting, stomach fullness, not able to finish normal sized meal, feeling excessively full after meals, loss of appetite, bloating, stomach or belly visibly larger, upper abdominal pain, upper abdominal discomfort, lower abdominal pain, lower abdominal discomfort, diarrhea, gas from above, and gas from below.

Statistical analysis

All pressures were calculated relative to baseline colonic

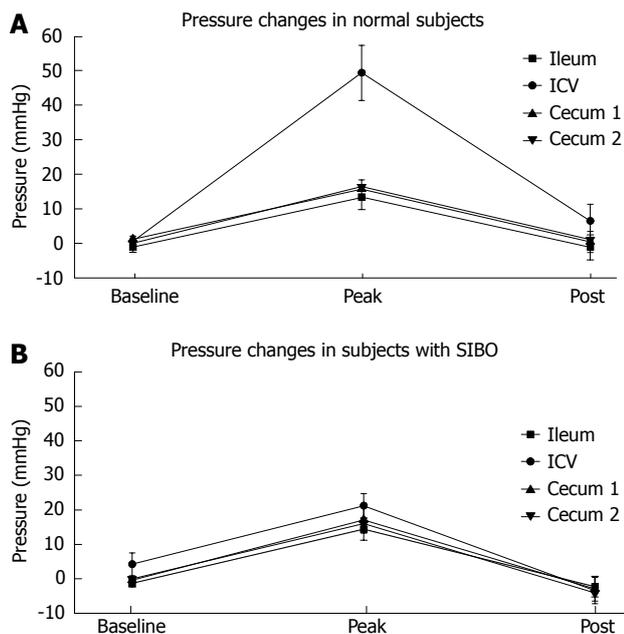


Figure 3 Average pressure changes (baseline, peak and post) in the colon, ileocecal valve and terminal ileum during and after air insufflation. A: The average response of the ileocecal valve during cecal distention by air insufflation in subjects with normal lactulose breath tests; B: The average response of the ileocecal valve during cecal distention by air insufflation in subjects with positive lactulose breath tests. ICV: Ileocecal valve; SIBO: Small intestinal bacterial overgrowth.

pressures. The analysis of pressures was performed using an unpaired Student's *t*-test, with a two tail distribution and equal variance. The questionnaires were evaluated using non-parametric tests (Mann Whitney *U*-test). The analysis was performed in a blinded manner. A Spearman correlation was used to compare ICV pressures to hydrogen and methane excretion.

RESULTS

Nineteen subjects (56.4 years, 7 male and 12 female) underwent colonoscopy with measurements of the ICV pressures after intubation of the ICV with a colonoscope. Cecal pressures before air insufflation were considered as baseline pressures and all the pressures noted were relative to cecal pressures. If the recorded pressures were less than the baseline cecal pressures, than they were considered negative. The resting pressures in the subjects with normal lactulose breath tests were -1.14 ± 1.44 mmHg and 1.18 ± 0.73 mmHg in TI and ICV respectively. The resting pressures in the subjects with positive lactulose breath tests were -1.31 ± 1.07 mmHg and 1.70 ± 2.49 mmHg in TI and ICV respectively. Initial readings showed no statistical difference in the resting pressures of the TI or ICV between subjects with positive lactulose breath tests and normal lactulose breath tests.

Subjects with normal lactulose breath tests

Ten subjects (5 male and 5 female), (age: 55.8 ± 3.05 years, weight: 86.77 ± 7.29 kg). The average dose of Mid-

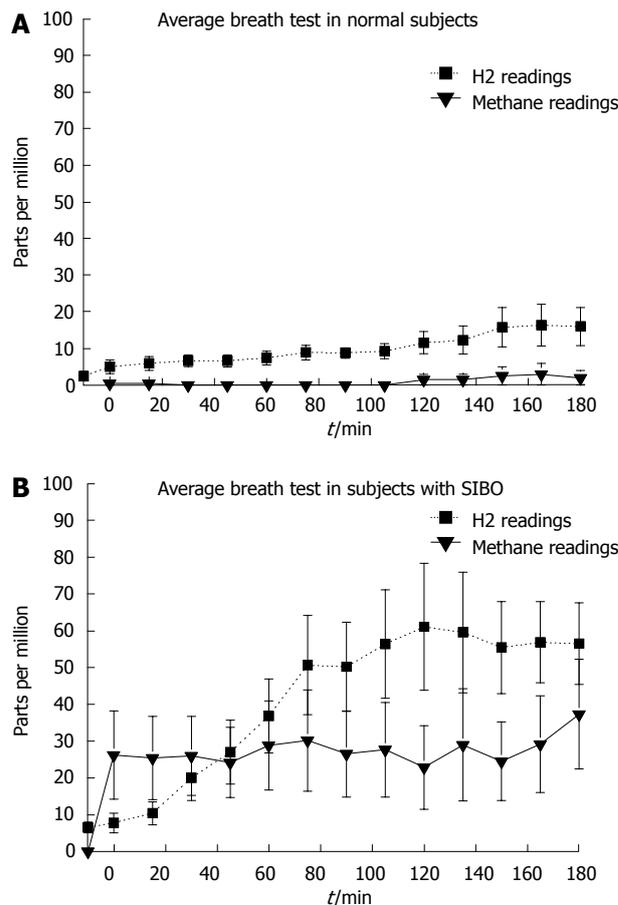


Figure 4 Average lactulose breath test results. A: Normal lactulose breath tests; B: Positive lactulose breath tests. ICV: ileocecal valve; SIBO: Small intestinal bacterial overgrowth.

azolam and Fentanyl per kg was 0.06 mg and 0.73 mg. The average peak ICV pressure during air insufflation into the cecum in subjects with normal lactulose breath tests was significantly higher than cecal pressures during air insufflation (49.33 ± 7.99 mmHg *vs* 16.40 ± 2.14 mmHg, $P = 0.0011$). The average percentage difference of the area under the pressure curve of the ICV from the cecum during air insufflations was significantly higher ($280.72\% \pm 43.29\%$ *vs* 100% ($P = 0.0006$)). The average symptom score for all subjects with normal lactulose breath tests was 13.30 ± 4.09 .

Subjects with positive lactulose breath tests

Nine subjects (2 male and 7 female), (age: 57 ± 4.92 years, weight: 84.35 ± 6.17 kg). The average dose of Midazolam and Fentanyl per kg was 0.08 mg and 0.50 mg. The average peak ICV pressure during air insufflation into the cecum in subjects with positive lactulose breath tests was not significantly different than cecal pressures during air insufflation (21.23 ± 3.52 mmHg *vs* 16.10 ± 3.39 mmHg). The average percentage difference of the area under the pressure curve of the ICV from the cecum during air insufflation was not significantly different $101.08\% \pm 7.96\%$ *vs* 100% . The average total symptom score for all subjects with positive lactulose breath tests

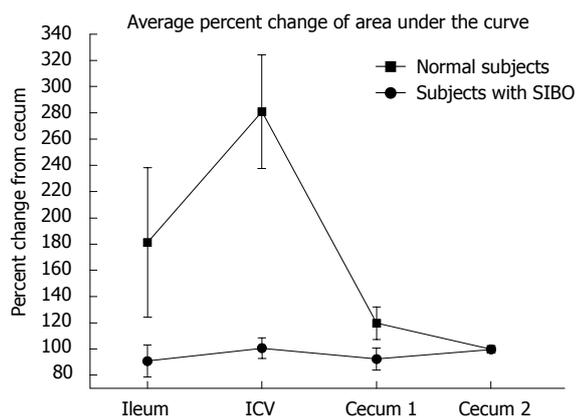


Figure 5 Average percent change of the area under the pressure curve during air insufflation into the cecum, in normal subjects (normal lactulose breath tests) and in subjects with small bowel bacterial over growth (positive lactulose breath tests), with respect to the cecum. ICV: ileocecal valve; SIBO: Small intestinal bacterial overgrowth.

was 24.14 ± 6.58 .

In some prior studies methane seems to influence smooth muscle function^[22]. Analyzing the breath test results, 3 of the subjects were methane producers' and one of these subjects was also positive for hydrogen. Six subjects were hydrogen producers. There was no significant difference in the peak ICV pressures between methane producers and hydrogen producers (23.7 ± 0.67 mmHg *vs* 26.8 ± 2.48 mmHg). There was only a weak correlation between the pressure measurements and the hydrogen excretion ($r = 0.19$) and the pressure measurements and the methane excretion ($r = 0.25$).

Comparison of subjects with normal lactulose breath tests to subjects with positive lactulose breath tests

There is no significant difference in age and weight between subjects with normal lactulose breath test and subjects with positive lactulose breath test. There is no significant difference in the drug dose/kg of Midazolam and Fentanyl between the two groups.

The ICV peak pressures during air insufflations were significantly higher in the subjects with normal lactulose breath tests than in the subjects with positive lactulose breath tests ($P = 0.005$) (Figures 3 and 4). The average percent difference of the area under the pressure curve within the ICV from cecum during air insufflations was significantly higher in subjects with normal lactulose breath tests than in subjects with positive lactulose breath tests ($P = 0.0012$) (Figure 5). The cecal peak pressures during air insufflations were not significantly different in the subjects with normal lactulose breath tests compared to the subjects with positive lactulose breath tests. The total symptom scores were not significantly different in subjects with normal lactulose breath tests than in subjects with positive lactulose breath tests. However, significantly higher symptom scores were observed in the subjects with positive lactulose breath tests in the individual symptoms of: "not able to finish normal sized meal" (P

$= 0.036$), "feeling excessively full after meals" ($P = 0.029$), "loss of appetite" ($P = 0.018$) and "bloating" ($P = 0.0536$).

DISCUSSION

In this study we explored whether patients with positive lactulose breath tests have an incompetent ICV. We tested this hypothesis by measuring ICV pressures during cecal distension in subjects with positive and negative lactulose breath tests.

In designing this study consideration was given to using a sleeve sensor to measure ICV pressures. However, this was rejected in favor of a four port pressure transducer because a sleeve sensor would have measured the peak pressure recorded and would not have been able to distinguish between the terminal ileum, the ICV or the cecum. Consideration was also given to using a barostat to distend the cecum. However, it was decided that a barostat would be very cumbersome to use during routine colonoscopy and would not distend the cecum in the correct position to reproducibly increase ICV pressure. We felt that insufflation of air, into the cecum, was closer to the actual physiology of gas being produced in the colon causing distension of the cecum. We did not use the same volume of gas in each subject. Rather we used a cutoff cecal pressure. We did this for safety purposes since we did not want to go above a certain threshold pressure and risk barotrauma to the subjects. A cutoff pressure of 40 mmHg was used during cecal distension. Finally consideration was given to collection of fluid from the small intestine to assay for SIBO. However, the colonoscopies were performed after a bowel preparation, which flushes bacteria out of the small intestine and this would have resulted in false negative results. In addition the colonoscope needs to pass through the colon before it can enter the small intestine and this could have contaminated the colonoscope with bacteria and could have resulted in false positive results for SIBO.

Yu *et al*^[23] have recently published an important study in which they combined oro-cecal scintigraphy and lactulose hydrogen breath testing demonstrating that breath testing may detect oro-cecal transit, not small intestinal bacterial overgrowth in patients with irritable bowel syndrome (IBS). Thus the lactulose hydrogen breath test may measure small intestinal transit rather than SIBO in IBS-patients. However, the patient population in that study was different than in the current study and the lactulose breath test is still used around the world to diagnose SIBO. Since the results of the lactulose breath test are dependent on motility within the stomach, the small intestine and the colon, we attempted to control for variability in motility between subjects by eliminating patients with known motility disorders, such as gastroparesis and small bowel motility disorders. We eliminated subjects who were taking any medications that would affect motility, such as prokinetics, anticholinergics and narcotics. The dosages of medications given during the colonoscopies were not significantly different between

the two groups, so as not to influence motility during the colonoscopy.

We found that the baseline pressure measurements showed no difference in the pressures of the TI, and ICV between the two groups. These findings are similar to the findings of Quigley *et al*²⁴, “because ileocecal muscle behaves as a sphincter, we were surprised by the absence of clear tonic pressures across the ICV”. However, when tested for the ICV cecal distension reflex using air insufflation into the cecum, we recorded an increase in the pressure within the ICV in subjects with normal lactulose breath tests which were significantly higher than in subjects with positive lactulose breath test. Subjects, with a positive lactulose breath test commonly had a common cavity effect (equalization of the pressures across all of the compartments) during cecal air insufflation.

When the lactulose breath test is evaluated against the gold standard of jejunal aspiration with bacterial culturing for the diagnosis of SIBO the sensitivity and specificity of the breath test is much lower. We suggest a number of potential explanations for these results. First, perhaps not all SIBO is due to an incompetent ICV. For example, in cases of scleroderma in which there are diverticulum in the small intestine it has been suggested that SIBO is due to bacteria growing within the diverticulum. Second and probably more important is the fact that these cultures were taken from the jejunum which is a great distance from the ICV and TI. Reflux of colonic contents would not be expected to wash backwards beyond the ileum due to the propulsive motion of peristalsis within the small intestine, which is presumably normal in these subjects. Normal peristalsis should keep the jejunal bacterial count relatively low even when refluxing colonic contents into the ileum¹⁴. Although we did not design this study to determine if subjects undergoing screening colonoscopy with a positive lactulose breath test have gastrointestinal (GI) symptoms, we did find that many of the subjects in our study had GI symptoms. While these symptoms were more common in the group with positive lactulose breath tests, they were not uncommon in the group with negative lactulose breath tests and we found that there was no significant difference in the overall symptom scores between the two groups. On the other hand, significantly higher symptom scores were demonstrated in the subjects with positive lactulose breath tests for the individual symptoms of, “not able to finish a normal sized meal”, “feeling excessively full after meals”, “loss of appetite” and “bloating”.

The Initial studies evaluating the treatment of IBS patients with poorly absorbed antibiotics, were based on the hypothesis that a significant proportion of these patients actually had occult SIBO²⁵. A number of studies have suggested that there are beneficial effects when poorly absorbed antibiotics are used to treat patients with IBS^{26,27}. Initial studies reported the presence of SIBO in up to 80% of IBS patients, on the basis of a rapid rise in breath hydrogen during lactulose breath testing. In a recent issue of the *New England Journal of*

Medicine, Pimentel *et al*²⁸ reported the results of two large, double-blind, placebo-controlled trials of rifaximin, in patients with IBS without constipation. They found that patients in the group treated with rifaximin had adequate relief of IBS symptoms or bloating. Similarly significant results were obtained in an analysis of relief of symptoms during the 10-wk period after the end of the double-blind treatment phase. Harder *et al*²⁹ showed that gas is less well tolerated in the small intestine than in the large bowel. Based on these studies the most likely mode of action of rifaximin is a reduction in overall small bowel bacterial load or a decrease in colonic flora. This may lead to decreased bacterial fermentation and less bloating, possibly in combination with decreased secretion of bacterial products or host responses to bacterial products that contribute to the generation of symptoms. In the future, we plan to perform a study on subjects with documented irritable bowel syndrome, by the Rome III criteria. In order to determine if a subgroup of patients with IBS symptoms is due to a defective ileocecal valve/cecal distension reflex with reflux of fecal flora or gas into the small intestine.

The results of our study suggest that some GI symptoms may be due to a defective ICV cecal distension reflex which either allows gas from the colon to distend the small intestine or lead to reflux of colonic contents into the small intestine, the colonization of the small intestine with fecal flora and the development of SIBO.

We hypothesize that even though a course of non-absorbable antibiotics may clear the small intestine or the colon of colonic flora, the symptomatic effect may not be durable because the underlying defect, the defective ICV cecal distension reflex, is still present and colonic flora or colonic gas may eventually reflux back into the small intestine. The results of the current study suggest that a restoration of the demonstrated dysfunction (pharmacological, endoscopic or surgical) by preventing coloileal reflux may have a more durable effect than antibiotic therapy. Indeed, prevention of coloileal reflux by constructing an artificial ileocolonic valve was suggested by Kellogg in 1913. Bakkevold³⁰ demonstrated that the nipple valve anastomosis may prevent recurrence of Crohn's disease, after ileocolic resection.

Pimentel *et al*²⁸ suggested that some IBS like symptoms, afflicting many millions of patients, might be due to SIBO and might respond to antibiotic treatment usually used to treat SIBO. In summary, the current study demonstrates that subjects with a positive lactulose breath test have a defective ICV cecal distension reflex. In addition it demonstrates that subjects with a defective ICV cecal distension reflex more commonly have certain GI symptoms. We propose that this pathophysiologic mechanism (defective ICV/cecal distension reflex) which may cause SIBO and IBS like symptoms due to reflux of fecal flora and the production of gas within the small intestine and/or the production of gas within the colon, with reflux of the gas into the small intestine.

ACKNOWLEDGEMENTS

We would like to thank Dilek Yazar for her contribution for this project.

COMMENTS

Background

Small intestinal bacterial overgrowth (SIBO) refers to a condition in which abnormally large numbers of bacteria are present in the small intestine and the types of bacteria in the small intestine resemble the bacteria of the colon. Irritable bowel syndrome (IBS) is a disorder that leads to abdominal pain and cramping, changes in bowel movements, and other symptoms. SIBO and some forms of IBS may be due to the distension of the small intestine with gas produced by bacteria within the small intestine and/or colon. Recent studies strongly suggest that some IBS like symptoms, afflicting many millions of patients, might be due to small intestinal bacterial overgrowth SIBO.

Research frontiers

A percentage of patients with IBS like symptoms have relief of their symptoms when they are treated with non-absorbable antibiotics which suppress the intestinal microbiota. It is thought that suppression of the microflora, within the colon or the small intestine is the mechanism of this therapy.

Innovations and breakthroughs

The initial studies evaluating the treatment of IBS patients with poorly absorbed antibiotics, were based on the hypothesis that a significant proportion of these patients actually had occult SIBO. A number of studies have suggested that there are beneficial effects when poorly absorbed antibiotics are used to treat patients with IBS. Initial studies reported the presence of SIBO in up to 80% of IBS patients, on the basis of a rapid rise in breath hydrogen during lactulose breath testing. The current study is the first study to evaluate the role of ileocecal valve (ICV)/cecal distension reflex in patients suffering with small bowel bacterial overgrowth.

Applications

These findings imply that repair of the ICV (pharmacologic, endoscopic or surgical) or restoration of the ICV/cecal distension reflex may be used to treat patients with SIBO and in patients with IBS like symptoms in the future.

Terminology

The ileocecal valve is a sphincter muscle situated at the junction of the ileum (last portion of your small intestine) and the colon (first portion of your large intestine). Its function is to allow digested food materials to pass from the small intestine into your large intestine. The ileocecal valve also blocks these waste materials from backing back up into your small intestine. It is intended to be a one-way valve, only opening up to allow processed foods to pass through.

Peer review

The authors examined the ICV/cecal distension reflex in patients with positive lactulose breath test and in subjects with negative lactulose breath test. The authors found that subjects with a positive lactulose breath test have a defective ICV/cecal distension reflex. This defective ICV/cecal distension reflex may cause SIBO and IBS like symptoms due to reflux of fecal flora and the production of gas within the small intestine and/or the production of gas within the colon, with reflux of the gas into the small intestine.

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Schizandra arisanensis extract attenuates cytokine-mediated cytotoxicity in insulin-secreting cells

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Abstract

AIM: To explore the bioactivity of an ethanolic extract of *Schizandra arisanensis* (SA-Et) and isolated constituents against interleukin-1 β and interferon- γ -mediated β cell death and abolition of insulin secretion.

METHODS: By employing BRIN-BD11 cells, the effects of SA-Et administration on cytokine-mediated cell death and abolition of insulin secretion were evaluated by a viability assay, cell cycle analysis, and insulin assay. The associated gene and protein expressions were also measured. In addition, the bioactivities of several peak compounds collected from the SA-Et were tested against cytokine-mediated β cell death.

RESULTS: Our results revealed that SA-Et dose-de-

pendently ameliorated cytokine-mediated β cell death and apoptosis. Instead of suppressing inducible nitric oxide synthase/nitric oxide cascade or p38MAPK activity, suppression of stress-activated protein kinase/c-Jun NH2-terminal kinase activity appeared to be the target for SA-Et against the cytokine mix. In addition, SA-Et provided some insulinotropic effects which re-activated the abolished insulin exocytosis in cytokine-treated BRIN-BD11 cells. Finally, schiariisanrin A and B isolated from the SA-Et showed a dose-dependent protective effect against cytokine-mediated β cell death.

CONCLUSION: This is the first report on SA-Et ameliorating cytokine-mediated β cell death and dysfunction *via* anti-apoptotic and insulinotropic actions.

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Key words: *Schizandra arisanensis*; C₁₉ homolignans; Type 1 diabetes; Insulin-secreting cells; Interleukin-1 β ; Interferon- γ

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INTRODUCTION

The onset of type 1 diabetes occurs when approximately 60%-90% of insulin secreting cells are lost and/or are dysfunctional due to β -cell directed autoimmunity^[1].

During the progression of autoimmunity, heavy infiltrations of mononuclear cells lead to substantial damage to β cells by generating locally high concentrations of pro-inflammatory cytokines, perforin, and FasL-Fas interactions within the micro-environment of islets^[2,3]. As a result, deleterious outcomes include excessive proinsulin secretion, the abolition of glucose-induced insulin secretion, and ultimately β cell death^[4].

Currently, scientists have developed a wide range of approaches to prevent or intervene in type 1 diabetes by stimulating β -cell proliferation, attenuating the cytotoxic effects of inflammatory agents, or modulating autoimmune response, and so on^[1]. While many clinical trials are ongoing, the application of herbal medicine remains to be fully explored.

In cell culture systems, exposing β cells to an interleukin (IL)-1 β and interferon (IFN)- γ mixture can mimic the consequences of an immune attack in type 1 diabetes, which affects pro-insulin secretion^[5] and induces β cell death^[6]. By employing such a platform, potential β cell protective herbal material can be identified. *Schizandra arisanensis* (SA-Et) is one of the schizandraceous plants from Taiwan. This plant also contains various C₁₈ dibenzocyclooctadiene lignans and C₁₉ homolignans^[7,8]. The indications for this herb in traditional Chinese medicine include diabetes, hepatitis, immunomodulation, and cancer^[7,9-11]. Therefore, in the present study, we investigated the potential β cell protective bioactivity of the ethanol extract of SA-Et, including its isolated constituents against cytokine-mediated β cell cytotoxicity and dysfunction.

MATERIALS AND METHODS

Plant material and reagents

Stems of SA-Et were collected and authenticated by Dr. Kuo YH in October 2005 in Chiayi County, Taiwan. A voucher specimen (No. NRICM20051003) was deposited at the National Research Institute of Chinese Medicine. The following were used as positive controls in the various biological assays performed; recombinant cytokines (IL-1 β and IFN- γ ; PeproTech, NJ, United States); epigallocatechin gallate (EGCG; Fluka, MO, United States); nitro-L-arginine methyl ester (L-NAME), glucose, KCl and CaCl₂ (Sigma-Aldrich Corp., MO, United States). These reagents were obtained at the highest purity available (> 97%) from the suppliers indicated.

Preparation of the ethanol extract of SA-Et

Dried stems of SA-Et were ground and then extracted with 95% (v/v) EtOH at 45 °C three times for 48 h each time. Under reduced pressure, the combined ethanol extracts were concentrated to an ethanolic extract residue of SA-Et. DMSO was employed as the dissolving reagent, and aliquots were prepared and stored at -20 °C before the biological assays were carried out.

High-performance liquid chromatographic analysis of the SA-Et

A filtered volume (100 μ L) of the SA-Et solution (1.0

mg/mL in methanol) was prepared and injected into a reverse-phase high-performance liquid chromatography (HPLC) system. The HPLC analysis was performed using a WatersTM HPLC (cont 600 Pump, 996 photodiode array detector, 600 controller, and 717 plus autosampler; Milford, Massachusetts, United States), using a reverse-phase RP-18 column (4.6 mm \times 250 mm *i.d.*). The solvent system used was a gradient of water and CH₃CN. The gradient system was as follows: 0-60 min, 45%-60% CH₃CN, and 60-70 min, 60%-75% CH₃CN; with elution performed at a solvent flow rate of 1 mL/min. The detection chromatogram was recorded at 215 nm.

Isolation of C₁₉ homolignan and C₁₈ lignan compounds

Peak compounds including schiarianrin A (peak 5), schiarianrin B (peak 1), schiarianrin C (peak 4), schiarianrin E (peak 3), and macelignan (peak 6) were obtained from the SA-Et followed by the procedures described below. Isolation of taiwanschirin A (peak 2) was based on a previous method^[12].

Five grams of SA-Et was partitioned with water and *n*-hexane, to give the *n*-hexane residue (SA-Et-H). Further separations of SA-Et-H (100 mg) were performed on a WatersTM HPLC, using a preparative Cosmosil 5C₁₈ AR-II column (250 mm \times 10 mm *i.d.*; Nacalai Tesque, Kyoto, Japan), employing a gradient system [water (A) and CH₃CN (B), mobile phase was as follows: 0-2 min, 45%-50% (B); 2-20 min, 50%-55% (B), 20-55 min, 55%-60% (B), 55-60 min, 60%-70% (B), and 60-70 min, 70%-100% (B)], at a flow rate of 3 mL/min, under 215 nm UV, to yield schiarianrin A (4.6 mg), schiarianrin B (4.1 mg), schiarianrin C (5.6 mg), schiarianrin E (6.3 mg), macelignan (11.5 mg), and schizarin A (3.6 mg), which were identified by comparison with authentic samples and reported spectroscopic data (UR, infrared, and nuclear magnetic resonance) in the literature^[8,12]. Their purity was over 75% as analyzed by HPLC.

Cytotoxicity test

BRIN-BD11 cells were routinely cultured in RPMI-1640 containing 10% (v/v) fetal bovine serum and 2 g/L glucose^[13]. For the cytotoxicity test, BRIN-BD11 cells were seeded into 24-well plates at the density of 5×10^4 cells/well. After overnight attachment, cells were incubated with cytokines according to the figure legends. The viability at the end of treatment was measured by a neutral red assay as described previously^[14]. The absorbance under control conditions was set to 100% viability.

Cell cycle analysis

At the end of treatment, cells were trypsinized and collected by centrifugation. Following a phosphate-buffered saline (PBS) wash, cell pellets were resuspended in ethanol overnight at -20 °C. Fixed cells were then washed with PBS before being resuspended in 1% (v/v) Triton X-100 and incubated for 15 min. The cells were then resuspended in propidium iodide (PI) staining solution, consisting of 20 μ g/mL PI, 50 μ g/mL RNase A, and 0.0001% (v/v) Triton X-100 in PBS, and incubated at 4 °C for 30 min

Table 1 Sequence of primer sets for conventional reverse transcription-polymerase chain reaction

β -actin	
Forward	5'-CGTAAAGACCTCTATGCCAA-3'
Reverse	5'-AGCCATGCCAAATGTGTCAT-3'
Glucokinase	
Forward	5'-AAGGGAACATACATCGTAGGA-3'
Reverse	5'-CATTGGCGGTCTTCATAGTA-3'
Insulin	
Forward	5'-TGCCAGGCTTTGTCAAACAGCACCTT-3'
Reverse	5'-CTCCAGTGCCAAGGCTGAA-3'
iNOS	
Forward	5'-TTTTACGACACCCTTACC-3'
Reverse	5'-GACCTGATGTTGCCACTGTTAG-3'

iNOS: inducible nitric oxide synthase.

with protection from light. Finally, the cells were passed through a mesh prior to cell cycle analysis by employing FACSsan (BD Bioscience, San Jose, California, United States).

Western blot analysis

This procedure followed a previous methodology^[15]. In brief, cells were washed with ice-cold PBS and scraped into ice-cold lysis buffer. Cell debris was removed by centrifugation. Equal amounts of protein (40 μ g) were subjected to separation on sodium dodecylsulfate 10% polyacrylamide gels. Following transfer to nitrocellulose membranes, blots were blocked with 5% (w/v) non-fat milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 for 1 h, and incubated with primary antibodies at 4 °C overnight prior to incubation for 1 h at room temperature with the secondary antibody. Finally, results were visualized after development of films with the aid of an enhanced chemiluminescence kit (Amersham Biosciences, Uppsala, Sweden).

Measurement of gene expression

Total RNA was extracted using the TRI-reagent according to the manufacturer's instructions. Total RNA (1 μ g) was reverse-transcribed to generate templates. Complementary (c) DNA at 50 ng was employed for the polymerase chain reaction (PCR). The sequences of primers are listed in Table 1. The annealing temperatures for amplification of β -actin (57 °C), glucokinase (57 °C), insulin (52 °C), and inducible nitric oxide synthase (iNOS) (55 °C) were employed to generate respective sequences of 349 bp, 130 bp, 187 bp, and 441 bp. Once the reaction was complete, PCR products were separated by gel electrophoresis, visualized, photographed using a digital camera, and quantified with Genetools 3.06 (Syngene, Cambridge, United Kingdom). When iNOS mRNA was determined by real-time reverse transcription-PCR, 1 μ g of total RNA was reverse transcribed into cDNA using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Maryland, United States) before being applied to the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Warrington, United Kingdom). Each reaction was carried out based on the description of the TaqMan®

Gene Expression Assay kit with pre-designed probes for iNOS (Rn00561646_m1) and β -actin (Rn00667869_m1). Data were ct values (i.e., cycle number where logarithmic PCR plots cross a calculated threshold line). For comparative quantification, Δ ct values were firstly calculated using the formula: Δ ct = (ct of iNOS) - (ct of β -actin). Then the $\Delta\Delta$ ct value was determined using the following formula: $\Delta\Delta$ ct = (Δ ct of each sample) - (average Δ ct of control samples). Fold increase in iNOS level under each condition was presented as $2^{-\Delta\Delta$ ct}.

Acute insulin-secretion test

The acute insulin-secretion test was as previously described^[14]. In brief, the culture medium was discarded and the cells were washed twice with 1 mL Krebs-Ringer bicarbonate (KRB) buffer. The cells were then pre-incubated in KRB for 40 min at 37 °C. Pre-incubation buffer was then poured off followed by the addition of 1 mL of test medium per well at a constant rate. After 20 min, the test medium was collected at a constant rate and stored at -20 °C prior to insulin determination by a rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Linco Research, St. Charles, Missouri, United States). The limit of sensitivity of the assay was 0.2 ng/mL (35 pmol/L) insulin using a 10 μ L sample. The appropriate range of the insulin assay was 0.2 ng/mL to 10 ng/mL insulin (10 μ L sample size). Samples with results greater than this range were diluted with sample buffer in order to fit the range of the standard curve.

Determination of nitrite production

Test samples at 100 μ L were mixed with 1.32% sulfanilamide (50 μ L) in 60% acetic acid and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride HCl (50 μ L) in distilled water. After 10 min incubation, the absorbance was read on a spectrophotometer at 540 nm. Actual concentrations were calculated from an absorbance vs nitrite (μ mol/L) standard curve.

Statistical analysis

The significance of various treatments was determined by the Student's unpaired *t*-test under non-parametric statistical conditions. Results are expressed as the mean \pm SE. Differences were considered significant at $P < 0.05$.

RESULTS

As shown in Figure 1, HPLC analysis of the ethanolic extract of SA-Et was carried out, and seven major peaks were obtained and identified as schiarisanrin B, taiwanschirin A, schiarisanrin E, schiarisanrin C, schiarisanrin A, macelignan, and schizanthrin A, respectively, by matching them to authentic samples. In addition, the model of cytokine-mediated β cell destruction was established using BRIN-BD11 cells. As shown in Figure 2A, a synergistic effect on cytotoxicity was only observed when IL-1 β was mixed with IFN- γ . Maximum cell death occurred at 48 h when cells were treated with IL-1 β (2 ng/mL) and IFN- γ (100 ng/mL). As shown in Figure 2B, the pres-

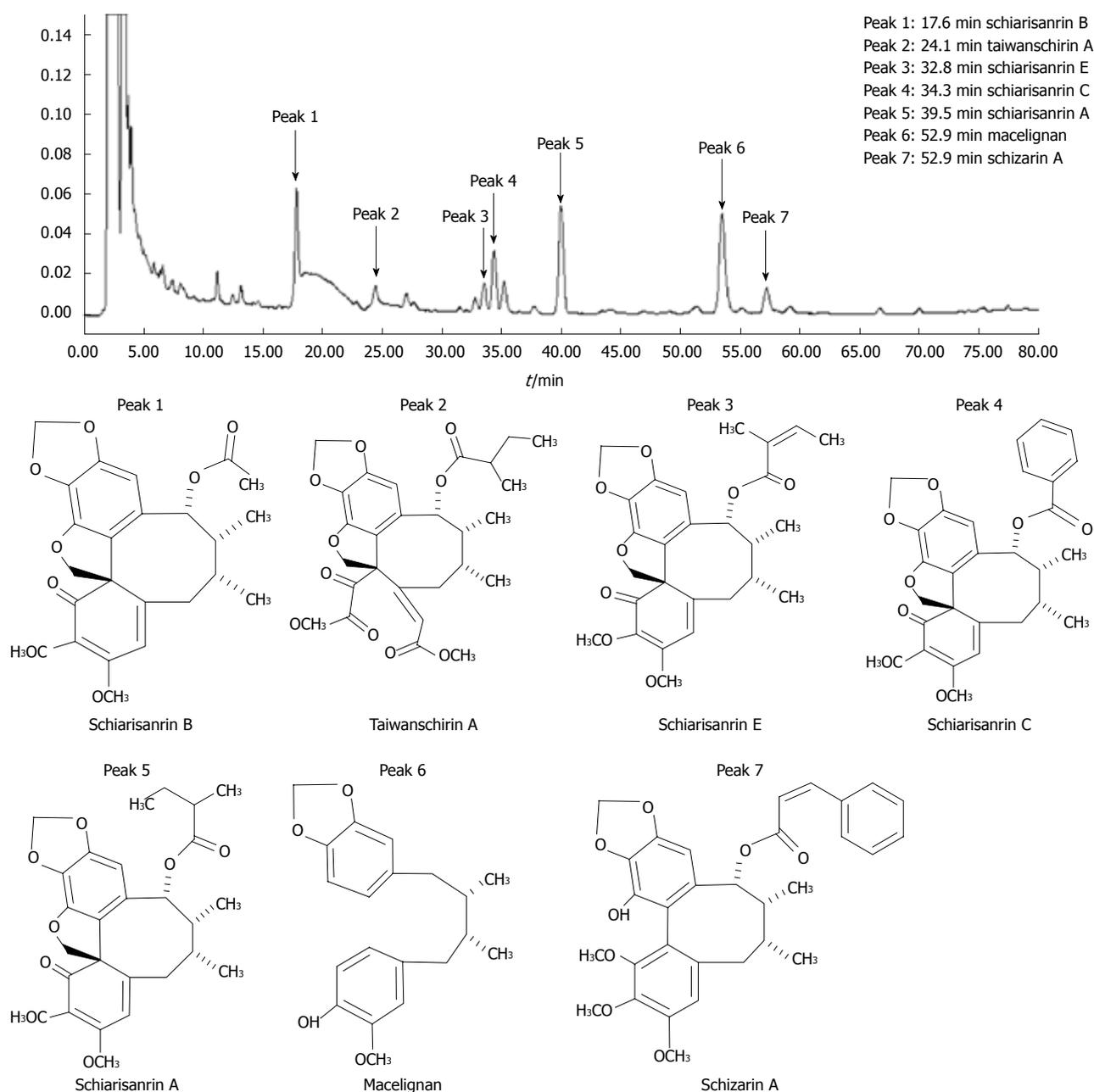


Figure 1 Chemical fingerprinting of the ethanolic extract of *Schizandra arisanensis*. The ethanolic extract of *Schizandra arisanensis* (SA-Et) (1.0 mg) was subjected to a high-performance liquid chromatography system to obtain chemical fingerprints. According to the retention times, the chemical identities and structures of seven major peaks are listed.

ence of IFN- γ alone resulted in significant G₁ arrest ($P < 0.05$). In contrast, the presence of IL-1 β alone caused significant inhibition at S phase. However, when IFN- γ was combined with IL-1 β , the accumulated G₁ arrested cells appeared to have progressed into the subG₁ phase ($P < 0.01$) at the end of cytokine treatment. By affecting the mechanisms of IL-1 β + IFN- γ using various inhibitors, our results showed that IL-1 β + IFN- γ -mediated cytotoxicity could be attenuated by SP600125 and SB203580. On the other hand, the presence of U0126 and L-NAME had no beneficial effects on cell viability. Moreover, the viability of cytokine-treated cells was worse in the presence of wortmannin (Figure 2C).

By employing our cell model, as shown in Figure 3A,

the SA-Et at 20 $\mu\text{g}/\text{mL}$ provided β cell protective activity as shown by an increase of approximately 1.3-fold ($P < 0.01$) in the viability in the cytokine treated condition. Consistently, induction of the subG₁ phase in the presence of IL-1 β + IFN- γ was attenuated by the SA-Et (Figure 3B). Finally, full-length caspase-3 degradation in the presence of IL-1 β + IFN- γ was observed and was parallel to the increase in cleaved caspase-3 (active form) of about 1.2-fold at 24 h post-treatment with IL-1 β + IFN- γ (Figure 3C). However, while SA-Et alone reduced the cleaved form of caspase-3 by 30%, the protease-mediated caspase-3 activation by IL-1 β + IFN- γ was also ameliorated by SA-Et.

Following determination of the protective effect of

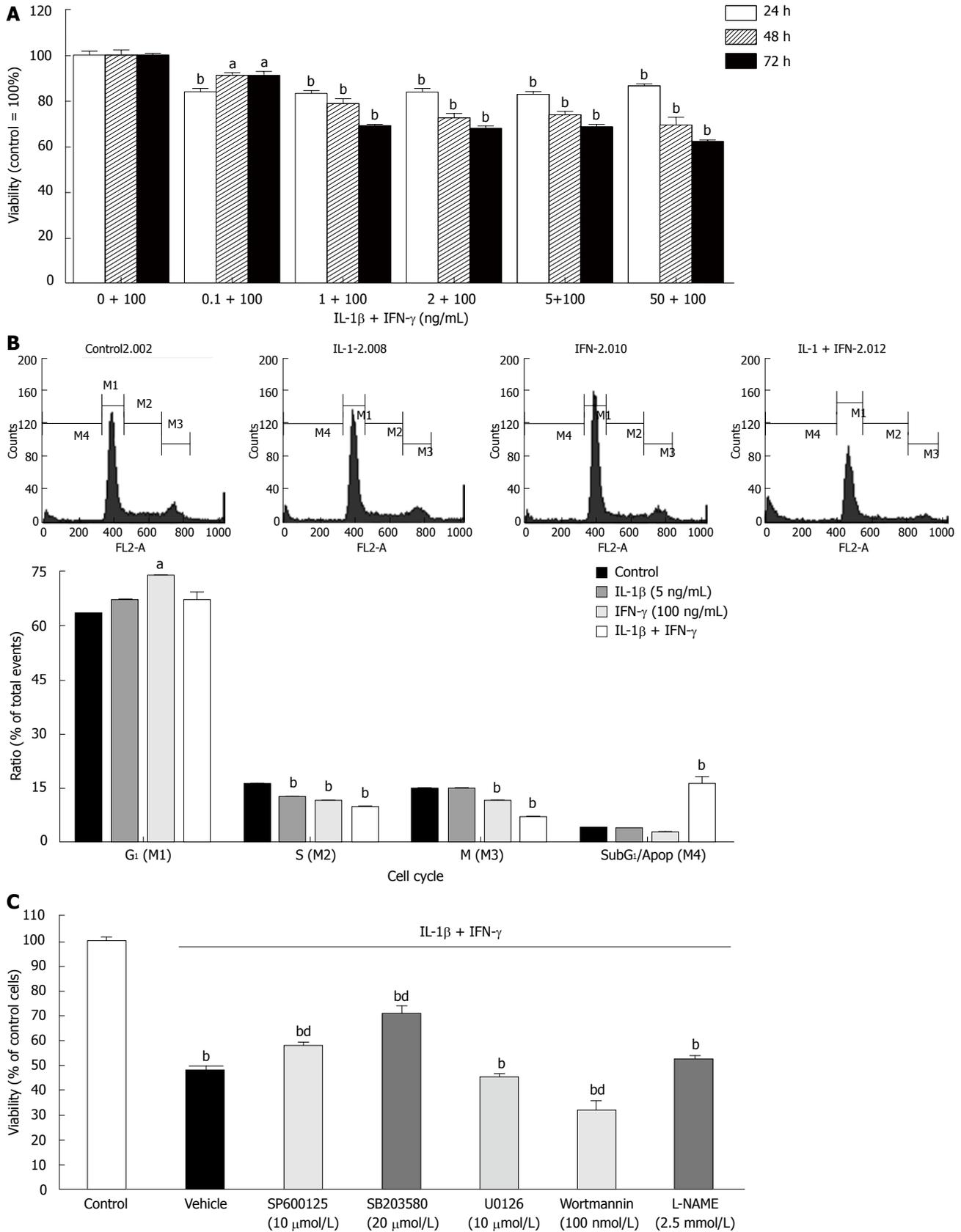


Figure 2 Interleukin-1 β and interferon- γ -mediated BRIN-BD11 cell apoptosis is attenuated by p38MAPK and stress-activated protein kinase/c-Jun NH2-terminal kinase inhibitors. **A:** The viability of interferon (IFN)- γ (100 ng/mL)-treated cells in the presence of various concentrations of interleukin (IL)-1 β for 24 h, 48 h and 72 h was measured. Data are presented as the mean \pm SE, $n = 4$. ^a $P < 0.05$, ^b $P < 0.01$ vs the viability of untreated cells; **B:** Cell cycle analysis was carried out at 48 h post-treatment with IL-1 β (5 ng/mL), IFN- γ (100 ng/mL), or a mixture of the two. Data are presented as the mean \pm SE, $n = 4$. ^a $P < 0.05$, ^b $P < 0.01$ vs the control; **C:** The viability of cytokine-treated BRIN-BD11 cells in the presence of inhibitors was measured. Data are presented as the mean \pm SE, $n = 4$. ^b $P < 0.01$ vs the viability of untreated cells; ^d $P < 0.01$ vs the viability of cells with the cytokine mix.

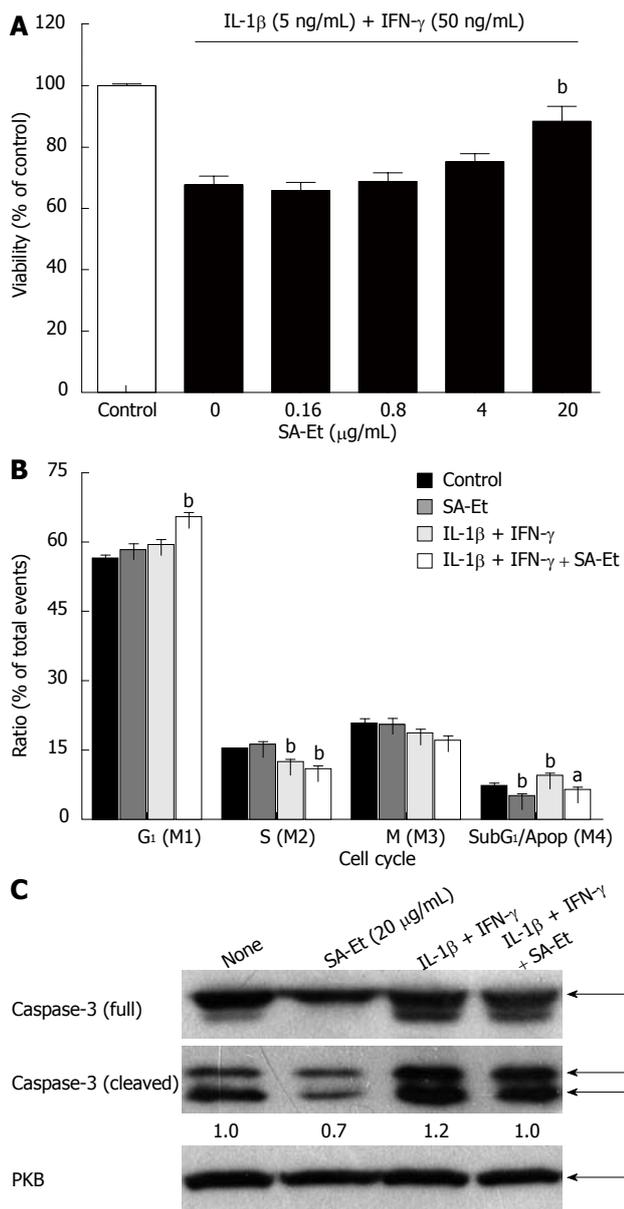


Figure 3 The anti-apoptotic effect of the ethanolic extract of *Schizandra arisanensis*. A: The viability of BRIN BD11 cells was determined after 48 h of cytokine treatment in the presence or absence of the ethanolic extract of *Schizandra arisanensis* (SA-Et); B: Cell cycle of BRIN BD11 cells was determined after 48 h of cytokine treatment in the presence or absence of the SA-Et. Data are presented as the mean \pm SE, $n = 4$. ^a $P < 0.05$, ^b $P < 0.01$ vs the control; C: Both full and cleaved forms of caspase-3 protein were analyzed by Western blot after 24 h of cytokine treatment in the presence or absence of the SA-Et. A representative from three experiments is shown. IL-1 β : Interleukin 1 β ; IFN- γ : Interferon- γ .

SA-Et, we further examined the impact of SA-Et on signal transduction of IL-1 β + IFN- γ . As shown in Figure 4A, stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK), p38MAPK, and STAT-1 α activation in cytokine-treated cells was evident, while ERK1/2 was not activated. In the presence of SA-Et, the phosphorylation of SAPK/JNK (at T183/Y182) and its substrate c-Jun (at S63) was attenuated by this treatment, while other pathways appeared to be unaffected by SA-Et.

In addition, cytokine treatment also promoted I κ B α degradation which was unaffected by the addition of SA-

Et (Figure 4B). Consistently, the presence of SA-Et (20 μ g/mL) was unable to inhibit cytokine-mediated iNOS mRNA through cytokine induction (Figure 4C and D). Interestingly, SA-Et administration up to 20 μ g/mL provided minor, but significant inhibition of cytokine-mediated NO formation. However, such inhibition did not reach the level of the known iNOS inhibitor, L-NAME, which provided > 50% of the inhibition of cytokine-mediated NO production (Figure 4E).

In terms of SA-Et treatment on β -cell function with or without cytokine challenge (Figure 5A), SA-Et treatment enhanced the insulinotropic effects of glucose and extracellular Ca²⁺, and modestly but significantly affected the insulinotropic effect of extracellular K⁺. As shown in Figure 5B, glucose responsiveness of BRIN-BD11 cells was unaffected by SA-Et alone. On the other hand, a detectable amount of secreted insulin under a basal or high concentration of glucose was not detected in surviving BRIN-BD11 cells following cytokine treatment. In contrast, we detected partial secretion of insulin from cytokine-treated BRIN-BD11 cells in the presence of SA-Et ($P < 0.01$).

When determining the insulin level in BRIN-BD11 cells under the indicated conditions, there was a modest reduction in insulin mRNA expression in the presence of SA-Et or the cytokine mix 48 h post-treatment (Figure 5C). In addition, insulin protein expression and the intracellular insulin content of BRIN-BD11 cells were consistent with each other (Figure 5D). Cellular insulin protein level appeared to be similar regardless of the addition of SA-Et. In contrast, cellular insulin contents in cytokine-treated cells were significantly elevated ($P < 0.001$). Interestingly, insulin content in cytokine-treated BRIN-BD11 cells with SA-Et treatment was significantly reduced ($P < 0.05$) compared with that in untreated cells.

Finally, we compared the biological activities of four major peaks as shown in Figure 6. Epigallocatechin gallate (EGCG) was employed as a reference drug which has been shown to protect against cytokine-mediated β cell death^[16]. Our results indicated that both peak 1 (schiarisanrin B) and peak 5 (schiarisanrin A) provided β cell protective bioactivity similar to EGCG (20 μ g/mL). The protective potency of schiarisanrin B reached its maximum at a concentration of 5 μ g/mL, and higher concentrations led to a deleterious outcome. On the other hand, schiarisanrin A provided a dose-dependent protective effect starting at a concentration of 5 μ g/mL ($P < 0.01$). Peak 4 (schiarisanrin C) led to cell death in a dose-dependent manner. Different from C₁₉ homolignans, the C₁₈ lignan, peak 6 (macelignan), played no role in cytokine-mediated cytotoxicity at any concentration.

DISCUSSION

Conventional therapy in patients with type 1 diabetes does not allow minute-to-minute control of blood glucose and does not prevent complications associated with the disease. Whole pancreas or islet transplantation facilitates glucose control on a real-time basis but the lack of

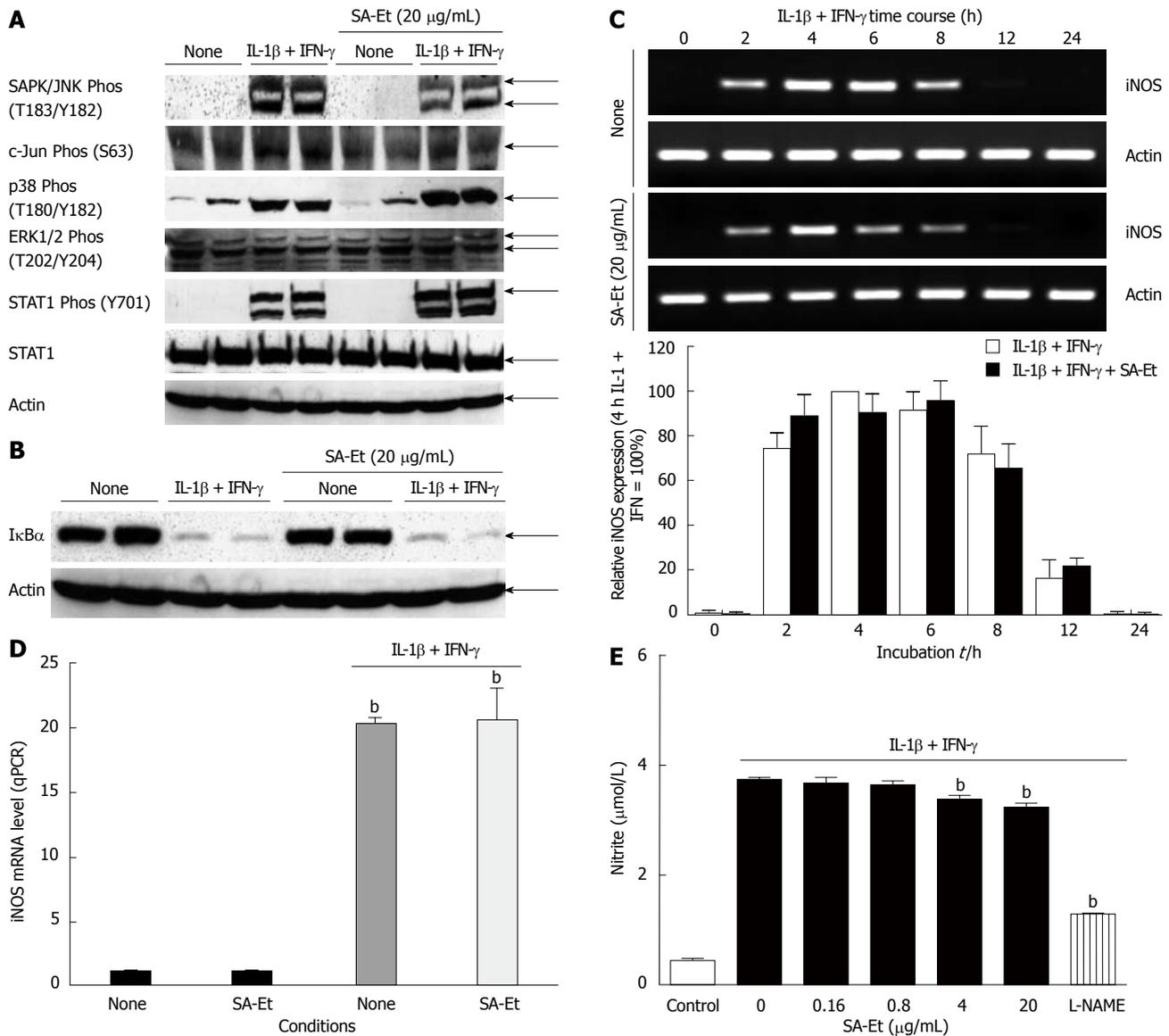


Figure 4 Characterizing the impact of ethanolic extract of *Schizandra arisanensis* on cytokine signal transduction. A: BRIN-BD11 cells stimulated with the cytokine mix in the presence or absence of ethanolic extract of *Schizandra arisanensis* (SA-Et) (20 μ g/mL) were harvested after 30 min treatment for Western blot with the indicated antibodies. A representative Western blot from three experiments is shown; B: BRIN-BD11 cells stimulated with the cytokine mix in the presence or absence of SA-Et were harvested after 60 min treatment to measure I κ B α protein level. A representative Western blot from three experiments is shown; C: Cytokine-induced inducible nitric oxide synthase (iNOS) mRNA levels in BRIN-BD11 cells during 24 h were determined by an reverse transcription-polymerase chain reaction. Relative iNOS gene expression was calculated by employing endogenous β -actin mRNA level as an internal control. The maximum iNOS gene expression at 4 h post-treatment with the cytokines alone was set to 100%. Data are presented as the mean \pm SE, $n = 4$; D: Changes in cytokine-induced iNOS mRNA levels in BRIN-BD11 cells at 4 h in the presence or absence of the SA-Et (20 μ g/mL) were also determined by real-time reverse transcription-polymerase chain reaction. β -actin was used as an internal control. The relative quantification of iNOS mRNA was presented as $2^{-\Delta\Delta Ct}$. Data are presented as the mean \pm SE, $n = 3$. $^bP < 0.01$ vs cells under control conditions; E: Nitric oxide production in cytokine-treated BRIN-BD11 cells in the presence or absence of the SA-Et (0-20 μ g/mL) or nitro-L-arginine methyl ester (L-NAME) (0.5 mmol/L) was determined. Data are presented as the mean \pm SE, $n = 4$. $^bP < 0.01$ vs cells treated with the cytokine mixture alone. IL-1 β : Interleukin 1 β ; IFN- γ : Interferon- γ .

sufficient organs for transplantation is a major obstacle. Employing toxin-resistant cells for type 1 diabetes therapy is another developing approach in cell-based therapies. However, disassociation between toxin resistance and insulin-secretory functions may occur during the induction of toxin resistance^[17]. Moreover, to our knowledge, no “master” toxin-resistant cells have been created so far^[17-19]. As a result, supplementation with β cell protective phytochemicals to enhance the survival rate and secretory functions of cell-based therapeutics for curing type 1 diabetes is an interesting and flexible approach^[20,21].

To pursue this objective, we created a simple platform by employing BRIN-BD11 cells. The use of a cytokine mix successfully promoted β cell death and abolished glucose-stimulated insulin secretion after 48 h of treatment. Through cell cycle analysis, it was apparent that only the combination of IL-1 β and IFN- γ elicited apoptosis as judged by caspase 3 cleavage and a significant increment of the subG₁ population in BRIN-BD11 cells under such conditions. In the present investigation, the anti-apoptotic effect of SA-Et against cytokine-mediated β cell death was clearly demonstrated. Consistent with the restoration

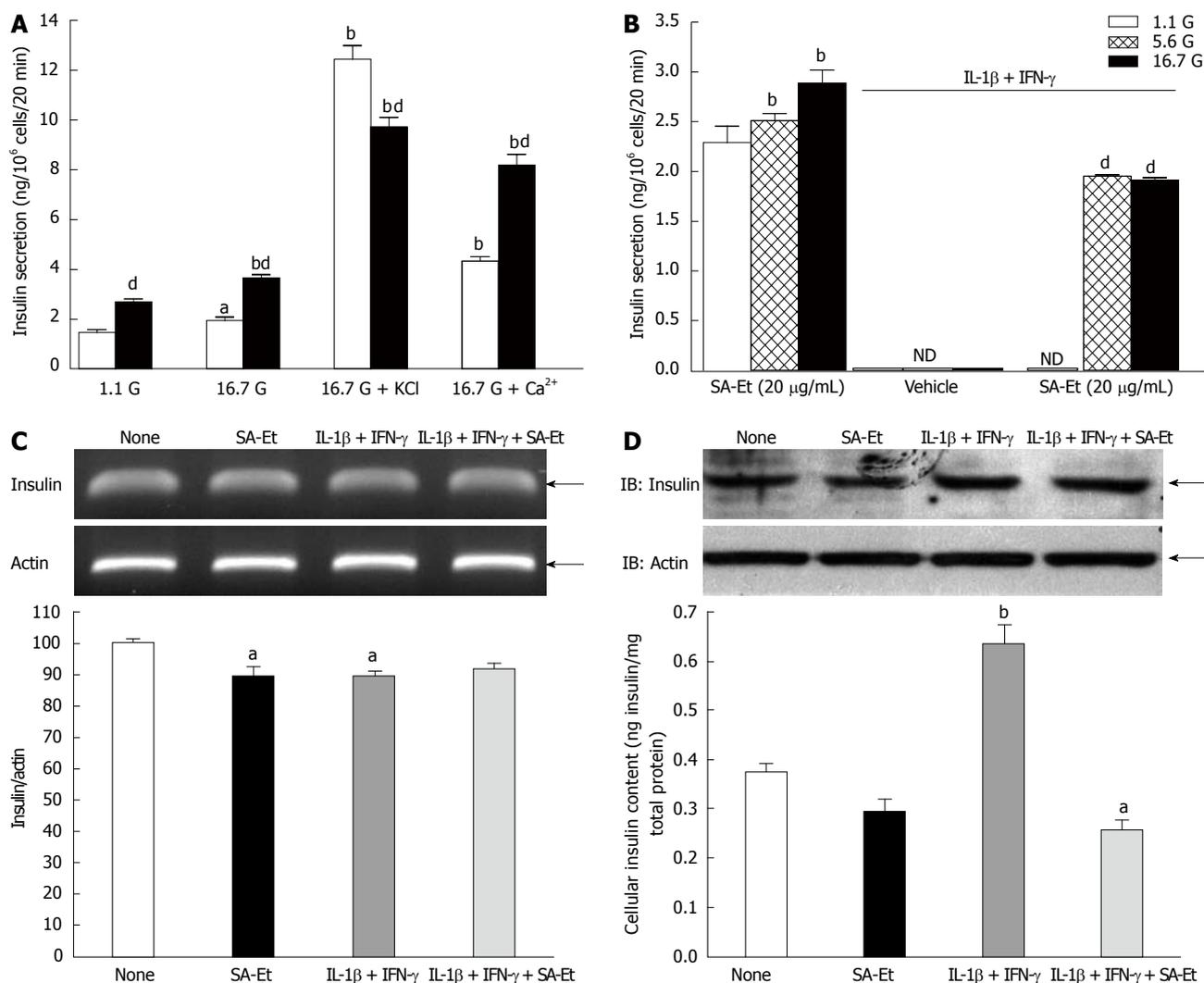


Figure 5 Ethanolic extract of *Schizandra arisanensis* treatment partially rescued the abolished insulin secretion in cytokine-treated BRIN-BD11 cells. **A:** Acute insulin release in response to glucose, KCl and Ca²⁺ in the presence or absence of the ethanolic extract of *Schizandra arisanensis* (SA-Et) was evaluated. Data are presented as the mean \pm SE, $n = 4$. ^a $P < 0.05$, ^b $P < 0.01$ vs insulin released from cells treated with 1.1 mmol/L glucose; ^d $P < 0.01$ vs control cells under the same conditions; **B:** Cultured BRIN-BD11 cells were treated with the cytokine mixture in the presence or absence of the SA-Et. At 48 h post-treatment, treated cells were evaluated for glucose responsiveness. Data are presented as the mean \pm SE, $n = 4$. ^b $P < 0.01$ vs insulin released from cells treated with 1.1 mmol/L glucose; ^d $P < 0.01$ vs SA-Et-treated cells under the same glucose conditions. **C:** In addition, a portion of the treated cells was harvested to measure insulin mRNA level using reverse transcription-polymerase chain reaction. Relative insulin gene expression was calculated by employing endogenous β -actin mRNA level as an internal control. ^a $P < 0.05$ vs control conditions (none); **D:** Protein level and cellular insulin content were independently measured by Western blot and enzyme-linked immunosorbent assay. Data are presented as the mean \pm SE, $n = 4$. ^a $P < 0.05$, ^b $P < 0.01$ vs control conditions (none). IL-1 β : Interleukin 1 β ; IFN- γ : Interferon- γ ; ND: Not determined.

of cell viability, the ratio of the subG₁ phase and protein level of cleaved caspase-3 in the cytokine-treated condition was blocked or reduced by SA-Et.

According to previous research, NF κ B, p38MAPK, and SAPK/JNK pathways all play a role in cytokine-mediated cytotoxicity in β cells. Interestingly, unlike other cell lines, NF κ B downstream iNOS/NO induction is not responsible for apoptosis of BRIN-BD11 cells. Instead, insulin secretion in response to glucose was significantly affected by NO production in BRIN-BD11 cells^[22]. Our results are consistent with previous findings and further demonstrated that p38MAPK and JNK/SAPK play important roles in cytokine-mediated BRIN-BD11 cell death.

Using this cell model, we screened several anti-diabetic herbal extracts and discovered that SA-Et has

protective bioactivity. According to the HPLC profile of the SA-Et, seven major peaks were identified: five C₁₉ homolignans (schiarisanrins) and two C₁₈ lignans (schizanrin A and macelignan). Through bioactivity screening, it was evident that some C₁₉ homolignans could account for the β cell protective effects of the SA-Et against the cytokine challenge. To our knowledge, this is the first report to reveal such bioactivity in C₁₉ homolignans. The results also implied that C₁₉ homolignans which possess an acetoxyl group at C-5 exhibited more-potent bioactivity than did those with the benzoic acid ester at C-5, regardless of the different C₁₉ homolignan skeletons as shown in Figure 1. Notably, schiarisanrin C exhibited dose-dependent negative protection activity, which was consistent with a previous report which found that schiarisanrin C exhibited cytotoxicity against other cell lines^[8]. The structure-

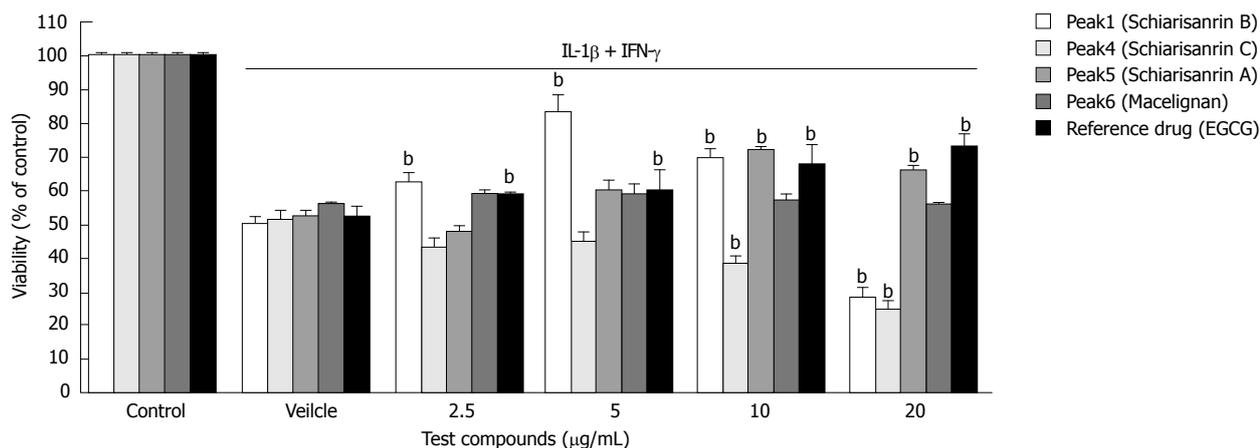


Figure 6 Identification of bio-active compounds isolated from *Schizandra arisanensis*. After major peak compounds were collected, BRIN-BD11 cells were treated with cytokine mix in the presence of each peak compounds for 48 h. The viability of cells was then measured by neutral red assay. Epigallocatechin gallate (EGCG) is used as reference drug. Data are presented as the mean \pm SE, $n = 8$. ^b $P < 0.01$ vs the viability of cells with no treatment.

activity relationship will likely be more obvious when more types of C₁₉ homolignan derivatives are compared.

To further elucidate the potential drug target of SA-Et, we evaluated the effects of SA-Et on key players in each pathway involved in cytokine-mediated β cell death. The results indicated that SA-Et appeared to impact on JNK/SAPK kinase activities and downstream substrate, i.e., c-Jun, to facilitate its protective activity against the cytokine mix. Recent research discovered that the JNK/c-Jun cascade could transactivate the expression of Bcl-2 homology 3 (BH3)-only member death protein 5 (DP5)/harakiri (Hrk) leading to β cell apoptosis^[23]; therefore, our results suggest that SA-Et treatment might intervene in such a cytokine pathway to facilitate β cell protection.

Interestingly, our results also demonstrated that SA-Et treatment had no inhibitory effect on cytokine-mediated NF- κ B release judged by a similar pattern of cytokine-mediated I κ B degradation and iNOS mRNA expression in the presence of SA-Et. NF- κ B is an important player in abolishing glucose-stimulated insulin secretion (GSIS) by affecting the glucose-induced influx of Ca²⁺^[24,25]. In addition, IL-1 β -mediated NO production also plays a negative role in insulin secretion in BRIN-BD11 cells^[22]. Therefore, whether SA-Et can not only preserve the viability of cytokine-treated BRIN-BD11 cells, but also rescue the impaired insulin secretion of the cytokine-treated cells was our next question.

In terms of the insulinotropic action of SA-Et, our results indicate that SA-Et acutely enhanced both glucose- and calcium-stimulated insulin secretion, although K⁺-stimulated insulin secretion appeared to be slightly reduced by SA-Et. As a result, we speculated that the insulinotropic effect of SA-Et might be associated with calcium-mediated insulin exocytosis, an overall proximal step in insulin secretion. In contrast, under cytokine treatment, insulin exocytosis rather than insulin mRNA expression appeared to be affected in BRIN-BD11 cells. This was based on the observations that insulin secreted by cytokine-treated BRIN-BD11 cells was undetectable using the insulin ELISA kit; however, the insulin mRNA

and intracellular insulin content (protein level) of BRIN-BD11 cells remained intact. When SA-Et was present, the abolished insulin exocytosis by the cytokine mix might be counteracted; therefore, resulting in partial recovery of glucose-stimulated insulin secretion and relief of accumulated insulin content in cytokine treated BRIN-BD11 cells.

In conclusion, the current investigation revealed, for the first time, that C₁₉ homolignans isolated from SA-Et possessed protective bioactivity against cytokine-induced β cell death. The cytokine-impaired insulin secretory function was also partially restored. However, iNOS/NO inhibition appeared to be an important factor if intact β cell insulin secretory function is to be preserved after cytokine challenge. In the future, continued research on potent β cell-protective phytochemicals should substantially contribute to the development of islet/stem cell transplantation or cell-based therapies for type 1 diabetes.

COMMENTS

Background

Conventional therapy (i.e., insulin injection) in patients with type 1 diabetes does not allow minute-to-minute control of blood glucose and does not prevent complications associated with the disease. However, the employment of whole pancreas or islet transplantation suffers from a lack of sufficient organs for transplantation. Therefore, the application of cell-based therapeutics for curing type 1 diabetes is an interesting and hot topic in the field.

Research frontiers

Employing toxin-resistant cells for type 1 diabetes therapy is a developing cell-based approach for curing type 1 diabetes. At least 5 toxin resistant β cell lines have been generated and investigated for defensive mechanisms. However, disassociation between toxin resistance and insulin-secretory functions may occur during the induction of toxin resistance. To our knowledge, no "master" toxin-resistant cells have been created so far. As a result, employing β -cell protective agent(s) including phytochemicals from traditional Chinese medicine to protect cell-based transplants has become an alternative and practical approach in the field.

Innovations and breakthroughs

By employing the glucose-responsive, insulin-secreting cell line, BRIN-BD11 cells, the authors successfully established a platform mimicking hormonal part of immune-attack in type 1 diabetes. In addition, the authors noted disassociation not only between cytokine-mediated nitric oxide and cell death, but also

between cell survival rate and secretory function. Moreover, the NF κ B-induced inducible nitric oxide synthase-nitric oxide pathway is not necessarily a master regulator for cell viability and function. *Schizandra arisanensis* (SA-Et) is one of the schizandraceous plants from Taiwan. The indications for this herb in traditional Chinese medicine include diabetes, hepatitis, immunomodulation and cancer. The current investigation is likely to be the first report on C₁₉ homolignans from SA-Et which possess β -cell protective effects. Different to previous β cell protective phytochemicals, such as epigallocatechin gallate and silymarin, the action of C₁₉ homolignans is not due to the blockage of NF κ B-inducible nitric oxide synthase-nitric oxide.

Applications

By employing this glucose-responsive, insulin-secreting cell line to generate several platforms which represent key steps in autoimmune mediated β -cell destruction. The authors will be able to design a formulation which can be used to enhance the survival rate and maintain secretory functions in cell-based therapeutics to achieve needle-free control of type 1 diabetes in the future.

Terminology

Type 1 diabetes (T1D) is an autoimmune disease which occurs when approximately 60%-90% of insulin secreting cells are lost and/or are dysfunctional due to β -cell directed autoimmunity. During the progression of T1D, heavy infiltrations of mononuclear cells lead to substantial damage to β cells by generating locally high concentrations of pro-inflammatory cytokines, perforin, and FasL-Fas interactions within the micro-environment of islets. As a result, deleterious outcomes including excessive pro-insulin secretion, the abolition of glucose-induced insulin secretion, and ultimately β cell death are observed.

Peer review

The article is very interesting and shows novel findings in insulin secreting cells. The article is well written. Furthermore, it correctly cites the most important articles in the field.

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Moxibustion inhibits interleukin-12 and tumor necrosis factor alpha and modulates intestinal flora in rat with ulcerative colitis

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testinal flora and release of interleukin-12 (IL-12) and tumor necrosis factor- α (TNF- α) from the colon in rat with ulcerative colitis (UC).

METHODS: A rat model of UC was established by local stimulation of the intestine with supernatant from colonic contents harvested from human UC patients. A total of 40 male Sprague-Dawley rats were randomly divided into the following groups: normal (sham), model (UC), herb-partition moxibustion (HPM-treated), and positive control sulfasalazine (SA-treated). Rats treated with HPM received HPM at acupuncture points ST25 and RN6, once a day for 15 min, for a total of 8 d. Rats in the SA group were perfused with SA twice a day for 8 d. The colonic histopathology was observed by hematoxylin-eosin. The levels of intestinal flora, including *Bifidobacterium*, *Lactobacillus*, *Escherichia coli* (*E. coli*), and *Bacteroides fragilis* (*B. fragilis*), were tested by real-time quantitative polymerase chain reaction to detect bacterial 16S rRNA/DNA in order to determine DNA copy numbers of each specific species. Immunohistochemical assays were used to observe the expression of TNF- α and IL-12 in the rat colons.

RESULTS: HPM treatment inhibited immunopathology in colonic tissues of UC rats; the general morphological score and the immunopathological score were significantly decreased in the HPM and SA groups compared with the model group [3.5 (2.0-4.0), 3.0 (1.5-3.5) vs 6.0 (5.5-7.0), $P < 0.05$ for the general morphological score, and 3.00 (2.00-3.50), 3.00 (2.50-3.50) vs 5.00 (4.50-5.50), $P < 0.01$ for the immunopathological score]. As measured by DNA copy number, we found that *Bifidobacterium* and *Lactobacillus*, which are associated with a healthy colon, were significantly higher in the HPM and SA groups than in the model group (1.395 ± 1.339 , 1.461 ± 1.152 vs 0.045 ± 0.036 , $P < 0.01$ for *Bifidobacterium*, and 0.395 ± 0.325 , 0.851 ± 0.651 vs 0.0015 ± 0.0014 , $P < 0.01$ for *Lactobacillus*). On the

Abstract

AIM: To investigate the effect of moxibustion on in-

other hand, *E. coli* and *B. fragilis*, which are associated with an inflamed colon, were significantly lower in the HPM and SA groups than in the model group (0.244 ± 0.107 , 0.628 ± 0.257 vs 1.691 ± 0.683 , $P < 0.01$ for *E. coli*, and 0.351 ± 0.181 , 0.416 ± 0.329 vs 1.285 ± 1.039 , $P < 0.01$ for *B. fragilis*). The expression of TNF- α and IL-12 was decreased after HPM and SA treatment as compared to UC model alone (4970.81 ± 959.78 , 6635.45 ± 1135.16 vs 12333.81 ± 680.79 , $P < 0.01$ for TNF- α , and 5528.75 ± 1245.72 , 7477.38 ± 1259.16 vs 12550.29 ± 1973.30 , $P < 0.01$ for IL-12).

CONCLUSION: HPM treatment can regulate intestinal flora and inhibit the expression of TNF- α and IL-12 in the colon tissues of UC rats, indicating that HPM can improve colonic immune response.

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Key words: Ulcerative colitis; Herb-partition moxibustion; Intestinal flora; Immune regulation

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INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory disease of the gastrointestinal tract characterized by increased stool frequency, bleeding and abdominal pain, and ulceration limited to the colon mucosa. The role of immunological pathogenesis in UC has been widely recognized^[1,2]. In recent years, the involvement of pathogen-mediated immune dysfunction has received extensive attention. Studies have shown that UC patients have altered intestinal flora species. For example, *Bifidobacterium*, *Lactobacillus* and fusobacterium *Escherichia coli* (*E. coli*) desulfovibrio were significantly decreased, while *Bacteroides fragilis* (*B. fragilis*), were significantly increased^[3-6]. In hosts with altered intestinal flora, the immune system recognizes intestinal flora and its metabolites as pathogenic antigens that can cause an abnormal immune response and stimulate bowel epithelial cells in UC patients with genetic susceptibility. This activity then destroys the structure and function of colonic mucosa, leading to the ongoing pathology associated with UC^[7]. Alteration of the intestinal flora has been considered to be the initiating and continuous factor in the onset of UC^[7-10]. Therefore, the modulation of intestinal flora has been suggested as a potential approach to treat UC.

Previous studies have shown that herb-partition moxibustion (HPM) can be anti-inflammatory^[11]. It can also improve symptoms in mild-to-moderate UC patients, such as relieving abdominal pain and decreasing bloody diarrhea^[12-14]. Experiments have also demonstrated that HPM can down-regulate the protein and mRNA expression of interleukin (IL)-8 and intercellular adhesion molecule-1 in colon tissues of UC patients, indicating that HPM can inhibit chemotaxis and cell migration into inflamed tissues^[14]. HPM can also promote neutrophil apoptosis and down-regulate cytokines, such as IL-1 beta, IL-6, and tumor necrosis factor-alpha (TNF- α), further suggesting an anti-inflammatory role for HPM treatment^[15]. Moreover, a recent report shows that intestinal injury was decreased and TNF- α was also found to be decreased after HPM treatment in a Crohn's disease model^[16]. Taken together, these results indicate that HPM can regulate immune function in UC. However, whether HPM can also alter the intestinal flora composition is still unknown.

A large number of bacteria comprise the human intestinal flora, outnumbering host cells by a ratio of 10:1. Various types of bacteria constitute the gut flora, including symbiotic and pathogenic bacteria whose pathogenicity is hidden from the immune system from various barrier and immune-mediated mechanisms. For this study, we observed the relative proportions of both the symbiotic - and most prevalent - bacteria, such as bifidobacteria and lactobacilli, and the conditioned pathogenic flora, such as *E. coli* and *B. fragilis*, as readouts to explore the effects of HPM on modulating the intestinal flora of rats in a model of UC. We also investigated whether HPM regulates the expression of pro-inflammatory cytokines, such as TNF- α and IL-12, in colon tissues of UC rats. This study would aid in understanding the mechanisms of how HPM can alleviate symptoms of UC.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats were obtained from the Department of Experimental Animal Science of Shanghai Medical College at Fudan University [No. SCXK(SH)2009-0019]. All protocols were performed in strict accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of the People's Republic of China^[17]. This study received permission from the Ethics Committee in Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, which is affiliated with Shanghai University of Traditional Chinese Medicine, China.

Establishment of the UC rat model

Forty neonatal, male Sprague Dawley rats weighing 183 ± 10 g were randomly divided into the following experimental groups: normal, model, HPM, and sulfasalazine (SA). The UC rat model was established according to an

Table 1 General morphological scoring

General morphological manifestation		Score
Colon adhesion	No adhesion	0
	Mild adhesion	1
	Severe adhesion	2
Ulcer and inflammation	No ulcer and no inflammation	0
	Local congestion, no ulcer	1
	1 ulcer without congestion or bowel wall thickening	2
	1 ulcer with inflammation	3
	> 2 ulcer and inflammation	4
	> 2 ulcer and/or inflammation area > 1 cm	5
> 2 ulcer and/or inflammation area > 2 cm, one more damage, plus 1	6-8	

Table 2 Immunopathological scoring

Immunopathological manifestation		Score
Ulcer	No ulcer	0
	Ulcer area < 3 cm	1
	Ulcer area > 3 cm	2
Inflammation	No inflammation	0
	Mild inflammation	1
	Severe inflammation	2
Granuloma	No granuloma	0
	Granuloma	1
Lesion depth	No lesion	0
	Submucosa	1
	Muscular layer	2
	Serosa layer	3
Fibrosis	No fibrosis	0
	Mild fibrosis	1
	Severe fibrosis	2

immunological method and local stimulation^[15,18-20]. In brief, fresh human colonic mucosa was obtained from surgical colonic specimens, homogenized in normal saline and centrifuged for 30 min at 3000 rpm in order to remove cells and bacteria. The supernatant, containing antigens released from UC colon patients, was diluted into an appropriate protein concentration and mixed with Freund's adjuvant (Shanghai Chemical Reagent Company, Shanghai, China). One mL of the antigen plus adjuvant mixture, containing a total of 6 mg protein, was injected into the front footpad at day 0. Following this initial dose, the same mixture was then injected into the back footpad, dorsa, inguina, and abdominal cavities on days 10, 17, 24 and 31, respectively. On day 38, rats were anesthetized intraperitoneally with 2% pentobarbital sodium (30 mg/kg) and 3 mL enema of 3% formalin were administered to the rats - in order to stimulate the colon immune response - for 1 h following a saline wash after a 2 mL enema of antigen fluid (without Freund's adjuvant) for 2 h following a saline wash.

Treatment

After the UC model was established in the rats, HPM was performed during days 39-46 of UC model in the treatment groups. Moxa cones (0.5 cm in diameter and

0.6 cm high) (Nanyang Hanyi Moxa Co., Ltd. China) were placed vertically on a medicinal formula composed of radix aconite, cortex, radix, carthami and salviae miltiorrhizae. The medicinal formula was then placed on acupoints ST25 (located on a horizontal line 2 cun laterally to the midline and 5 cun above the symphysis pubis, a point of hand Yangming, the mu point of the large intestine meridian, and it regulates the function of the intestine and stomach)^[21,22] and RN6 (located on ventral midline 3.5 cun above the symphysis pubis and 1.5 cun below the navel, a point of the conception meridian, and it strengthens original qi and improves immune function); the moxa cones were then ignited and each acupoint was treated twice in 15 min increments. This treatment was repeated once daily for a total of 8 d^[23]. As a positive control for inhibition of UC symptoms, the immunosuppressant SA was used. For the SA treatment group, salicylazosulfapyridine solution (0.25 g/tablet) was intragastrically administered to rats (Sanwei Pharmaceutical, Shanghai, China; Batch No. 200807C30) twice daily for a total of 8 d. The salicylazosulfapyridine solution concentration was 20 mg/mL with a daily dose of 100 mg/kg, which is equivalent to 0.1 g/kg in a human patient^[24].

Morphological observation of fixed colon samples

Following treatment and sacrifice by cervical dislocation, samples were collected from the descending colon (5 cm above the anus), cleaned with normal saline, and general morphology was then scored (Table 1). The samples were fixed in 10% formalin, dehydrated, embedded in paraffin, and sectioned into 4 µm thick slices. These sections were then stained by hematoxylin-eosin for pathological observation and the histological grade was scored with the bland method^[25,26] (Table 2).

TNF-α and IL-12 immunohistochemistry

Paraformaldehyde-fixed and paraffin-embedded samples were sliced into 4 µm slices. Paraffin slides were deparaffinized in xylene I, xylene II, and xylene III; dehydrated in 95%, 90% and 70% ethanol; and then incubated with primary rabbit antibodies at 4 °C for 18 h (anti-TNF-α was diluted to 1:150; IL-12 was diluted to 1:200) (Abcam Co., United Kingdom). The tissues were then visualized with 0.5 g/L diaminobenzidine and 0.3 g/L H₂O₂ in distilled water, and rinsed in phosphate buffered saline for 10 min. A known positive sample was used as a positive control for all slices, and phosphate buffered saline was substituted for the primary antibody and isotype controls as negative controls. All the samples were analyzed by a Motic Med 6.0 image analysis system (Motic Group Co., Ltd.). Three fields were randomly selected under an optical microscope (Olympus Co., Ltd.) at 400 × magnification to calculate the positive target value of the integral optical density.

16S rRNA real-time quantitative polymerase chain reaction

Rat feces taken directly from inoculated rats was placed

Table 3 Oligonucleotide primers and TaqMan fluorescent probe sequences used for real-time polymerase chain reaction assays

Primer or probe name	Primer sense	Sequence (5'→3')	Amplification product (bp)
<i>Bifidobacterium</i>	Forward	5'-ACTGGAATTCCTGGTGAAC-3'	85
	Reverse	5'-GTCAGTAACGGCCAGAGAC-3'	
<i>Lactococcus lactis</i>	Forward	5'-CAACATTGGAAACGAATGC-3'	134
	Reverse	5'-CCTTGGTGAGCCTTACCTC-3'	
<i>Bacteroides</i>	Forward	5'-ATTGCAGTGGGAATGATGTGG-3'	106
	Reverse	5'-TATGGCACTTAAGCCGACAC-3'	
<i>Bacillus vallismortis</i>	Forward	5'-ACCGCATGGTTCAGACATAA-3'	88
	Reverse	5'-AGCCGTACCTCACCAACT-3'	

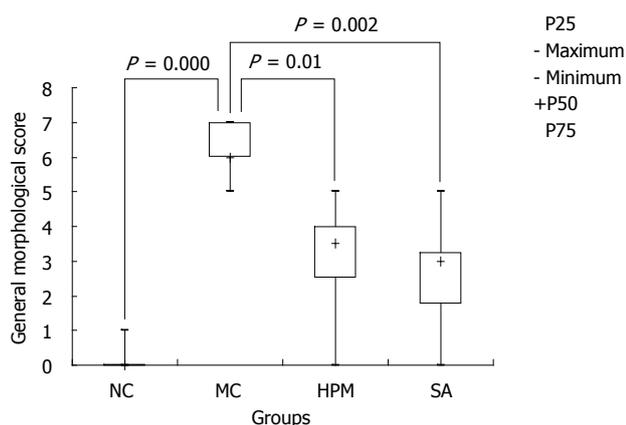


Figure 1 Herb-partition moxibustion inhibits tissue damage in colonic tissues of rats with ulcerative colitis. The general morphological score of the colonic tissue in the model control (MC) group was significantly higher than the normal control (NC) group ($P = 0.000$). After treatment, the scores were lower in both the herb-partition moxibustion (HPM) group ($P = 0.01$) and the control sulfasalazine (SA) group ($P = 0.002$).

into 5-10 mL of nutrient broth and was shaken and cultivated overnight at 37 °C. To extract DNA, 50 μ L of the bacterial culture fluid was placed in a 1.5 ml sterile centrifuge tube, and 50 μ L of DNA extract was added. This was mixed well at a constant temperature of 100°C for 10 min. It was then centrifuged at 12 000 rpm for 5 min and saved for later use. For the quantitative reverse transcription-polymerase chain reaction, the 7500 Sequence Detection System was used (ABI Co., USA). The sequence of the forward primer (F) and reverse primer (R) are shown in Table 3. DNA was prepared for PCR amplification in the following way: 10 μ L 5 \times PCR buffer, 0.5 μ L forward primer F, 0.5 μ L reverse primer R, 0.5 μ L dNTPs, 0.5 μ L TaqMan fluorescent probe, 1 μ L Taq enzyme, 32 μ L dH₂O, and 5 μ L cDNA template. We used the following amplification conditions: (1) 50 °C 2 min, (2) 95 °C 5min, (3) 95 °C 15 s, and (4) 60 °C 45 s, for 40 cycles.

Statistical analysis

All data were analyzed using SPSS 10.0 statistical software (SPSS Inc., United States), and all data were expressed as mean \pm SD for normally distributed continuous variables and as median (QL-QU) for abnormal variables. A one-way analysis of variance was used for normal

distributions, LSD/SNK-q for homogeneity of variance, and Dunnett's T3 for heterogeneity of variance. A non-parametric test was used for when the data did not follow Gaussian distribution, and the Spearman method was used for correlation analysis. A value of $P < 0.05$ was considered statistically significant.

RESULTS

HPM inhibits tissue damage in colonic tissues of UC rats

When the colonic tissues were observed for morphological changes upon treatment, we observed that our UC model group exhibited edematous colonic mucosa and there was serious congestion, erosion, and ulcer formation as compared to the normal group, which exhibited a smooth colonic mucosa surface, clear vascular texture, and no erosion or ulcers. Interestingly, HPM treatment was able to decrease the observed changes in UC treated rats and return the colon to a more normal state, as the colonic mucosa surface of these rats was smooth, there was accidental edema, the vascular texture was clear, and congestion, edema and erosion were significantly reduced compared to the model group. As shown in Figure 1, the general morphological score of the colonic tissue in the model group was significantly higher than the normal group [6.0 (5.5-7.0) *vs* 0.0 (0.0-0.0), $P = 0.000$]. After treatment, the scores were lower in both the HPM group [3.5 (2.0-4.0) *vs* 6.0 (5.5-7.0), $P = 0.01$] and the control SA group [3.0 (1.5-3.5) *vs* 6.0 (5.5-7.0), $P = 0.002$]. This data suggests that HPM is able to dampen the tissue-damaging effects of UC.

HPM inhibits immunopathology in colonic tissues of UC rats

To evaluate whether HPM was also able to regulate the inflammatory cell infiltration that causes tissue damage in UC, we evaluated the extent of immunopathology by looking for inflammatory cell tissue infiltration by HE staining. As shown in Figure 2A-D, the colonic mucosa and mucosa villi were damaged or missing in the UC model group, and large mononuclear cell and macrophage infiltration appeared in the mucosa or submucosa with congestion, edema, and ulceration as compared to the normal group, where the colonic mucosa epithelium was complete and the colonic gland was regularly arranged with inconspicuous inflammatory cell infiltration. However, HPM treatment decreased the inflammatory

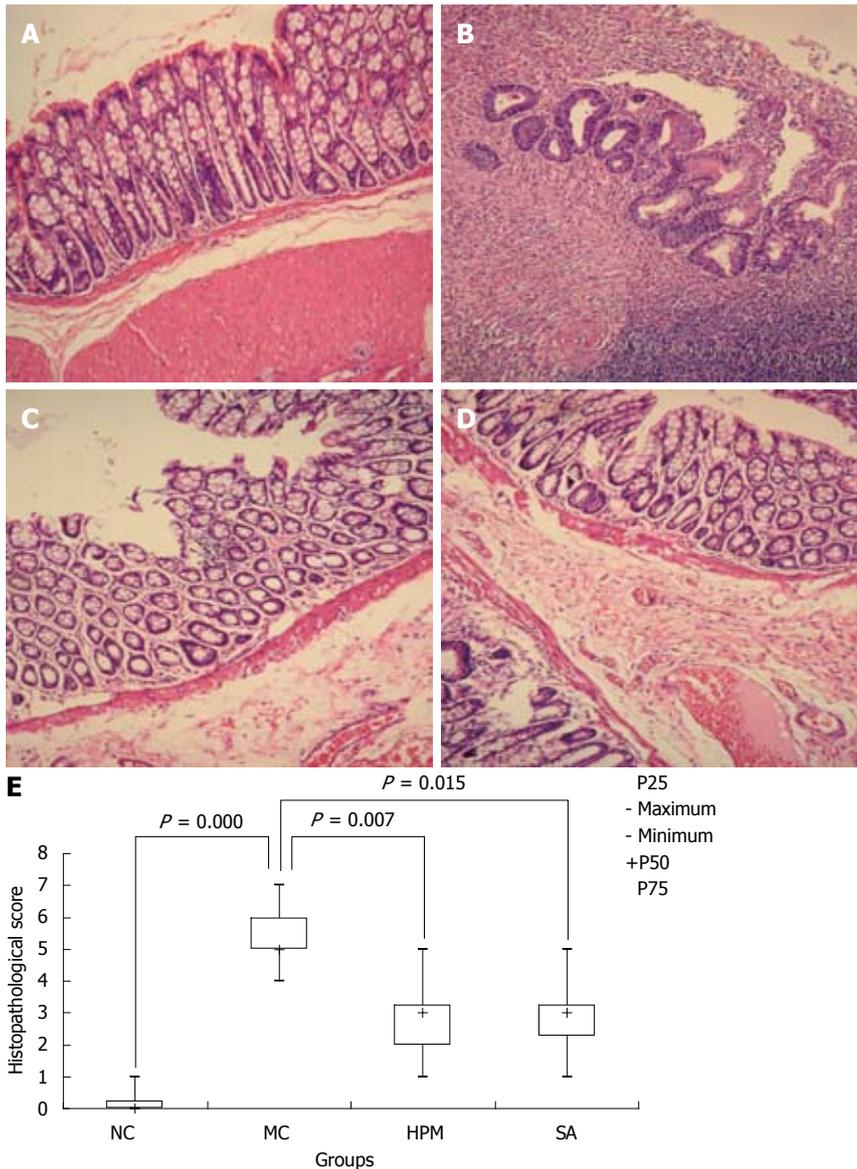


Figure 2 Herb-partition moxibustion inhibits immunopathology and decreases the histopathological scores in colonic tissues of rats with ulcerative colitis. A: Normal; B: Ulcerative colitis (UC); C: Herb-partition moxibustion (HPM); D: Sulfasalazine (SA); E: The histopathological scores for the colonic tissue in the UC model control (MC) group were significantly higher than the normal control (NC) group ($P = 0.000$). After treatment, the scores were lower in both the HPM group ($P = 0.007$) and SA group ($P = 0.015$). The colonic mucosa was damaged in the UC model group with cell infiltration, congestion, edema, and ulceration. After HPM treatment, there are only slight submucosal edema and inflammatory cell infiltration, and the colonic mucosa epithelium and the colonic gland were more regularly arranged than in the model group, new epithelial cells were observed to be covering the ulcers. Positive control SA treatment showed similar recovery of the UC model as the HPM group.

cell infiltration and the ensuing immunopathology, as we observed only slight submucosal edema and inflammatory cell infiltration, and the colonic mucosa epithelium and the colonic gland were more regularly arranged than in the model group. Additionally, new epithelial cells were observed to be covering the ulcers that developed under UC conditions, indicating that HPM treatment could induce recovery of these ulcers. Positive control SA treatment showed similar recovery of the UC model as the HPM group. As shown in Figure 2E, the histopathological scores for the colonic tissue in the model group were significantly higher than the normal group [5.00 (4.50-5.50) *vs* 0.00 (0.00-0.50), $P = 0.000$]. After

treatment, the scores were lower in both the HPM group [3.00 (2.00-3.50) *vs* 5.00 (4.50-5.50), $P = 0.007$] and SA group [3.00 (2.50-3.50) *vs* 5.00 (4.50-5.50), $P = 0.015$]. Taken together, these data indicate that HPM treatment can inhibit inflammatory cell infiltration under UC conditions and therefore limit the resulting immunopathology in the colon tissue.

HPM treatment rebalances the colonic intestinal flora of UC rats

Because the deregulation of intestinal flora is now understood to be a major component of UC pathogenesis, we tested whether HPM could restore the balance of

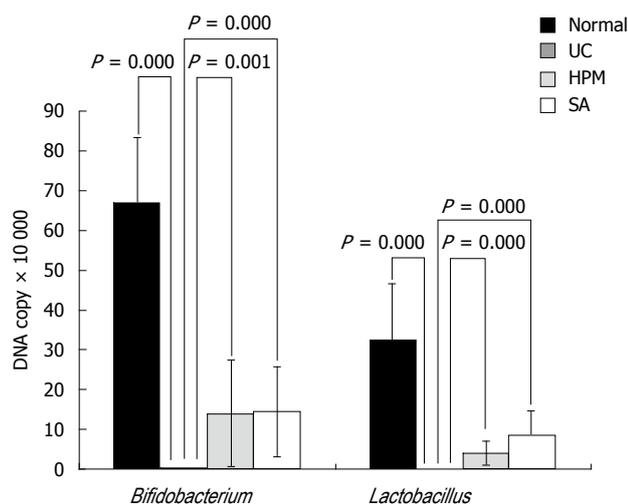


Figure 3 Herb-partition moxibustion treatment increases the colonic *Bifidobacterium* and *Lactobacillus* of rats with ulcerative colitis. The DNA copies of the symbiotic groups *Bifidobacterium* and *Lactobacillus* were both significantly decreased in the ulcerative colitis (UC) group compared to the normal control (NC) group ($P = 0.000$). After herb-partition moxibustion (HPM) treatment, the DNA copies of *Bifidobacterium* and *Lactobacillus* were both significantly increased in the HPM group ($P = 0.001$ and $P = 0.000$) and sulfasalazine (SA) group ($P = 0.000$ and $P = 0.000$) compared with the UC model group.

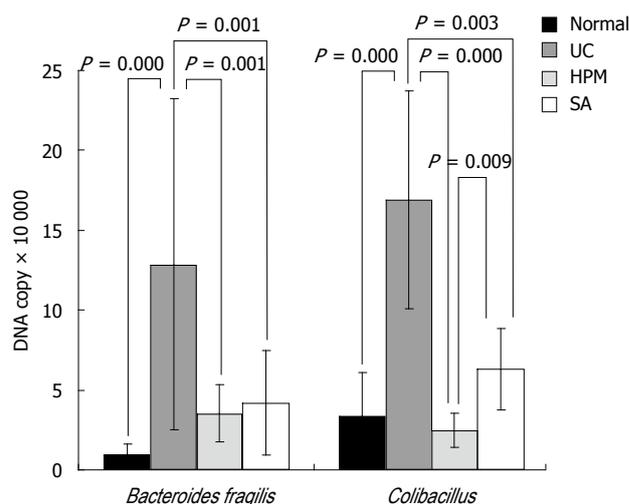


Figure 4 Herb-partition moxibustion treatment decreases the colonic pathogenic bacteria *Bacteroides fragilis* and *Escherichia coli* of rats with ulcerative colitis. The DNA copies of the pathogenic bacteria *Bacteroides fragilis* (*B. fragilis*), and *Escherichia coli* (*E. coli*) were both significantly increased in the ulcerative colitis (UC) rats compared to the normal control (NC) rats ($P = 0.000$). After herb-partition moxibustion (HPM) treatment, the DNA copies of *B. fragilis* and *E. coli* were both significantly decreased in the HPM group ($P = 0.001$ and $P = 0.000$) and sulfasalazine (SA) group ($P = 0.001$ and $P = 0.003$) compared with the UC model group.

the bacteria species back to normal by using the readout of the levels of symbiotic and pathogenic bacteria. To do this, we tested the levels of species-specific bacterial DNA by detecting 16S RNA from rat feces among the different groups. As shown in Figure 3, DNA copies of the symbiotic groups *Bifidobacterium* and *Lactobacillus* were both significantly decreased in the UC group compared to the normal group ($0.045 \pm 0.036 \times 10^5$ vs $6.707 \pm 1.595 \times 10^5$ for *Bifidobacterium*, $0.0015 \pm 0.0014 \times 10^7$ vs $3.254 \pm 1.395 \times 10^7$ for *Lactobacillus*, $P = 0.000$). After HPM treatment, the levels were restored, because DNA copies of *Bifidobacterium* and *Lactobacillus* were both significantly increased in the HPM group and SA group compared with the model group (1.395 ± 1.339 , 1.461 ± 1.152 vs $0.045 \pm 0.036 \times 10^5$, $P = 0.001$ in HPM group and $P = 0.000$ in SA group for *Bifidobacterium*, and 0.395 ± 0.325 , 0.851 ± 0.651 vs $0.0015 \pm 0.0014 \times 10^7$, $P = 0.000$ for *Lactobacillus*). As shown in Figure 4, the DNA copies of the pathogenic bacteria *B. fragilis* and *E. coli* were both significantly increased in the UC rats compared to the normal rats (1.285 ± 1.039 vs $0.097 \pm 0.063 \times 10^5$ for *B. fragilis*, 1.691 ± 0.683 vs $1.691 \pm 0.683 \times 10^5$ for *E. coli*, $P = 0.000$). After HPM treatment, the DNA copies of *B. fragilis* and *E. coli* were both significantly decreased in the HPM group and SA group compared with the UC model group ($0.351 \pm 0.181 \times 10^5$, $0.416 \pm 0.329 \times 10^5$ vs $1.285 \pm 1.039 \times 10^5$, $P = 0.001$ for *B. fragilis* and $0.244 \pm 0.107 \times 10^5$, $0.628 \pm 0.257 \times 10^5$ vs $1.691 \pm 0.683 \times 10^5$, $P = 0.000$ in HPM group and $P = 0.003$ in SA group for *E. coli*). These data indicate that HPM treatment can rebalance the intestinal flora toward more normal levels after alteration under UC conditions.

HPM inhibits the secretion of the pro-inflammatory mediators TNF- α and IL-12 in the colon tissue of UC rats

To determine whether the HPM-induced reduction of inflammation was due to an effect of HPM on the secretion of pro-inflammatory cytokines, we measured TNF- α and IL-12 levels in colon tissue by immunohistochemistry. As shown in Figure 5A1-E1, TNF- α expression was significantly increased in the UC model group compared to the normal group ($650\ 313.82 \pm 65\ 996.76$ vs $48\ 384.84 \pm 9438.98$, $P = 0.000$). After treatment, TNF- α expression was significantly decreased in both the HPM group and SA group compared with the UC model group ($231\ 783.33 \pm 50\ 222.65$, $283\ 668.65 \pm 44\ 978.06$ vs $650\ 313.82 \pm 65\ 996.76$, $P = 0.000$). There was a difference in TNF- α expression between the HPM group and positive control SA group ($231\ 783.33 \pm 50\ 222.65$ vs $283\ 668.65 \pm 44\ 978.06$, $P = 0.034$). As shown in Figure 5A2-E2, IL-12 expression was significantly increased in the model group compared to the normal group ($901\ 708.26 \pm 215\ 867.35$ vs $76\ 799.88 \pm 15\ 270.78$, $P = 0.000$). HPM treatment inhibits IL-12 expression because it was significantly decreased in both the HPM group and positive control SA group compared with the model group ($333\ 652.88 \pm 121\ 428.18$, $512\ 202.17 \pm 95\ 369.17$ vs $901\ 708.26 \pm 215\ 867.35$, $P = 0.001$ in the HPM group and $P = 0.012$ in the SA group). There was a difference in IL-12 expression between the HPM group and the SA group ($333\ 652.88 \pm 121\ 428.18$ vs $512\ 202.17 \pm 95\ 369.17$, $P = 0.033$). This data indicates that HPM treatment exerts its anti-inflammatory effects through the inhibition of pro-inflammatory cytokine secretion, such as TNF- α and IL-12.

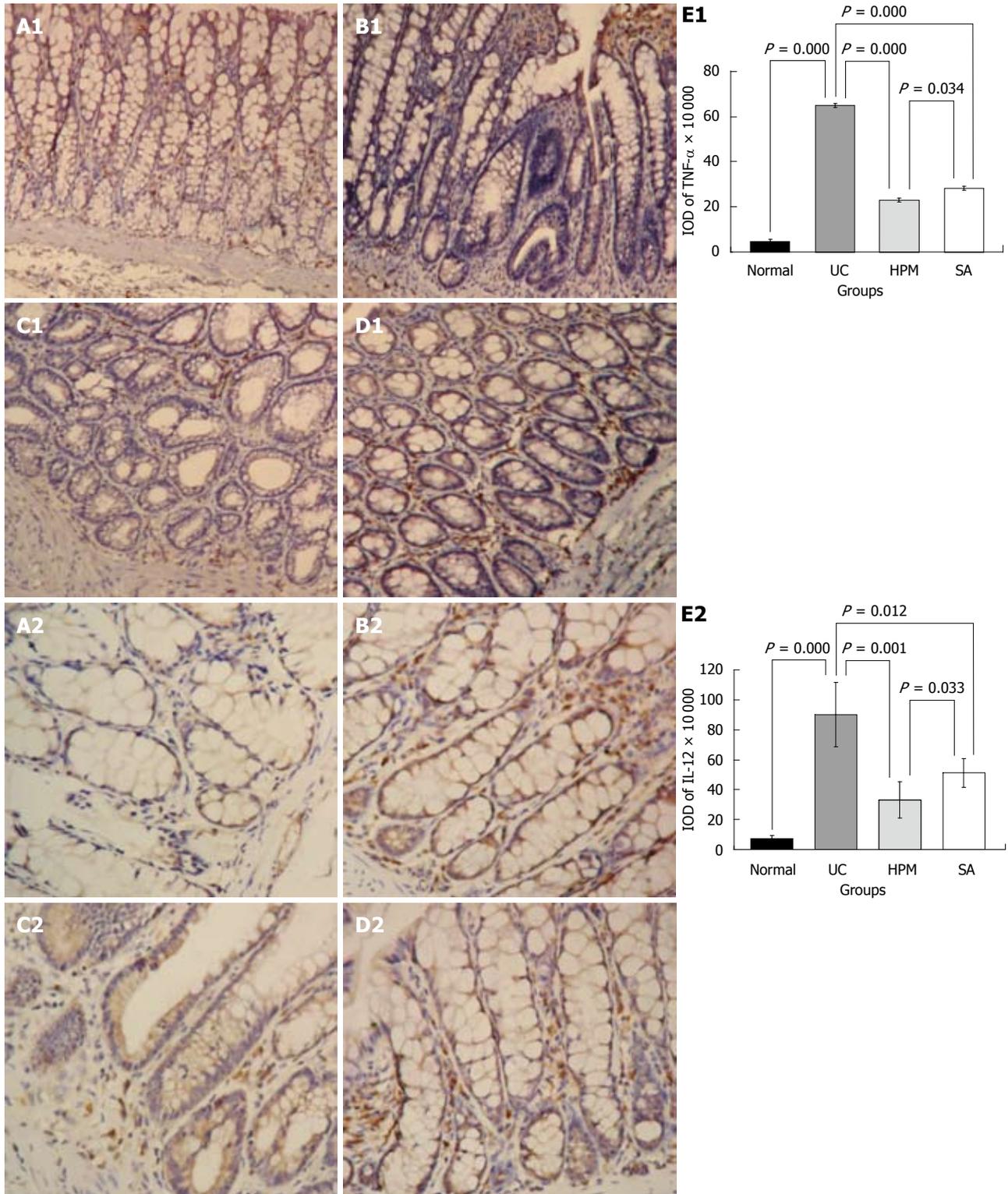


Figure 5 Herb-partition moxibustion inhibits the secretion of the pro-inflammatory mediators tumor necrosis factor- α and interleukin-12 in the colon tissue of rats with ulcerative colitis ($\times 400$). A1, A2: Normal control; B1, B2: Ulcerative colitis (UC); C1, C2: Herb-partition moxibustion (HPM); D1, D2: Sulfasalazine (SA). A1-E1: Tumor necrosis factor- α (TNF- α) expression was significantly increased in the UC model group compared to the normal group ($P = 0.000$). After treatment, TNF- α expression was significantly decreased in both the HPM group ($P = 0.000$) and SA group ($P = 0.000$) compared with the UC model group. There was a difference in TNF- α expression between the HPM group and positive control SA group ($P = 0.034$); A2-E2: Interleukin-12 (IL-12) expression was significantly increased in the model group compared to the normal group ($P = 0.000$). HPM treatment inhibits IL-12 expression because it was significantly decreased in both the HPM group ($P = 0.001$) and positive control SA group ($P = 0.012$) compared with the model group. There was a difference in IL-12 expression between the HPM group and SA group ($P = 0.033$). IOD: Integrated optical density per dye.

DISCUSSION

In normal physiological conditions, the overgrowth of pathogenic microorganisms is kept in check by the balance of gut flora species as well as the interaction between microbes and the host's immune system. These mechanisms limit the pathogenic microorganisms from sticking to the intestinal mucosa and act as a biological barrier to the host^[7,27,28]. Altered bacterial composition and function of the gut flora in UC results in increased immune stimulation, epithelial dysfunction, and enhanced mucosal permeability^[7]. Moreover, this barrier dysfunction leads to increased recognition of pathogens and their metabolites by the immune system and cause an abnormal intestinal immune response^[7] that constantly stimulates intestinal epithelium cells, among other effects. This immune response leads to the production of a large amount of cytokines, such as TNF- α , IL-12 and IL-6, that further aggravate the local immune response of the intestinal mucosa and cause continual damage of the intestinal mucosa^[29,30]. In recent years, studies have widely focused on the relationship between intestinal bacteria and UC. Changes in gut flora have been considered as key in the initiation and maintenance stages of the inflammatory processes in UC^[7,10,31-35]. There is evidence to support that UC patients undergo alterations in intestinal flora: the amount of *Enterobacter*, *Enterococcus*, and small-bowel *Clostridium* increased significantly, while *Bifidobacteria* and *Lactobacilli* decreased significantly in acute UC. In patients undergoing remission from UC, bacteroid and *Bifidobacteria* were increased markedly and small-bowel *Clostridium* was decreased significantly^[36,37]. In some studies, bacteroids were significantly increased^[38] and *Bifidobacteria* and *Lactobacilli* in the gut were both decreased in acute and remission UC^[39]. Other studies have indicated that *Bifidobacteria*, *Lactobacilli*, and *Fusobacterium* in the gut of UC patients decreased significantly, while *Bacteroides*, *E. coli*, and *Desulfovibrio* increased remarkably^[3-6]. The results of the present study showed that *Bifidobacteria* and *Lactobacilli* decreased significantly, while *E. coli* and *B. fragilis* significantly increased by testing the bacterial DNA copy numbers in the stool of UC rats as compared to normal rats. These results indicate that gut flora alterations also occur in the rat model of UC used in these studies and provide further evidence that alterations in gut flora play an important role in the pathogenesis of UC.

In recent years, modulation of gut flora has been suggested as an approach to treat UC. The balance between beneficial and detrimental bacterial species determines homeostasis *vs* inflammation. This balance can be manipulated by antibiotics, probiotics, and prebiotics to treat and prevent relapses of UC^[40]. Additionally, alternative treatments or complementary therapy, such as the use of probiotics, have been considered good candidates for treatment of UC because there are less problems with resistance, it means less risk for the development of bacterial resistance to antibiotic treatment, and there are less potential side effects and fewer ecological concerns

than using antibiotic drugs^[41]. Our previous studies in humans have indicated that HPM treatment is safe and has a therapeutic effect on UC patients^[12-14]. To determine the mechanism of how HPM might control UC, the aim of this study was to analyze whether HPM can modulate the gut flora in a rat model of UC. The results shown here demonstrate that HPM treatment does indeed alter the intestinal flora bacterial composition, as *Bifidobacteria* and *Lactobacilli* increased significantly, while *E. coli* and *B. fragilis* decreased remarkably in the stool of HPM-treated UC rats as compared to the stool of UC rats without treatment. Damage to the colonic mucosa was also remarkably improved after HPM treatment of UC rats, which indicated that the alteration of the gut flora back towards normal rat flora correlated with increased recovery of gut pathology in the UC rats treated with HPM. Therefore, HPM could modulate the alteration of gut flora in the UC rat model, recover intestinal micro-ecological balance, and inhibit the development and/or maintenance of gut pathology. The resultant modulation of gut flora by HPM is similar to that of probiotic supplements^[29], and therefore deserves further study as an alternative treatment for UC symptoms.

IL-12 and TNF- α are pre-inflammation factors and play an important role in the pathology of intestinal bowel disease (IBD). IL-12 from dendritic cells induces the differentiation of CD4⁺ T cells to Th1 cells. IL-12 also stimulates natural killer cells and T cells to secrete γ -interferon and TNF- α cytokines to mediate the inflammation. TNF- α cooperates with γ -interferon and can change the function of the intestinal barrier; it can also enhance the permeability of the mucous membrane or induce apoptosis of bowel epithelial cells. This tissue damage is the key process in the colonic inflammatory response in UC^[3,39] and is closely related to the pathogenesis of UC^[4,5]. Studies have revealed that IL-12 protein and mRNA expression is increased and correlates with the active index score of UC^[5,6]. IL-12 and TNF- α are significantly increased in the peripheral blood of patients with IBD^[40-43]. These previous studies indicate the relevance of IL-12 and TNF- α cytokines in the initiation and maintenance of UC pathogenesis.

Previous studies showed that HPM could rectify abnormal mucosal immune responses^[43], regulate the expression of IL-1 and IL-1 β ^[44,45], and improve intestinal mucus damage by down-regulating the expression of TGF- β 1 and IGF-1 in the inflamed tissue of UC^[46,47]. The results of the present study indicate that TNF- α and IL-12 are expressed at low levels in the colonic mucosa and the submucosa of normal rats. However, in UC rats, TNF- α and IL-12 were expressed at high levels that correlated with the observed higher morphological and histopathological scores. After HPM treatment, however, the expression of TNF- α and IL-12 decreased and the general morphological and histopathological scores were lower than those of the UC group, indicating that HPM treatment had an anti-inflammatory effect and improved the UC-mediated pathology. These results further support the previous reports that suggest that HPM can

improve the inflammatory response induced by UC, and that the mechanism may be regulation of the expression of inflammatory cytokines IL-6, IL-1 β , TNF- α , and IL-12 in the colon mucosa of UC rats.

Additionally, the intestinal flora and their products have been found to trigger cytokine expression, such as inducing TNF- α in macrophage and epithelial cell systems of inflammatory bowel disease^[48]. In addition to restoring beneficial intestinal flora, probiotics may enhance host protective immunity such as down-regulation of pro-inflammatory cytokines, IL-12 and TNF- α in colitis^[49,50]. Rifaximin administration decreased the protein and mRNA levels of IL-12 and TNF- α , and caused a significant reduction of colon bacterial translocation towards mesenteric lymph nodes, in colon of 2, 4, 6-trinitrobenzene sulfonic acid-induced colitis in mice^[51]. The results of this study showed that HPM can modulate intestinal flora towards a more normal flora and inhibit the expression of TNF- α and IL-12 in UC rats. These indicate that there may be a relationship between the release of IL-12 /TNF- α and the modulation of bacterial flora.

In conclusion, we suggest that HPM can modulate intestinal flora towards a more normal flora and inhibit the expression of the pro-inflammatory mediators TNF- α and IL-12 in UC rats. Therefore, whether HPM can improve the intestinal inflammation response of UC by modulating intestinal flora deserves further study as a treatment for patients suffering with UC.

COMMENTS

Background

Intestinal flora plays an important role in human health. Previous studies indicated that herb-partition moxibustion (HPM) has a beneficial effect on ulcerative colitis (UC), not just to relieve the symptoms, but also to improve the stool property, but whether the effect of HPM is related to intestinal flora remains unknown.

Research frontiers

More and more data show that the key role of bacteria in the pathogenesis of inflammatory bowel disease, which has become a hot spot of study.

Innovations and breakthroughs

This study is the first to report on the effects of HPM on intestinal flora of UC. HPM treatment can regulate intestinal flora of the UC model rat.

Applications

The experimental data can be used in the further study of moxibustion therapy in the treatment of inflammatory bowel disease.

Peer review

This is a good original study in which authors observed the relative proportions of both the symbiotic and most prevalent bacteria, and the conditioned pathogenic flora to explore the effects of HPM on modulating the intestinal flora of rats in a model of UC. They also investigated whether HPM regulates the expression of pro-inflammatory cytokines in colon tissues of UC rats. They concluded that HPM treatment regulates intestinal flora and inhibit the expression of interleukin-12 and tumor necrosis factor, indicating that HPM can improve colonic immune response.

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Minimally invasive treatment of pancreatic necrosis

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Abstract

AIM: To systematically review these minimally invasive approaches to infected pancreatic necrosis.

METHODS: We used the MEDLINE database to investigate studies between 1996 and 2010 with greater than 10 patients that examined these techniques. Using a combination of Boolean operators, reports were retrieved addressing percutaneous therapy (341 studies), endoscopic necrosectomy (574 studies), laparoscopic necrosectomy *via* a transperitoneal approach (148 studies), and retroperitoneal necrosectomy (194 studies). Only cohorts with at least 10 or more patients were included. Non-English papers, letters, animal studies, duplicate series and reviews without original data were excluded, leaving a total of 27 studies for analysis.

RESULTS: Twenty-seven studies with 947 patients total were examined (eight studies on percutaneous approach; ten studies on endoscopic necrosectomy; two studies on laparoscopic necrosectomy *via* a transperitoneal approach; five studies on retroperitoneal necrosectomy; and two studies on a combined percutaneous-retroperitoneal approach). Success rate, complications, mortality, and number of procedures were outcomes that were included in the review. We found that most published reports were retrospective in na-

ture, and thus, susceptible to selection and publication bias. Few reports examined these techniques in a comparative, prospective manner.

CONCLUSION: Each minimally invasive approach though was found to be safe and feasible in multiple reports. With these new techniques, treatment of infected pancreatic necrosis remains a challenge. We advocate a multidisciplinary approach to this complex problem with treatment individualized to each patient.

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Key words: Acute pancreatitis; Pancreatic abscess; Pancreatic necrosis; Necrosectomy; Laparoscopic necrosectomy

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Bello B, Matthews JB. Minimally invasive treatment of pancreatic necrosis. *World J Gastroenterol* 2012; 18(46): 6829-6835 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i46/6829.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i46.6829>

INTRODUCTION

Most cases of acute pancreatitis are self-limited and resolve without serious complications. However, severe acute pancreatitis is associated with the development of potentially life-threatening complications including pancreatic necrosis and pancreatic abscess. The 1992 international consensus conference held in Atlanta established uniform terminology for acute pancreatitis and its complications. According to the Atlanta Classification, pancreatic necrosis refers to diffuse or focal areas of nonviable pancreatic parenchyma, typically associated with peripancreatic fat necrosis, whereas pancreatic abscess is defined as a circumscribed intra-abdominal col-

lection of pus, usually in proximity to the pancreas arising as a consequence of acute pancreatitis or pancreatic trauma^[1]. Treatment for pancreatic necrosis has evolved considerably over the past decade with respect to both the timing of intervention and the development of alternatives to traditional open necrosectomy.

Pancreatic necrosis may be sterile or infected. The prognosis (with or without intervention) is much worse for infected than sterile necrosis. Historically, early surgical intervention was considered mandatory for cases of suspected infection. The 2002 International Acute Pancreatitis (IAP) guidelines recommended early fine-needle aspiration to discriminate between sterile and infected pancreatic necrosis, with continued non-operative management for stable or improving patients with sterile necrosis but surgical intervention for those with documented infection^[2]. The traditional surgical approach to infected necrosis was open necrosectomy with the goals of wide drainage of all infected compartments and complete removal of all necrotic tissue with the placement of drains for continuous postoperative closed lavage. Frequently, repeat laparotomy was needed to ensure complete debridement^[2]. Historically, open necrosectomy was associated with substantial morbidity and rates of perioperative mortality that exceeded 50% in some reports^[3,4], although mortality in some contemporary series has been 11% or lower^[5,6].

Because various studies showed high mortality with early operation for severe necrotizing pancreatitis, the IAP recommended avoidance of surgical intervention within the first 14 d after onset unless there was progressive multiple organ failure and clinical deterioration. Subsequent studies suggested that morbidity and mortality can be further reduced if operation is delayed beyond 28-30 d^[7], presumably because the extended interval allows sufficient demarcation between normal and necrotic tissue, thereby reducing the risk of inciting overwhelming postoperative septic and systemic inflammatory responses, as well as the risk of intraoperative injury to surrounding organs and hemorrhage.

In addition to the open approach for pancreatic necrosis and abscess, the last two decades have brought about alternative “minimally-invasive” techniques. Percutaneous drainage of infected pancreatic necrosis has been shown to be safe and effective in highly-selected patients^[8,9], but multiple procedures are often needed, and adjunctive treatment is often required.

Gagner^[10] first described minimally invasive surgical treatment of necrotizing pancreatitis in 1996, including laparoscopic retrocolic, retroperitoneoscopic, and transgastric procedures. Over the past 15 years, a number of other minimally invasive surgical, endoscopic, and radiological approaches to drain and debride pancreatic necrosis have been described. These alternatives appear to be feasible and safe, although comparisons among approaches have been difficult due to small numbers, lack of uniform reporting criteria, and varying degrees of operator dependence. The advent of some of these

alternatives has led to reconsideration of some of the fundamental tenets of open surgical necrosectomy, particularly with respect to the timing and completeness of debridement. In this report, we review current techniques of minimally invasive pancreatic necrosectomy.

MATERIALS AND METHODS

A literature search was performed of the MEDLINE database from April 1996 to November 2010 for each of four techniques well described for minimally invasive necrosectomy: percutaneous therapy (341 studies), endoscopic necrosectomy (574 studies), laparoscopic necrosectomy *via* a transperitoneal approach (148 studies), and retroperitoneal necrosectomy (194 studies). Only cohorts with at least 10 or more patients were included. Non-English papers, letters, animal studies, duplicate series and reviews without original data were also excluded.

For percutaneous drainage, a search was conducted using subject headings: “percutaneous”, “drainage”, “pancreatic necrosis”, and “necrotizing pancreatitis” with the aid of Boolean operators. There were 341 initial hits in MEDLINE. After exclusion using the above-mentioned criteria, eight studies were included in this review.

For endoscopic necrosectomy, a search was conducted using subject headings: “endoscopic”, “endotherapy”, “drainage”, “pancreatic necrosis”, and “necrotizing pancreatitis” with the aid of Boolean operators. There were 574 initial hits in MEDLINE. After exclusion using the above-mentioned criteria, ten studies were included in this review.

For laparoscopic necrosectomy *via* a transperitoneal approach, a search was conducted using subject headings: “laparoscopic”, “minimally invasive”, “necrosectomy”, “pancreatic necrosis”, and “necrotizing pancreatitis” with the aid of Boolean operators. There were 148 initial hits in MEDLINE. After exclusion using the above-mentioned criteria, two studies were included in this review.

For retroperitoneal necrosectomy, a search was conducted using subject headings: “laparoscopic”, “minimally invasive”, “retroperitoneal”, “necrosectomy”, “pancreatic necrosis” and “necrotizing pancreatitis” with the aid of Boolean operators. There were 194 initial hits in MEDLINE. After exclusion using the above-mentioned criteria, five studies were included in this review.

RESULTS

Percutaneous therapy

The results of the literature search for percutaneous therapy for pancreatic necrosis are summarized in Table 1. Technique was similar throughout the studies, access to the area of necrosis was obtained utilizing ultrasound and/or computed tomography to place percutaneous drains ranging in size from 10 to 28 French drains. Afterward, saline flushes were often used every 8 h.

Table 1 Percutaneous drainage, endoscopic, laparoscopic and retroperitoneal necrosectomy and combined approach

Study	n	Median delay to drainage, d (range)	Median procedures per patient (range)	Success (%)	Median follow-up, mo (range)	Complications (%)	Overall mortality (%)
Percutaneous drainage							
Freeny <i>et al</i> ^[8]	34	9 d (1-48 d)	4 (1-12)	16 (47)	NA	24 (71)	4 (12)
Van Santvoort <i>et al</i> ^[11]	43	NA	1-2	15 (35)	6	17 (40)	8 (19)
Rocha <i>et al</i> ^[12]	28	NA	NA	5 (18)	NA	3 (11)	8 (29)
Mortelé <i>et al</i> ^[13]	35	11 d (2-33 d)	3 (1-7)	17 (49)	NA	4 (11)	6 (17)
Bruennler <i>et al</i> ^[14]	80	3.5 d (1-40 d)	2 (1-9)	42 (53)	NA	23 (29)	27 (34)
Lee <i>et al</i> ^[15]	18	NA	NA	14 (78)	7.3	2 (11)	1 (6)
Baril <i>et al</i> ^[16]	38	NA	2.4 (2-4)	30 (79)	NA	1 (3)	2 (5)
Gambiez <i>et al</i> ^[17]	10	17 d (10-25 d)	NA	3 (30)	NA	6 (60)	2 (20)
Endoscopic necrosectomy							
Seifert <i>et al</i> ^[18]	93	41 d (4-158 d)	6.2 (1-35)	63 (68)	43	24 (26)	13 (19)
Coelho <i>et al</i> ^[19]	56	5 wk (4-10 wk)	4 (2-8)	49 (87)	21	11 (20)	2 (3.5)
Escourrou <i>et al</i> ^[20]	13	27.5 d (23-32 d)	1.8 (1-3)	13 (100)	19.5	6 (46)	0 (0)
Lopes <i>et al</i> ^[21]	26	NA	NA	24 (94)	9	2 (7.7)	0 (0)
Voermans <i>et al</i> ^[22]	25	84 d (21-385 d)	NA	23 (95)	16	10 (40)	0 (0)
Papachristou <i>et al</i> ^[23]	53	49 d (20-300 d)	3 (1-12)	41 (77)	5.7	26 (49)	3 (6)
Hookey <i>et al</i> ^[24]	17	23 d (10-45 d)	2 (1-2)	10 (59)	NA	2 (12)	NA
Charnley <i>et al</i> ^[25]	13	24 d (3-180 d)	4 (1-10)	11 (85)	16	0 (0)	2 (15)
Seewald <i>et al</i> ^[26]	13	NA	NA	9 (69)	8.3	3 (5.6)	0 (0)
Baron <i>et al</i> ^[27]	43	NA	2 (1-6)	31 (72)	25	16 (37)	NA
Laparoscopic necrosectomy							
Parekh ^[28]	19	65 d (22-154 d)	1 (1-3)	14 (74)	NA	11 (58)	2 (10.5)
Zhu <i>et al</i> ^[29]	10	1-3 wk (NA)	NA	9 (90)	NA	NA	1 (10)
Retroperitoneal necrosectomy							
Gambiez <i>et al</i> ^[17]	20	18 d (NA)	5 (1-9)	17 (85)	NA	6 (30)	2 (10)
Raraty <i>et al</i> ^[30]	137	32 d (1-181 d)	3 (1-9)	120 (86)	NA	75 (55)	26 (19)
Chang <i>et al</i> ^[31]	19	35 d (14-56 d)	NA	17 (89.5)	NA	4 (21)	3 (15.8)
Castellanos <i>et al</i> ^[32]	11	13 d (1-28 d)	5 (3-10)	11 (100)	2-60	0 (0)	0 (0)
Carter <i>et al</i> ^[33]	10	24 d (13-187 d)	3 (1-6)	8 (80)	NA	5 (50)	2 (20)
Combined approach							
Van Santvoort <i>et al</i> ^[11]	43	4 wk	1-7	41 (95)	3-6	17 (40)	8 (19)
Horvath <i>et al</i> ^[34]	40	4 wk	1 (1-2)	24 (60)	6	29 (72.5)	2 (5)

NA: Not available.

Eight studies included 286 patients ranging from 10 to 80 patients. There were six retrospective case series, one prospective case series^[15], and one randomized control trial^[11]. In the randomized control trial, percutaneous drainage was used as part of the minimally invasive “step-up” approach, in which a retroperitoneal necrosectomy was necessary in 65% of patients. This technique is further described later in the Results section under “combined approach”.

One hundred and twenty seven of 286 (44%) patients had successful percutaneous therapy and did not subsequently require surgical necrosectomy. Mortality was reported in 58 of 286 patients (20.2%). Complications were reported in 80 of 286 patients (28%) and included multiple organ failure^[8,11], colonic perforation^[16], intrabdominal bleeding^[8,11-13], gastrointestinal fistula^[8,11,12,14,15], biliary obstruction^[8], respiratory failure^[8], renal failure^[8], pancreatic fistula^[11,14], new-onset diabetes^[17], pseudocyst^[17], and use of pancreatic enzymes^[11].

Endoscopic necrosectomy

The results of the literature search for endoscopic necrosectomy for pancreatic necrosis are summarized in Table 1. Ten studies, all retrospective, included 352 pa-

tients ranging from 13 to 93 patients.

Two hundred and seventy four of 352 (78%) patients had successful endoscopic necrosectomy therapy and did not subsequently require surgical necrosectomy. Mortality was reported in 20 of 352 patients (5.6%). Complications were reported in 100 of 352 patients (28%) and included bleeding, fever^[20,23,24], gallbladder puncture^[23], hypotension^[23], deep-vein thrombosis/ pulmonary embolism^[23], peritonitis^[23], *Clostridium difficile colitis*^[25], perforation of necrosis into abdominal cavity^[18,22], pneumoperitoneum^[21,24,27], migration of stent into cyst^[21,23], post-endoscopic retrograde cholangiopancreatography pancreatitis^[24], fistula^[18,23], bowel obstruction^[23], and air embolism^[18]. Median follow up was reported as 5.7 mo to 43 mo.

The specific technique varied among the reports. Usually, a therapeutic or pediatric gastroscope or a viduodoscope was used. Endoscopic ultrasound was used often to locate the necroma cavity and guide initial access. Transmural drainage was then usually performed followed by dilation and irrigation of the debris. This was accomplished most often *via* the stomach, although the duodenum was used in approximately 10%-50% of these patients. The size of the endoscopic cystgastro-

tomy was typically 1.5 cm to 2.0 cm. Endoscopic biopsy forceps and baskets was used to remove solid debris into the stomach, allowing it to be eliminated by digestion and peristalsis. Multiple pigtail stents were left in place to maintain cystgastrostomy patency, and often a nasocystic tube was left in place for post-procedure irrigation. On average, 3.2 endoscopic debridement procedures were necessary to achieve resolution of necrosis.

Laparoscopic necrosectomy

The results of the literature search for laparoscopic necrosectomy for pancreatic necrosis are summarized in Table 1. Two studies included 29 patients ranging from 10 to 19 patients^[28,29]. Each was a retrospective study.

Twenty-three of 29 (79%) patients had successful laparoscopic necrosectomy therapy and did not subsequently require open necrosectomy. Mortality was reported in 3 of 29 patients (10.3%). Complications were reported in only the study by Parekh^[28] and included *Clostridium difficile* infection, reintubation, central line infection, delirium tremens, pseudomonas pneumonia, and minor wound complications. The study also notes 11 patients were diagnosed with a pancreatic fistula. Thus the complication rate was calculated as 11 of 19 patients (58%). Zhu *et al.*^[29] did not report complications of the procedure, only mortality. Median follow up was not available for either study.

Parekh^[28] described using three ports for access: a hand access device and two standard laparoscopic ports. Access to the retroperitoneum was obtained either through an infracolic approach or through the greater omentum between the stomach and colon. Gentle finger dissection was used for debridement and several drains were left for postoperative drainage. Four patients that were referred to the service were not considered for a laparoscopic approach because two patients had ileus and anasarca, and two patients had gastrointestinal perforations from prior interventions at other hospitals.

Zhu *et al.*^[29] described using at least four standard ports, and going through the gastrocolic ligament to approach the pancreas. A fan retractor was used to elevate the stomach for exposure. Four to six drainage tubes were used for postoperative lavage. Selection criteria were not described.

Retroperitoneal necrosectomy

The results of the literature search for endoscopic necrosectomy for pancreatic necrosis are summarized in Table 1.

Five studies included 197 patients ranging from 10 to 137 patients^[17,30-33]. There were three retrospective studies^[17,30,31] and two prospective case series^[32,33]. 173 of 197 (88%) patients had successful retroperitoneal necrosectomy therapy and did not subsequently require open necrosectomy. Mortality was reported in 33 of 197 patients (17%). Complications were reported in 90 of 197 patients (46%) and included colonic fistula^[17], gastric perforation^[31], duodenal perforation^[31], enteric fistula^[30],

bleeding^[17,30,33], pseudocyst^[17,30,33], pancreatic fistula^[17], incisional hernia^[17], myocardial infarct^[30], cerebrovascular event^[30], biliary stricture^[30], pulmonary embolus^[30], colonic necrosis^[30], hepatic portal/superior mesenteric/splenic vein thrombosis^[30], *Clostridium difficile* infection^[30], residual abscess^[31], pneumonia^[31], respiratory and liver failure^[33], and gastric ileus^[33].

Technique varied among the five studies. Gambiez *et al.*^[17] described using a short lumbotomy (6 cm in length) centered on the 12th rib. The spleen and descending colon (or ascending colon if on right side) were mobilized anteriorly and pancreas was accessed without violating the peritoneum. A 23 cm mediastinoscope was used for direct vision. Necrotic areas were removed with blunt dissection using a suction metal tube. A tube drain was left to facilitate later drainage. Similarly, Castellanos *et al.*^[32] described a left translumbar approach. However, the group used a flexible endoscope for access followed by flushing and aspiration under direct guidance.

Chang *et al.*^[31] described using a 5 cm skin incision below the costal margin, followed by blunt dissection. A Yankauer sucker was then used for probing the abscess cavity and/or removing necrotic discharge.

Raraty *et al.*^[30] described first placing a 12F pigtail catheter under CT-guidance. After moving the patient to the operating room, the catheter was exchanged over a guide wire with serial dilators to 30F size. A nephroscope was then used for access, and a metal forceps used for piecemeal removal. Two drains were placed for irrigations. Carter *et al.*^[33] used a similar technique with the nephroscope but also used a flexible endoscope for direct access.

Combined approach

Based on anecdotal success of percutaneous radiological and endoscopic necrosectomy used as primary therapy with avoidance of open necrosectomy, an increasing number of reports have utilized combinations of non-surgical approaches to treat pancreatic necrosis. A prospective randomized multicenter trial called the Minimally Invasive Step Up Approach Versus Maximal Necrosectomy in Patients with Acute Necrotising Pancreatitis (PANTER) was recently performed in the Netherlands^[11]. After the diagnosis of necrotizing pancreatitis or infected pancreatic necrosis was made, patients were randomly assigned to either a “step-up” approach or open necrosectomy. The step-up approach consisted of percutaneous drainage or endoscopic drainage, followed by a minimally invasive retroperitoneal necrosectomy if necessary. A video-assisted retroperitoneal debridement (VARD) with postoperative lavage was then performed three days after if there was no clinical improvement. Major complications or death occurred in 31 of 45 patients after open necrosectomy (69%) *vs* 17 of 43 patients after the step-up approach (40%) (risk ratio with the step-up approach, 0.57; 95% CI, 0.38 to 0.87; *P* = 0.006). About 35% of patients in the step up group were successfully able to be managed with percutaneous drainage only^[11].

Similar to the PANTER Trial, there is also a recent, prospective multicenter, single-arm study based out of the University of Washington. Percutaneous drainage was used as initial treatment for infected pancreatic necrosis. If there was a 75% reduction in size based on follow-up scan 10 d later, the remainder of their treatment would be percutaneous drains alone. If patients did not have a 75% reduction they were treated with a VARD. Twenty-three percent of patients were treated with percutaneous drains only. Sixty percent of patients were treated with a “minimally invasive intervention” (drains with or without VARD). Mortality at 30 d was 2.5%.

The results of these two studies are summarized in Table 1.

DISCUSSION

Infected pancreatic necrosis and pancreatic abscess are serious complications of severe acute pancreatitis. However, the last two decades have demonstrated much innovation and variety in minimally invasive techniques. These new techniques can potentially lower the significant morbidity and mortality of these complications. The less invasive approach can potentially keep an infection compartmentalized, specifically avoiding contamination of virgin spaces, such as the peritoneal cavity. It may reduce systemic inflammatory and septic response as a consequence of a major open operation and release of infected necrosis.

The percutaneous approach to infected pancreatic necrosis has been shown to be safe and feasible in multiple retrospective case series. It is significant to note, 44% of patients in the studies reviewed did not need surgical therapy. Conclusions are limited, however, since the studies are retrospective and small. We limited our review to include only studies that examined 10 or greater patients. Selection criteria was also not often reported and thus, it may be difficult to predict which subset of patients would most benefit from percutaneous drainage alone. In addition, repeat procedures were often needed, ranging from 1 to 12 procedures in the above-mentioned studies. What has become increasingly popular is the combination of percutaneous technique combined with a VARD as mentioned in the PANTER trial and the Horvath study^[11,34]. These studies not only confirmed that there is a subgroup of patients that can benefit from percutaneous drainage alone but also examined a combined technique in a prospective fashion with a relatively larger amount of patients.

The endoscopic approach to necrosectomy has also been shown to be safe and feasible with an acceptable procedure-related morbidity. The endoscopic ultrasound has allowed this approach to evolve allowing a better definition of the fluid collection and surrounding vasculature. The approach can also be used in poor risk surgical candidates. However, this technique though is not available at all institutions and often there is a need for repeated procedures for maximal drainage. Furthermore, not all pancreatic abscesses and pancreatic necrosis are

accessible *via* a transgastric or transduodenal approach. Like percutaneous drainage alone, often repeat procedures are needed.

Laparoscopic necrosectomy may provide better access to fluid collections not amenable to endoscopic approach. This may facilitate a more thorough debridement of the cavity. The laparoscopic approach has also been demonstrated to be safe in several small case series. In the two studies reported 23 of 29 (79%) patients did not require an open necrosectomy. As previously discussed, these are highly selected patients: these were likely stable patients that were able to tolerate pneumoperitoneum. Morbidity still exists in this approach including enterocutaneous and pancreatic fistulas.

Similar to the laparoscopic approach, retroperitoneal necrosectomy has been shown to be effective in select patients: 173 of 197 (88%) patients did not need an open necrosectomy. The approach has the theoretical advantage of not having transmission of intraperitoneal infection from the necrotic area compared to laparoscopy. This technique allows access to areas not accessible *via* endoscopy and the potential to remove all necrotic tissue from the area.

Because of their relatively rare presentation, reports on these minimally invasive approaches to pancreatic necrosis and abscess have mostly small numbers of patients and are mostly retrospective in nature. Selection and publication bias is inherent in these studies and the low morbidity and mortality demonstrated in these studies have to be interpreted as such. There is also a significant amount of heterogeneity and variety of technique within each type of different approach.

In addition, several studies utilize a combined technique using percutaneous drainage first followed by endoscopic, laparoscopic, or retroperitoneal necrosectomy. Percutaneous drainage likely temporizes the patient in early phases of infected necrosis.

In the future, we look for more prospective studies that use definitions as laid out clearly by the Atlanta classification. Several of the above studies do not strictly adhere to those classifications. Furthermore, complications, specifically major ones including pancreatic or enteric fistula and major hemorrhage should separately be reported. Additionally, some studies do not report the amount of days from presentation to time of necrosectomy. It is well-accepted now that a delay of 28 d between onset of symptoms and intervention can potentially lower morbidity and mortality^[7].

Up until recently, there has been no comparison of results between open necrosectomy and these minimally invasive techniques. The only randomized control trial to date comparing a minimally invasive approach versus an open approach is the PANTER trial recently published. The minimally invasive approach is actually a “step-up” approach in which the first step is either a percutaneous or endoscopic transgastric approach. A VARD procedure was then performed if those approaches were unsuccessful or if the patient did not clinically improve in 72 h. The step-up approach reduced the rate of the

composite end point of major complications or death. In addition, the step-up approach yielded less incisional hernias and new-onset diabetes. The authors hypothesize that in open necrosectomy, there is potentially viable pancreatic parenchyma that is unintentionally debrided leading to diabetes as a late complication. This is potentially the reason why other minimally invasive techniques have shown good outcomes.

It is important to note, that the PANTER trial is not a direct comparison between open necrosectomy and minimally invasive necrosectomy, rather a comparison between treatment strategies. Perhaps this is a better model moving forward in contrast to doing direct comparisons between open necrosectomies or among the different types of minimally invasive approaches. It is likely that these pancreatic fluid collections are amenable to multiple approaches.

Complications of severe acute pancreatitis remain a complex problem to treat. We advocate a multidisciplinary approach with interventional radiologists, gastroenterologists, intensivists, and hepatobiliary surgeons at tertiary care centers. Since comparison data is limited, the minimally invasive approach should be based on location of lesion and individual patient presentation. Challenges moving forward will be learning curves, technology improvements, and product cycles of new techniques. This report provides a brief overview of the evolving minimally invasive approaches as alternatives to open necrosectomy.

COMMENTS

Background

A variety of minimally invasive approaches have been described for treatment of infected pancreatic necrosis and pancreatic abscess.

Research frontiers

Early, open necrosectomy for infected pancreatitis was the traditional mainstay of surgical therapy for these critically ill patients. Current practice has shifted toward, with intervention ideally delayed for a minimum of two and preferably four weeks from the onset of illness. Moreover, various minimally invasive surgical, endoscopic, and percutaneous drainage/debridement strategies have emerged as alternatives to open operation.

Innovations and breakthroughs

This systematic review summarized studies published between 1996 and 2010 with greater than 10 patients. The quality of these published reports is limited by their retrospective design, selection bias, and possible publication bias.

Applications

Treatment algorithms for infected pancreatic necrosis should be multidisciplinary, reflective of local expertise and experience, and tailored to the individual presenting circumstances of each patient.

Terminology

Pancreatic necrosectomy refers to the debridement and drainage of necrotic pancreatic and peripancreatic tissue.

Peer review

An exhaustive review of the literature leading to the best summary of minimally invasive surgical approaches to infected pancreatic necrosis.

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Dynamic magnetic resonance defecography in 10 asymptomatic volunteers

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Abstract

AIM: Evaluation of the wide range of normal findings in asymptomatic women undergoing dynamic magnetic resonance (MR) defecography.

METHODS: MR defecography of 10 healthy female volunteers (median age: 31 years) without previous pregnancies or history of surgery were evaluated. The rectum was filled with 180 mL gadolinium ultrasound gel mixture. MR defecography was performed in the supine position. The pelvic floor was visualized with a dynamic T2-weighted sagittal plane where all relevant pelvic floor organs were acquired during defecation. The volunteers were instructed to relax and then to perform straining maneuvers to empty the rectum. The pubococcygeal line (PCGL) was used as the line of reference. The movement of pelvic floor organs was mea-

sured as the vertical distance to this reference line. Data were recorded in the resting position as well as during the defecation process with maximal straining. Examinations were performed and evaluated by two experienced abdominal radiologists without knowledge of patient history.

RESULTS: Average position of the anorectal junction was located at -5.3 mm at rest and -29.9 mm during straining. The anorectal angle widened significantly from 93° at rest to 109° during defecation. A rectocele was diagnosed in eight out of 10 volunteers showing an average diameter of 25.9 mm. The bladder base was located at a position of +23 mm at rest and descended to -8.1 mm during defecation in relation to the PCGL. The bladder base moved below the PCGL in six out of 10 volunteers, which was formally defined as a cystocele. The uterocervical junction was located at an average level of +43.1 mm at rest and at +7.9 mm during straining. The uterocervical junction of three volunteers fell below the PCGL; described formally as uterocervical prolapse.

CONCLUSION: Based on the range of standard values in asymptomatic volunteers, MR defecography values for pathological changes have to be re-evaluated.

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Key words: Magnetic resonance imaging; Defecography; Standard values

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INTRODUCTION

As a result of the complex anatomy and synergism of pelvic organs and their muscular structures, there is a wide variety of static and functional disorders. Outlet obstruction, anism, dyskinesia of the puborectal muscle, intussusception, prolapse of the anus and rectum, vaginal prolapse, rectocele, cystocele, and enterocele represent common diagnoses in proctology, urology and gynecology. Besides constipation, fecal or urinary incontinence is the most common symptom, with serious problems for the patients that have a major negative impact on quality of life. Women are affected at a significantly higher rate with a ratio of 9:1^[1]. Patients usually attend gynecologists, urologists and proctologists. Therefore, a comprehensive interdisciplinary approach for diagnosis and therapy is the most promising strategy.

A systematic medical history as well as a thorough proctological examination (inspection, palpation, rectoscopy, and proctoscopy) along with manometry and endosonography is the basic diagnostic approach for complex pelvic floor disorders. Furthermore, radiological imaging with evacuation proctography can give important additional information^[2].

With the development of magnetic resonance imaging (MRI) technology, dynamic MRI of the pelvic floor has become an important alternative for the diagnosis of complex combined pelvic floor disorders. Since its first introduction by Yang *et al*^[3] and Kruyt *et al*^[4] in 1991, MRI has increasingly replaced evacuation proctography, which was first described in 1952 by Walldén^[5], for evaluation of outlet obstruction.

For the evaluation of extraluminal pelvic disorders such as enterocele or utero-vaginal prolapse, MRI of the pelvic floor is favorable in several aspects compared to clinical examination and conventional evacuation proctography, which does not depict extraluminal structures^[3,4,6-10]. Additionally, MRI examination can be repeated because of the total lack of ionizing radiation. This may increase the chances of detecting pathological findings in some patients^[9]. The horizontal position of the patient during MR defecography may be a disadvantage, because it could influence the pelvic floor physiology as well as the dynamic defecation process. Therefore, some authors consider that videoproctography is still superior to MRI for assessment of an enterocele or rectocele^[11,12].

So far, patient preparation, examination technique, as well as reference lines for the evaluation of MRI are still not standardized and findings differ widely in the current literature. Most of the previously performed studies did not examine the defecation process itself. Normal find-

ings and values were defined in small control groups and therefore were only applicable within the particular study setting.

The aim of the present study was to show the wide range of normal findings in asymptomatic female volunteers and therefore the necessity of obtaining common standards not only in terms of patient preparation, but also in the evaluation of numeric values for the definition of pathological and normal dynamic MR examinations of the pelvic floor.

MATERIALS AND METHODS

Volunteers

Ten healthy female volunteers (median age: 31 years, range: 22-40 years) without previous pregnancy or history of any gynecological, urological or proctological surgery were evaluated by dynamic pelvic MRI. The volunteers had no symptoms of incontinence, constipation or other stool evacuation problems. None of the volunteers had any contraindications for MRI. Informed consent was obtained from all volunteers. We applied a uniform admission questionnaire for standardized documentation of patient history. The study was approved by the local ethics committee.

Dynamic MRI of the pelvic floor

All healthy volunteers were asked to empty their bladder 3 h before the examination to achieve a medium filling of the urinary bladder during MRI. The rectum was filled with 180 mL ultrasound gel mixture (1% Gd-DTPA-GEL-Mixture). MRI (1.5 Tesla MRI, Magnetom Symphony; Siemens, Erlangen, Germany) was performed in the supine position with hips and knees bent at 45°. The pelvic floor was visualized in three planes (transversal, coronal, sagittal, T1 and T2) to find the appropriate sagittal plane in which all relevant pelvic floor organs were acquired during defecation over 55 s at a frequency of one shot per 1.1 s [True FISP (True Fast Imaging with Steady State Precession), TR: 5.8 ms, TE: 2.8 ms]. Slice thickness was 7 mm (field of view: 270 mm × 270 mm, matrix 256 × 256). During the examination, healthy volunteers were instructed via headphones. They first were asked to relax and then to perform straining maneuvers to empty the rectum as completely as possible. The sequences were acquired digitally and analyzed cinematographically.

MRI evaluation

The pubococcygeal line (PCGL) was defined as the line of reference (Figure 1). Movement of pelvic floor organs was measured as the vertical distance with reference to this line. The location of structures above the PCGL were marked with positive values (+), structures located below this line were marked negative (-).

Data were recorded in the resting position as well as during the defecation process with maximal straining. All data represents mean values with corresponding SD.

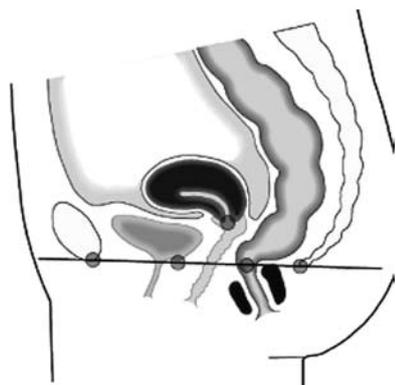


Figure 1 The pubococcygeal line (black) according to Yang *et al*^[3] from the most inferior part of the pubic symphysis to the last coccygeal joint; furthermore, gray dots show (from left to right) bladder base, uterocervical junction, and anorectal junction.

The anorectal junction was defined as the intersection point of the central axis of the anal canal and a line along the posterior rectal wall. The anorectal angle was measured between these two lines^[3,7,9].

According to the current literature, based on different evaluation techniques, a rectocele is defined as a bulge of the anterior rectal wall of > 20-30 mm^[11,13]. For our data analysis we applied the method described by Delemarre, who defined the distance from the anorectal junction to the tip of the protrusion of the anterior rectal wall as the correct measurement^[11].

MRI revealed data about the position of the bladder base, the uterocervical junction and the vagina. Additionally the location of small bowel in relation to the PCGL was assessed.

A cystocele and a uterovaginal prolapse were diagnosed if the bladder base or the uterocervical junction fell below the PCGL during defecation^[9,10]. Widening of the rectovaginal space or a descent of mesenteric parts, small bowel or sigmoid colon beyond the PCGL during defecation was defined as an enterocele^[8,10].

All examinations were performed and evaluated in consensus by two experienced board-certified abdominal radiologists without any knowledge of the volunteers history or age.

RESULTS

All 10 volunteers were able to hold the rectal enema and perform defecation within the MR scanner according to the examination protocol. The average position of the anorectal junction was located at -5.3 mm (\pm 9.9 mm) at rest and descended to -29.9 mm (\pm 10.3 mm) during maximal straining. The average anorectal angle widened significantly from 93° (\pm 4.8°) at rest to 108.7° (\pm 14.7°) at maximal straining during defecation. A rectocele based on the current definition of an anterior bulging of the anterior rectum wall of at least 20 mm was diagnosed in eight of 10 volunteers, showing an average size of 26 mm (\pm 6 mm).

The bladder base was located at an average position

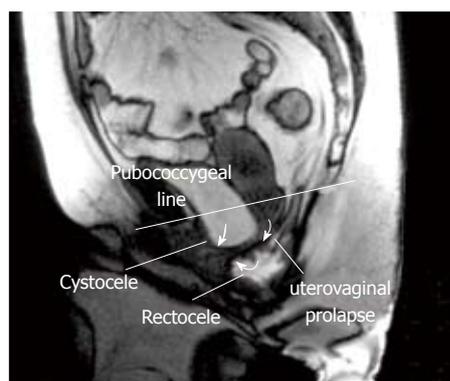


Figure 2 Dynamic magnetic resonance imaging of a 36-year-old healthy female volunteer. At the end of defecation, a rectocele, cystocele and uterovaginal prolapse were visualized.

Table 1 Results of magnetic resonance defecography of 10 healthy asymptomatic volunteers

Anatomic structure	Rest	Straining	Relative movement
Anorectal junction (mm)	-5.3 (\pm 9.9)	-29.9 (\pm 10.3)	-24.6
Anorectal angle (°)	93.0 (\pm 4.8)	108.7 (\pm 14.7)	15.7
Bladder base (mm)	23.0 (\pm 4.6)	-8.1 (\pm 11.1)	-14.9
Uterovaginal junction (mm)	43.1 (\pm 7.8)	7.9 (\pm 16.5)	-35.2

A positive value represents the lowest position of the pelvic structure above the pubococcygeal line, a negative value marks structures located below the pubococcygeal line. The values represent the median measured distance in millimeters; the numbers in brackets represent the range.

of +23 mm (\pm 4.6 mm) at rest and descended to -8.1 mm (\pm 11.1 mm) during defecation. The bladder base moved below the PCGL in six of 10 healthy volunteers during maximal straining, which is defined as a cystocele (Figure 2).

The uterocervical junction or vaginal vault was located at an average level of +43.1 mm (\pm 7.8 mm) at rest and at +7.9 mm (\pm 16.5 mm) during maximal straining. The uterocervical junction of three volunteers descended below the PCGL at maximal straining during defecation, described as uterocervical prolapse according to the common definition in the literature (Figure 2). No enterocele or other additional pathological findings were detected in any volunteers. Table 1 shows an overview of all results and findings of the dynamic MRI examinations.

DISCUSSION

Dynamic MRI of the pelvic floor, also known as MR defecography, is increasingly used for the diagnosis of complex pelvic floor disorders and replaces conventional videoproctography. Different medical specialties such as radiology, urology, gynecology and surgery are involved in the interpretation and evaluation of findings and therefore need an accepted definition of normal values and clinical practical reference lines for image assessment.

Back in 1991, Kruyt *et al.*⁴ were the first authors using MRI to study functional aspects of the anorectal region. In their study, the anatomy of the anorectum and the anorectal angle was examined in 10 healthy volunteers placed in the prone position within the MRI scanner. The symphysiosacral line reaching from the most superior part of the pubic symphysis to the lower part of the sacrum, was taken as the reference line to evaluate the mobility of the pelvic organs. Dynamics were measured during contraction and relaxation of the pelvic floor and during straining. In this study, the defecation process itself was not visualized because no contrast medium or enema was applied to the rectum.

Also in 1991, Yang *et al.*³¹ introduced dynamic MRI as a new method for the diagnosis of the descending perineum in women. They compared 26 patients with 16 asymptomatic women in the supine position. Values were documented with various degrees of straining maneuvers. Again, defecation itself was not because the rectum was not filled with contrast medium. The PCGL was taken as the reference line to evaluate the degree of descent of the pelvic structures.

Another study comparing clinical examination, videoproctography and dynamic MRI in the diagnosis of anterior rectoceles was published by Delemarre *et al.*¹¹¹. In this study patients were examined in the prone position without any rectal enema, which again made evaluation of the defecation process itself impossible. The pubosacral line reaching from the most inferior part of the pubic symphysis to the lower part of the sacrum was chosen as the reference line for MRI. Measurements were performed at rest and during straining for both imaging techniques.

The pubosacral line was also used by Goodrich *et al.*⁶ who examined five female patients with descending perineum syndrome pre- and postoperatively, as well as 10 asymptomatic female volunteers undergoing MRI. In contrast to the previous study, patients were placed in the supine position. The authors did not state whether the rectum, vagina or bladder had been contrasted.

Healy *et al.*^{8,14} have analyzed various aspects of pelvic floor disorders in patients and healthy volunteers comparing videoproctography with dynamic MRI with patients placed in the supine position. The defecation maneuver was not acquired. In contrast to other studies^{9,10,12}, instead of contrast medium, a tampon was placed in the rectum. Measurements were performed during maximal straining, while the lower PCGL was the reference landmark.

Tacke¹⁵ has tested a new method of dynamic MRI with radial real-time imaging and a reduced image area for defecography. Patients in the supine position were asked to void a condom filled with a gadolinium-based contrast gel. The authors discussed this form of rectal filling critically as it may mask intussusception or latent incontinence.

Vanbeckevoort *et al.*¹² compared colpocystoproctography (videoproctography with opacification of vagina

and bladder) and dynamic MRI in supine position. For MRI, the rectum was filled with 100 mL of ultrasound gel, which was not meant to be voided. Measurements were taken during maximal straining using the PCGL as a reference line.

Lienemann *et al.*¹⁰ compared colpocystoproctography and MRI for the diagnosis of enteroceles. In MRI, patients and healthy volunteers were placed in a supine position and the rectum was filled with 200 mL of ultrasound gel, which was to be defecated during imaging. The study does not reveal data on frequency and completeness of the defecation process. Reference line was the PCGL.

In a technically equal approach, Lienemann *et al.*⁹ examined 44 female patients and five asymptomatic volunteers for descent of the pelvic floor. Patients and volunteers were asked not to void the gel. Additionally, there was no discussion of the findings of the healthy volunteers in this study.

Hilfiker *et al.*¹⁶ and Schoenberger *et al.*¹⁷ presented an “open system” MRI where patients were examined in an upright position analogous to videoproctography. In the study of Schoenberger *et al.*¹⁷, the findings of 15 patients examined with videoproctography and this new form of open configured MRI were compared. Five healthy volunteers were included for the definition of normal values, which are not mentioned in the paper.

This overview illustrates the problems arising with the introduction of a new examination technique. So far, dynamic MRI is widely accepted as a promising technique for the diagnosis of the pelvic floor^{3,4,6,9,10}, particularly for functional aspects of pelvic floor disorders because pelvic organs and muscles can be visualized and evaluated without invasive opacification and without any exposure to ionizing radiation. Still, without standardization of patient preparation, examination technique and evaluation of the data according to standardized reference lines and landmarks, it may not yet replace a well-established technique like videoproctography or colpocystoproctography. We believe that a comparison of both procedures has to take place under standardized conditions. In this context, the documentation of the defecation process is most important for subsequent comparability of both techniques. Vanbeckevoort *et al.*¹² have compared the results of 35 patients examined with colpocystoproctography with and without defecation with the findings of dynamic MRI without defecation. For colpocystoproctography the urinary bladder and the small bowel were filled with a contrast medium. In their analysis, colpocystoproctography including defecation was by far superior to the same technique without defecation. Based on the observation that the pelvic floor reached its maximum downward movement only during defecation, and supported by the fact that patients are placed in a horizontal position in MRI, the authors concluded that colpocystoproctography including defecation may also be superior to MRI without defecation. Our results also revealed that the formation of a rectocele and

Table 2 Patient preparation, evaluation techniques, normal values and findings as described by other authors

	Yang <i>et al.</i> ^[3]	Kruyt <i>et al.</i> ^[4]	Vanbeckevoort <i>et al.</i> ^[12]	Lienemann <i>et al.</i> ^[9]	Healy <i>et al.</i> ^[14]	Paetzel <i>et al.</i> ^[19]
Defecation	No	No	No	No	No	Yes
Rectal contrast	No	No	100 mL µg	200 mL µg	Plastic tube	180 mL µg
Bladder contrast	No	No	No	60 mL sodium chloride	No	No
Vaginal contrast	No	No	No	50 mL µg	Plastic tube	No
Position	Horizontal	Prone	Horizontal	Horizontal	Horizontal	Horizontal
Reference line	Pubococcygeal	Symphysisacral	Pubosacral	Pubococcygeal	Pubococcygeal	Pubococcygeal
Anorectal junction	-25 mm	-30 mm to -40 mm	-25 mm	No information	-20 mm	-5.3 mm
Bladder base	-10 mm	No information	± 0	± 0	-10 mm	+23 mm
Uterocervical junction	+10 mm	No information	No information	+0	± 0	+43 mm
Rectocele (evaluation)	No information	No information	Yoshioka	Yoshioka	Yoshioka	Delemarre
Rectocele (size)	No information	no information	< 30 mm	< 30 mm	no information	26 mm
Anorectal angle	No information	< 130° (rest)	No information	No information	No information	93° (rest)

a cystocele as well as the maximal descent of the anorectal junction and of the uterocervical junction was only completely visible at the end of the defecation process but not during straining alone.

In our study, the rectum was filled with 180 mL ultrasound gel with a gadolinium-based contrast medium. Ikenberry *et al.*^[18] have shown that varying viscosity of the contrast medium does not significantly influence the findings in videoproctography. Due to the natural differences in signal intensity of the urinary bladder, vagina, small bowel, and peritoneum, the procedure can be kept on a low level of invasivity.

The lower PCGL was used to visualize relative movements of the pelvic organs during defecation. The anorectal junction and anorectal angle were determined by the central axis of the anal canal and a line along the posterior wall of the rectum^[8,10,14]. According to Delemarre *et al.*^[11], rectoceles were measured from the anorectal junction to the tip of protrusion of the anterior rectum wall. Table 2 gives an overview of different techniques and normal values as described in other studies.

The position of the anorectal junction related to the PCGL at rest and during defecation is one parameter in the evaluation of a descent of the perineum. In the present study the anorectal junction moved 30 mm on average below the PCGL during defecation. In a previously published study by our group we evaluated symptomatic patients with pelvic floor disorders. In this study the anorectal junction moved an average 49 mm below the PCGL, while in patients suffering from a rectal incontinence, an average of 51 mm was measured^[19].

Besides the descent of the anorectal junction, changes in the anorectal angle are commonly used as an indicator for the functional status of the pelvic floor^[20]. A narrowing of the anorectal angle may indicate a disorder of the puborectal muscle^[21]. This may lead to constipation with subsequent straining leading to rectal intussusception, rectocele and mucosal prolapse with a solitary ulcer of the rectum^[21,22]. If the anorectal angle is already widened at rest, this may be a sign of weakness of the pelvic floor and is commonly observed along with incontinence and rectal prolapse^[23-25].

Standard values in the literature vary enormously. For

videoproctography, Hardcastle *et al.*^[26] described normal values between 60° and 105° at rest whereas the findings of other groups^[27-29] relying on control groups with up to 150 volunteers show values between 90° and 104° at rest and 103° to 137° during defecation. Comparing the data with our study we determined an average angle of 93° at rest and 108.7° during straining. Accordingly, the use of changes in the anorectal angle as a diagnostic parameter is difficult not only because data vary significantly but also because findings of patients and asymptomatic volunteers tend to overlap^[4,7,8,15,29].

A patient with an anterior rectocele often presents with symptoms of incomplete defecation and are observed along with a descent of the pelvic floor^[13]. However, a rectocele can also frequently be found in asymptomatic patients. Therefore, some authors assume that symptoms depend on the diameter of the rectocele^[9,29,30]. Due to different approaches in the attempt to measure the expansion of a rectocele, there are still no well-defined normal values available. Delemarre *et al.*^[11] used videoproctography and MRI to examine 38 patients in the prone position without rectal filling and without defecation. He concluded that videoproctography was superior to MRI for diagnosis of rectoceles. He postulated that a symptomatic rectocele > 20 mm needs to be operated. Lienemann *et al.*^[9] and Yoshioka *et al.*^[13] have defined a rectocele as a protrusion of the anterior rectal wall of > 30 mm. Yoshioka *et al.*^[13] have found that, in comparison to clinical examination, MRI was superior to colprocystoproctography in detecting rectoceles. Patients were examined in the supine position with rectal filling during maximal straining but without defecation. In contrast, Healy *et al.*^[8] rated videoproctography as superior to MRI. They have defined expansion of > 20 mm as pathological. In addition, they found that a rectocele < 13 mm measured by videoproctography was totally missed by MRI. MRI examination again was performed without defecation.

According to our observations the horizontal position does not seem to be a disadvantage because a rectocele was found in eight out of 10 healthy volunteers, with an average size of 26 mm (Table 1). This was in accordance with data from the literature^[9,14] where the

incidence of a rectocele in asymptomatic volunteers was about 80%, although these are described as “small” rectoceles.

Incidence and degree of a cystocele and an uterocervical prolapse is usually connected with the number of vaginal deliveries, preceding hysterectomy, and chronic constipation with increased straining maneuvers^[12]. Besides clinical examination, imaging techniques make quantification of findings possible^[9].

Yang *et al.*^[3], Lienemann *et al.*^[9], Vanbeckevoort *et al.*^[12] and Healy *et al.*^[7,14] have used normal values for the descent of the bladder base and uterocervical junction during straining in relation to the PCGL. These values were raised partly in healthy volunteers and partly determined at random.

Due to the natural differences in signal intensity of the urinary bladder and the vagina there was no necessity to apply additional contrast media^[16]. In our study, six of 10 asymptomatic healthy female volunteers without a history of previous delivery or surgery showed the finding of a cystocele, and in three of 10 a vaginal prolapse was diagnosed.

In the studies of Lienemann *et al.*^[31] and Sprenger *et al.*^[32], 20 and 39 healthy females were examined with dynamic MRI of the pelvic floor, including defecation. A cystocele or vaginal prolapse was detected in none of them.

An enterocele is defined as herniation of the peritoneum into the rectovaginal space^[33], which may contain small bowel loops or sigmoid colon. They are often accompanied by severe defecation disorders and a sensation of pressure as well as downward movement of the pelvic floor^[34]. The prevalence of an enterocele in women is between 18% and 37%^[35,36]. They often occur after hysterectomy^[33]. No enterocele was detected in any volunteer. Lienemann *et al.*^[10] examined 55 patients and 11 asymptomatic volunteers with colpocystoproctography and dynamic MRI, without application of contrast medium into the peritoneum or small bowel. MRI had a clear advantage because the peritoneum and contents of the enterocele were easily identified. He concluded that MRI may replace colpocystoproctography in the diagnosis of an enterocele.

It proved to be useful to acquire the complete defecation process after a rectal enema to detect potential pathological changes. A rectocele can easily be identified by dynamic MRI. The pelvic organs of healthy volunteers show a relatively high mobility, therefore, presently suggested normal values for the position of pelvic organs in relation to the PCGL have to be redefined. It is necessary to evaluate normal values under standardized investigation conditions and with large groups of healthy volunteers.

physiology of the pelvic floor we have come across a wide variety of disorders.

Research frontiers

Imaging plays an important role in the diagnostic workup of complex combined pelvic floor disorders. Functional examinations like videoproctography provide valuable information but it has been increasingly replaced by dynamic magnetic resonance imaging (MRI) that can visualize intra- and extraluminal structures. Further strengths of MRI are excellent soft tissue contrast and the lack of ionizing radiation.

Innovations and breakthroughs

There have been several studies about the diagnostic value of dynamic MRI of the pelvic floor; most of them with symptomatic patients. This investigation is believed to be the first to document the range of normal findings in MR defecography of asymptomatic female volunteers.

Applications

The wide range of normal findings in this study emphasizes the necessity of obtaining common standards for the evaluation of MR defecography.

Terminology

MR defecography is a dynamic pelvic MRI examination. After optional application of a rectal enema, defecation is visualized by a series of fast MRI sequences.

Peer review

This is a good study but the authors should make some revision.

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COMMENTS

Background

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Improved techniques for double-balloon-enteroscopy-assisted endoscopic retrograde cholangiopancreatography

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Abstract

AIM: To investigate the clinical outcome of double balloon enteroscopy (DBE)-assisted endoscopic retrograde cholangiopancreatography (DB-ERCP) in patients with altered gastrointestinal anatomy.

METHODS: Between September 2006 and April 2011, 47 procedures of DB-ERCP were performed in 28 patients with a Roux-en-Y total gastrectomy ($n = 11$), Billroth II gastrectomy ($n = 15$), or Roux-en-Y anastomosis with hepaticojejunostomy ($n = 2$). DB-ERCP was performed using a short-type DBE combined with several technical innovations such as using an endoscope attachment, marking by submucosal tattooing,

selectively applying contrast medium, and CO₂ insufflations.

RESULTS: The papilla of Vater or hepaticojejunostomy site was reached in its entirety with a 96% success rate (45/47 procedures). There were no significant differences in the success rate of reaching the blind end with a DBE among Roux-en-Y total gastrectomy (96%), Billroth II reconstruction (94%), or pancreatoduodenectomy (100%), respectively ($P = 0.91$). The total successful rate of cannulation and contrast enhancement of the target bile duct in patients whom the blind end was reached with a DBE was 40/45 procedures (89%). Again, there were no significant differences in the success rate of cannulation and contrast enhancement of the target bile duct with a DBE among Roux-en-Y total gastrectomy (88%), Billroth II reconstruction (89%), or pancreatoduodenectomy (100%), respectively ($P = 0.67$). Treatment was achieved in all 40 procedures (100%) in patients whom the contrast enhancement of the bile duct was successful. Common endoscopic treatments were endoscopic biliary drainage (24 procedures) and extraction of stones (14 procedures). Biliary drainage was done by placement of plastic stents. Stones extraction was done by lithotomy with the mechanical lithotripter followed by extraction with a basket or by the balloon pull-through method. Endoscopic sphincterotomy was performed in 14 procedures with a needle precutting knife using a guidewire. The mean total duration of the procedure was 93.6 ± 6.8 min and the mean time required to reach the papilla was 30.5 ± 3.7 min. The mean time required to reach the papilla tended to be shorter in Billroth II reconstruction (20.9 ± 5.8 min) than that in Roux-en-Y total gastrectomy (37.1 ± 4.9 min) but there was no significant difference ($P = 0.09$). A major complication occurred in one patient (3.5%); perforation of the long limb in a patient with Billroth II anastomosis.

CONCLUSION: Short-type DBE combined with several technical innovations enabled us to perform ERCP in most patients with altered gastrointestinal anatomy.

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Key words: Double-balloon enteroscopy; Endoscopic retrograde cholangiopancreatography; Pathological anatomy; Pancreatobiliary disease

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INTRODUCTION

Patients who have undergone bowel reconstruction have been considered unsuitable for endoscopic retrograde cholangiopancreatography (ERCP) using conventional endoscopy^[1-6]. This is because the endoscopic approach to the afferent loop, blind end, and the choledochojunostomy site using a conventional endoscope is difficult because of the distance from the gastrojejunal anastomosis site, the unusual anatomical features of the intestine such as its winding form, and postoperative adhesions. Recently, double balloon enteroscopy (DBE) has been reported to be of significant benefit for the diagnosis and treatment of biliary and pancreatic diseases in such patients^[7-13]. DBE enables us to advance much deeper into the small intestine than using a conventional push-enteroscope^[14-16]. It is still difficult, however, not only to reach the papilla of Vater or the site of choledochojunostomy, but also to cannulate selectively into the pancreatic and/or biliary duct in ERCP using DBE (DB-ERCP). Therefore, further contrivances including both instrumental and technical innovations have been required to improve the outcome of DB-ERCP. One of the most significant instrumental innovations is a short-type DBE, which has been recently developed, and contributes to increased success rates not only for reaching the papilla and blind end but also for ERCP-related interventions because of its short working length and the availability of various accessory devices^[17,18]. There has still been a need for developing the technical innovations to obtain more favorable outcome of the DB-ERCP. This study describes several technical innovations and tips in the use of a short-type DBE to improve clinical outcomes of DB-ERCP.

MATERIALS AND METHODS

Patient population

Between September 2006 and April 2011, 48 ERCP procedures were analyzed in 28 patients (10 female, 18 male; age 54-91 years, mean 74.0 years) with pancreatobiliary diseases who had previously undergone bowel reconstruction. The bowel reconstruction methods included Roux-en-Y total gastrectomy in 11 patients (26 procedures), Billroth II gastrectomy in 15 patients (18 procedures), and Roux-en-Y anastomosis with pancreaticoduodenectomy in two patients (three procedures). Patients with Roux-en-Y total gastrectomy or Billroth II gastrectomy had naïve papilla ($n = 26$), and those with pancreaticoduodenectomy had hepaticojunostomy ($n = 2$). Twenty-eight consecutive postsurgical patients were included in this study after written informed consent was obtained.

Endoscope and accessories

We used short-type DBE, EC-450B15 (Fujifilm, Saitama, Japan). The DBE system is a high-resolution videoendoscope with a flexible overtube. The videoendoscope has a 2.8-mm working channel and a working length of 1520 mm, and a detachable balloon at its tip. It is used with a soft overtube measuring 1050 mm in length with another balloon at the distal end. The endoscope and overtube balloons are made from latex, which is 0.1 mm thick and very soft. The balloons can be inflated or deflated by a specially designed air pump controller with one-touch controls, while monitoring the air pressure. A soft transparent hood (DH-17EN; Fujifilm) was attached to the tip of a scope in all procedures.

DB-ERCP

All procedures were performed during conscious sedation by giving 35 mg pethidine hydrochloride and 0.5-2 mg of flunitrazepam intravenously. Scopolamine butylbromide was only used after reaching the end of the afferent loop. DBE was performed using a standard technique as described by Yamamoto *et al.*^[14], Kita *et al.*^[15] and May *et al.*^[16], and DB-ERCP was carried out as follows. When an endoscopist was not sure whether the endoscope was in the afferent loop, selective contrast enhancement in the intestine was performed with a balloon inflated on the endoscope tip, allowing the endoscopist to confirm the direction of the afferent loop under fluoroscopy^[17,18]. Although formerly we used standard air insufflations during the procedure, since April 2009 we have used CO₂ instead of standard air insufflations to improve intubation depth during DBE, as well as to reduce postprocedural pain^[19]. In the first procedure, the beginning of the Roux limb was marked by submucosal tattooing (Indian ink) in patients with a Roux-en-Y anastomosis (Figure 1). The time taken for this procedure and the whole procedure was recorded.

Once the endoscope reached the papilla or ductal anastomosis, appropriate stabilization of the endoscope with the overtube and/or enteroscope balloon

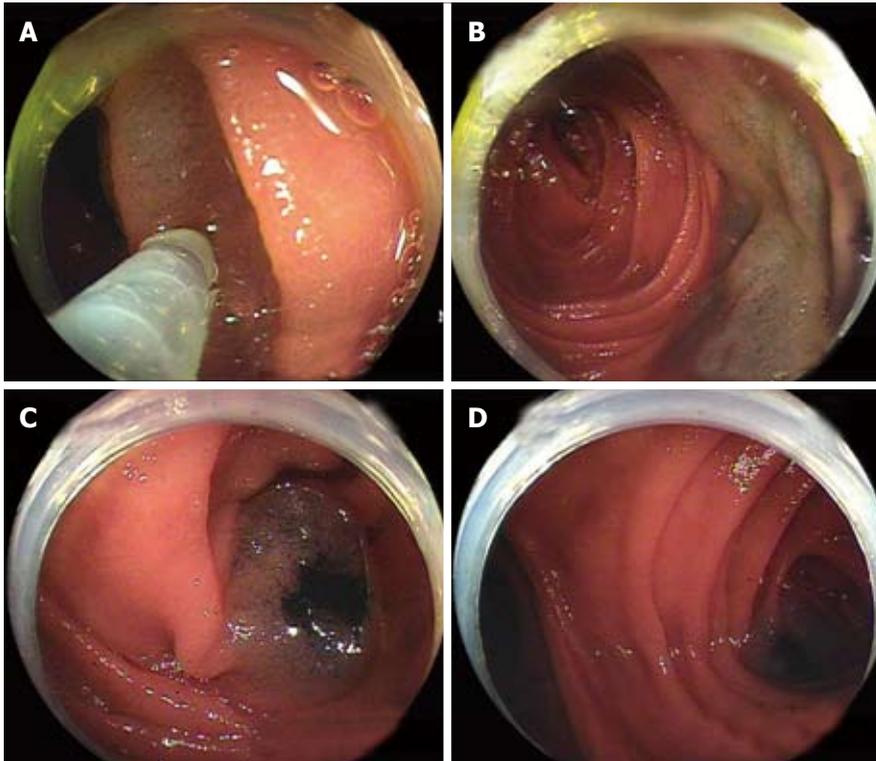


Figure 1 In the first procedure, the beginning of the Roux limb was marked by submucosal tattooing in patients with a Roux-en-Y anastomosis.

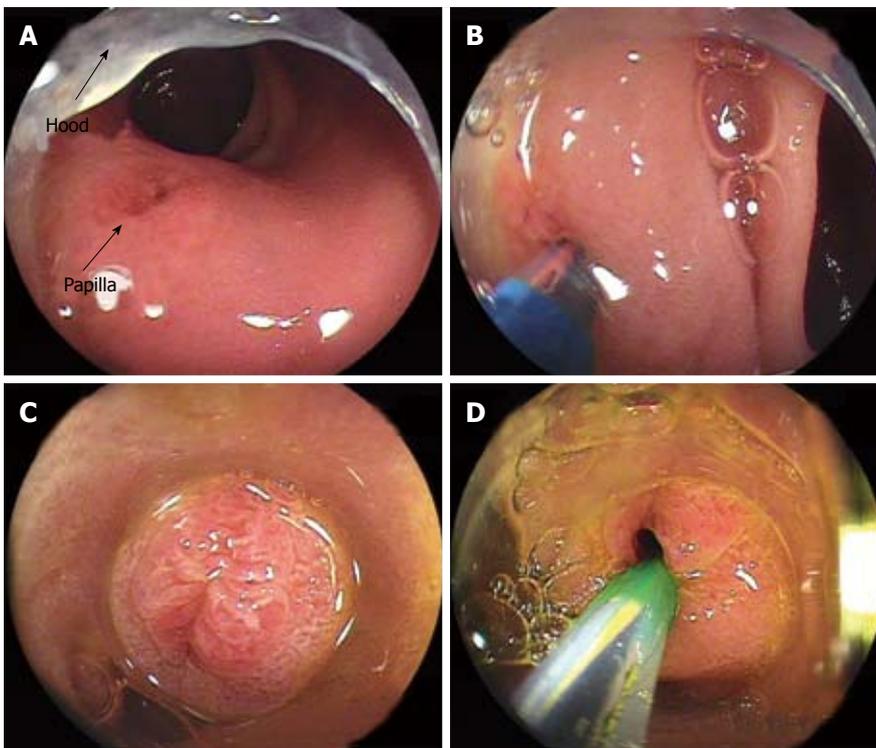


Figure 2 A soft transparent hood (DH-17EN; Fujifilm) was attached to the tip of a scope in all procedures.

was often required prior to ERCP. Using DBE, the endoscopist could stabilize the endoscope by the deep insertion of an overtube, and immobilize the intestine in a position that allowed the bile duct to run in the 11

o'clock direction, as in the conventional ERCP view. A soft transparent hood was attached to the tip of a scope in all procedures. The attachment hood was useful to fix the ampulla (Figure 2). Selective biliary cannulation

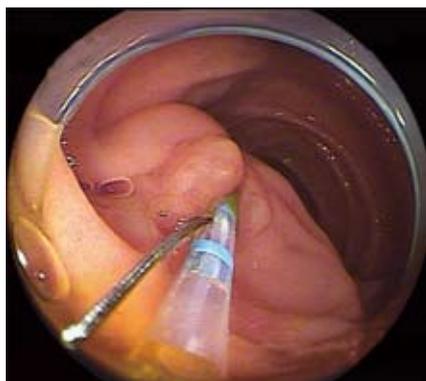


Figure 3 A sphincterotome with rotatable tip (Autotome RX49; Boston Scientific) was also useful for cannulation in some cases, in which biliary cannulation was unsuccessful with a standard cannula.

was achieved using a standard cannula or, if necessary, a sphincterotome (Autotome RX49; Boston Scientific, Tokyo, Japan) with a tip rotatable through 360° (Figure 3). Guidewire (Jagwire 0.035; Boston Scientific) was preloaded into the cannula or sphincterotome, and was placed in the bile duct after successful cannulation for the following procedure. Endoscopic sphincterotomy (EST) was performed using a sphincterotome with rotatable tip or a precutting knife (Single Use 3-Lumen Needle knife V KD-V441M; Olympus, Tokyo, Japan). When EST was difficult, endoscopic papillary balloon dilation was performed with an 8- or 10-mm balloon dilation catheter (Hurricane RX 4594; Boston Scientific). When the opening of the choledochojunostomy was stenotic, balloon dilation using either an 8-10-mm balloon dilation catheter or 12-mm balloon catheter (CRE Wireguided Balloon Dilatation Catheter 5842; Boston Scientific) was performed.

Stone extraction was performed with a six-wire basket (MTW) or a mechanical lithotripter (Xemex crusher catheter; Zeon Medical, Tokyo, Japan). After the stones were removed, a retrieval balloon catheter (Extractor; Boston Scientific) was used with a guidewire to confirm that no small stones remained.

In patients with the bile duct winding at sharp angles where the mechanical lithotripter could not be inserted, and in patients with a bulky stone that could not be extracted in one attempt, a 7-Fr plastic stent (Flexima Biliary Stent System 3920; Boston Scientific) was positioned.

Statistical analysis

All data are expressed as mean \pm SE, if applicable. Statistical analyses were performed with JMP statistical software (Version 9.0). Pearson's χ^2 test was performed to compare the success rate of DB-ERCP among Roux-en-Y total gastrectomy, Billroth II reconstruction, and pancreatoduodenectomy. An analysis of variance followed by Tukey's multiple comparison test was carried out to determine statistical significance among these three groups. A value of $P < 0.05$ was considered statistically significant.

Table 1 Success rate of double balloon enteroscopy-assisted endoscopic retrograde cholangiopancreatography n (%)

	Reaching the papilla of Vater	Contrast enhancement of target bile duct	Treatment of pancreatic and/or biliary duct
Roux-en-Y reconstruction	24 (96)	21 (88)	21 (100)
Billroth II gastrectomy	18 (95)	16 (89)	16 (100)
Pancreatoduodenectomy	3 (100)	3 (100)	3 (100)
Total	45 (96)	40 (89)	40 (100)

RESULTS

Reaching the blind end with a DBE

Twenty-eight patients had a total of 47 sessions of DBE. Deep insertion of an endoscope to the papilla of Vater was successful in 24 out of 25 procedures (96%) in patients with Roux-en-Y total gastrectomy, in 18 out of 19 procedures (94%) in patients with Billroth II reconstruction, and in all three procedures in patients with pancreatoduodenectomy (Table 1). There were no significant differences in the success rate of reaching the blind end with a DBE among Roux-en-Y total gastrectomy, Billroth II reconstruction, and pancreatoduodenectomy ($P = 0.91$). DBE can be a very useful modality for patients with altered gastrointestinal anatomy irrespective of surgical procedures.

Cannulation and contrast enhancement of the target bile duct in patients in whom the blind end was reached

Overall, the rate of successful cannulation and contrast enhancement of the target bile duct was 40 out of 45 (89%) in patients in whom the blind end was reached; this was made up of 21 out of 24 procedures (88%) in Roux-en-Y total gastrectomy patients, 16 out of 18 procedures (89%) in Billroth II patients, and all three procedures in patients with pancreatoduodenectomy. Again, there were no significant differences in the success rate of cannulation and contrast enhancement of the target bile duct in patients whom the blind end was reached with a DBE among Roux-en-Y total gastrectomy, Billroth II reconstruction, and pancreatoduodenectomy ($P = 0.67$).

Treatment of the pancreatic and/or biliary duct

Treatment was achieved in all 40 procedures (100%) in patients in whom contrast enhancement of the bile duct was successful. The 40 treatment procedures are listed in Table 2, and the details of treatment are given in Table 3. The most common endoscopic treatment was endoscopic biliary drainage (24 procedures), which was done by placement of plastic stents. Extraction of stones (14 procedures) was done by lithotomy with the mechanical lithotripter followed by extraction with a basket or by the balloon pull-through method. EST was performed with a needle precutting knife using a guidewire (14 procedures). The choledochojunostomy site was dilated by balloon in three procedures.

Table 2 Treatment procedures for double balloon enteroscopy-assisted endoscopic retrograde cholangiopancreatography

	Roux-en-Y reconstruction	Billroth II gastrectomy	Pancreatoduodenectomy
<i>n</i>	21	16	3
EST + drainage	2	1	
EST + SE		2	
EST + SE + drainage	2	1	
BD + SE	1	1	3
BD + SE + drainage	1		
EST + BD + drainage	1	1	
EST + BD + SE		2	
EST + BD + SE + drainage	1	1	
SE + drainage		1	
Drainage	12	5	
Other	1	1	

BD: Balloon dilation; SE: Stone extraction; EST: Endoscopic sphincterotomy.

Time of the procedure

The mean total duration of the procedure was 93.6 ± 6.8 min. The mean time required to reach the papilla was 30.5 ± 3.7 min. For a subanalysis, the time required to reach the papilla of Vater was 37.1 ± 4.9 min in Roux-en-Y total gastrectomy, 20.9 ± 5.8 min in Billroth II reconstruction, and 33.3 ± 13.4 min in pancreatoduodenectomy. It tended to be shorter in Billroth II reconstruction than that in Roux-en-Y total gastrectomy but there was no significant difference ($P = 0.09$). DB-ERCP seemed to be technically more difficult in patients with Roux-en-Y total gastrectomy than those with Billroth II reconstruction.

Complications

A major complication occurred in one patient (3.5%); perforation of the long limb in a patient with Billroth II anastomosis. Perforation occurred during insertion of DBE in this patient's second session. The perforation was successfully resolved surgically. No post-ERCP pancreatitis occurred.

DISCUSSION

The invention of DBE has dramatically changed the endoscopic management of pancreatobiliary diseases in patients who have undergone bowel reconstruction^[8-12]. DBE has made it possible to reach the papilla or bilio-pancreatoenteric anastomosis site even in patients with Roux-en-Y surgical reconstruction. Although patients who have undergone Billroth II reconstruction are considered capable of undergoing ERCP with a conventional endoscope, the endoscopic approach to the afferent loop, blind end, and the site of hepaticojejunostomy is difficult in cases where there are severe adhesions or a long afferent loop. In such patients with Billroth II reconstruction, DB-ERCP is effective.

DB-ERCP has three major steps; the first step is intubation to the papilla or the site of hepaticojejunostomy, the second is cannulation into the bile and/or

Table 3 Detailed treatment procedures for double balloon enteroscopy-assisted endoscopic retrograde cholangiopancreatography

	Roux-en-Y reconstruction	Pancreatoduodenectomy	Billroth II gastrectomy	Total
Stone extraction	5	2	7	14
EBD	18		6	24
EPD			3	3
Endoscopic sphincterotomy	6		8	14
Balloon dilation	2	3	5	10
Cytological diagnosis			1	1

EBD: Endoscopic biliary drainage; EPD: Endoscopic pancreatic duct drainage.

pancreatic duct, and the third is therapeutic manipulation such as sphincterotomy and/or stone extraction. There have been several reports of DB-ERCP. Although the success rates of reaching the papilla or of cannulation for intact papilla are high (50%-100%, 67%-100%, respectively)^[17,18,20-22], they are still lower than for conventional ERCP. Therefore, technical difficulties or instrument limitations are considered likely. Some useful techniques or endoscopic accessories have been reported for conventional endoscopy or DBE, however, there are few reports which describe in detail the technique or the instruments for each step of DB-ERCP. We describe these things in detail, because they may contribute to improving the outcome of DB-ERCP.

For the first step, intubation to the papilla or the site of hepaticojejunostomy, short-type DBE may be recommended. Originally, double balloon enteroscopes were 200 cm long, which did not cause a problem with insertion into the papilla, but limited the number of devices for the ERCP procedure^[17,18,20,21]. Short-type DBE, which was originally developed for colonic insertion, has resolved this problem^[17,18]. Shimatani *et al*^[18] have reported that because of its short working length and the availability of various accessory devices, short-type DBE increases the success rates for reaching the papilla and blind end, and for ERCP-related interventions. Cases in which double balloon enteroscope insertion may be difficult are patients who have jejunojunctionostomy such as Roux-en-Y anastomosis or pancreatoduodenectomy. Selective contrast enhancement in the intestine with a balloon inflated on the endoscope tip is useful to confirm the direction of the afferent loop^[17,18]. Once the correct way is confirmed, submucosal tattooing at the beginning of the Roux limb may be useful for later sessions (Figure 1)^[12,17,21,23,24].

In our series, the blind end was reached in 45 out of 47 procedures (95.7%) with short-type DBE, comprising 18 out of 19 procedures in Billroth II patients, all three in pancreatoduodenectomy, and 24 out of 25 procedures in Roux-en-Y patients. One procedure in Billroth II patients was aborted because of perforation of the long limb, and one in the Roux-en-Y group failed because of

strong adhesion. We have used CO₂ instead of standard air insufflations since April 2009 to improve intubation depth during DBE, as well as to reduce postprocedural pain^[19]. The mean duration for reaching the blind end using CO₂ insufflation in Roux-en-Y patients tended to be shorter than that using air insufflation, although the difference was not significant ($P = 0.18$, 40.9 min *vs* 29.4 min, air *vs* CO₂, respectively). CO₂ insufflation could be useful especially in patients with Roux-en-Y total gastrectomy where DB-ERCP seems to be technically difficult compared to Billroth II reconstruction.

The second step, deep cannulation into the bile and/or pancreatic duct, is an important precondition for later therapeutic manipulations. In patients with Roux-en-Y reconstruction, the position of the papilla may vary and may be observed by moving the screen in various directions. The manipulation of both the overtube and the endoscope makes it possible to change the position of the papilla in the view field. Double balloon enteroscopes are not provided with an elevator, therefore, cannulation may be difficult, but the attachment hood is useful to fix the ampulla and to align the axes of the cannula and the bile duct, enabling safe endoscopic procedures and selective pancreatic and/or biliary cannulation (Figure 2). We usually use a normal ERCP catheter. If the biliary axis does not fit, we use a sphincterotome with a rotatable tip (Figure 3). Needle-knife precut papilotomy was performed when the conventional biliary cannulation promised to be difficult, as described by Fukatsu *et al.*^[25]. In this study, cannulation was successful and contrast enhancement of the target bile duct was achieved in 40 out of 45 (89%) patients in whom the blind end was reached. There were difficult cases in Roux-en-Y patients (21/24, 88%) and in Billroth II patients (16/18, 89%). It was easy in all three patients with pancreatoduodenectomy.

The third therapeutic step is the endpoint of the DB-ERCP procedure. Deep insertion and placing the guidewire into the biliary and/or pancreatic duct is indispensable for the following procedures. Guidewire stabilizes the papilla and makes it easy to perform sphincterotomy or insert the devices into the duct. Treatment was achieved in all 40 procedures in patients in whom contrast enhancement of the bile duct and guidewire placement were successful. Endoscopic biliary drainage by placement of plastic stents was not difficult. However, the diameter of the stents was limited to < 7 Fr. Sphincterotomy using a sphincterotome is sometimes difficult in DB-ERCP. A rotatable sphincterotome or needle precutting knife may be useful in such cases. If sphincterotomy is impossible or insufficient, balloon dilation should be performed. Extraction of stones was done by lithotomy with the mechanical lithotripter followed by extraction with a basket or by the balloon pull-through method. A few types of mechanical lithotripters and baskets that can go through the DBE working channel are commercially available at the present time. However, most of them do not have the wire-guided mechanism,

which means it is sometimes difficult to insert into the duct.

DB-ERCP complications in patients who have undergone bowel reconstruction have featured in a limited number of reports. Perforations or emphysema at the time of insertion of an endoscope or endoscopic sphincterotomy have been reported in 2.3%-11.1% of cases. Raithel *et al.*^[21] have reported post-ERCP pancreatitis in 2.3% and post-interventional bleeding in 1.1% of patients. In our study, complications occurred in only one patient (3.5%); perforation of the long limb in a patient with Billroth II anastomosis^[17,18,21]. With regard to the insertion of an endoscope and shortening of the intestinal tract, attention should be paid to avoid forced insertion or shortening, which may cause perforation.

In conclusion, short-type DBE combined with several technical innovations enabled us to perform ERCP and ERCP-related interventions in a number of patients for whom it was previously impossible. Endoscopic tattooing at the beginning of the limb, using an attachment hood, and CO₂ insufflation might facilitate intubation of the papilla and reduce the duration of the procedure. Cannulation into the bile or pancreatic duct is the most difficult part of DB-ERCP. Using an attachment hood and/or a rotatable sphincterotome may facilitate the procedure. Further instrumental improvements such as the widening of the DBE working channel or development of the accessories for use in balloon enteroscope systems are necessary to improve the outcome of DB-ERCP.

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COMMENTS

Background

Patients who have undergone bowel reconstruction have been considered to be difficult for endoscopic retrograde cholangiopancreatography (ERCP) using conventional endoscopy. Double balloon enteroscopy (DBE) has brought a significant benefit for performing ERCP in patients with such an altered gastrointestinal anatomy. However, there are still some technical difficulties to be resolved.

Research frontiers

Recently developed short DBE contributes to increased success rates for reaching the papilla and for ERCP-related interventions in DBE-assisted ERCP (DB-ERCP). In the area of DB-ERCP, the research hotspot is how to improve its results for completing therapeutic interventions such as stone extraction and stent insertion of the pancreatobiliary system.

Innovations and breakthroughs

Although a short DBE is more useful than a conventional long DBE, clinical outcomes of DB-ERCP are still unsatisfactory compared to conventional ERCP. DB-ERCP has three major steps: intubation to the papilla or the site of hepaticojejunostomy, cannulation into the bile and/or pancreatic duct, and therapeutic manipulation. To improve intubation, the authors used CO₂ instead of standard air insufflations, selective contrast enhancement in the intestine with a balloon inflated on the endoscope tip to confirm the right direction, and submucosal tattooing in patients with Roux-en-Y anastomosis for the next session. To improve cannulation, we attached a soft transparent hood to the tip of a scope, and used a sphincterotome with a rotatable tip. To improve therapeutic manipulation, we performed guidewire placement into the biliary/pancreatic duct, and needle-

knife precutting and/or balloon dilation to make a large enough opening of the orifice.

Applications

In this study, the authors showed the possibility that short-type DBE combined with several technical innovations enabled us to perform ERCP and ERCP-related interventions more efficiently in a number of patients with altered gastrointestinal anatomy for whom it was previously impossible.

Terminology

DBE has been developed for visualization of and intervention in the entire small intestine. Currently, two types of double-balloon endoscope are available including a conventional type (200 cm working length) and a short type (152 cm). Short-type DBE has great practical use because of its short working length and the availability of various accessory devices. Roux-en-Y reconstruction of the small bowel is a general surgical technique in gastrointestinal oncology surgery, hepatobiliary and pancreatic surgery, and bariatric surgery. For the endoscopist, accessing the ampulla is technically difficult in a patient needing Roux-en-Y reconstruction, because of the long length of bowel that the endoscope must pass through, and the acute angle of the afferent limb and Roux limb anastomosis.

Peer review

This is an important paper describing difficult ERCP cases after gastric surgery. This is a useful report of DB-ERCP techniques. The authors' modifications of the technique are particularly helpful.

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Is laparoscopy equal to laparotomy in detecting and treating small bowel injuries in a porcine model?

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Abstract

AIM: To evaluate the safety and effectiveness of laparoscopy compared with laparotomy for diagnosing and treating small bowel injuries (SBIs) in a porcine model.

METHODS: Twenty-eight female pigs were anesthetized and laid in the left recumbent position. The SBI model was established by shooting at the right lower quadrant of the abdomen. The pigs were then randomized into either the laparotomy group or the laparoscopy group. All pigs underwent routine exploratory laparotomy or laparoscopy to evaluate the abdominal injuries, particularly the types, sites, and numbers of SBIs. Traditional open surgery or therapeutic laparoscopy was then performed. All pigs were kept alive within the observational period (postoperative 72 h). The postoperative recovery of each pig was carefully observed.

RESULTS: The vital signs of all pigs were stable within 1-2 h after shooting and none of the pigs died from gunshot wounds or SBIs immediately. The SBI model was successfully established in all pigs and definitively

diagnosed with single or multiple SBIs either by exploratory laparotomy or laparoscopy. Compared with exploratory laparotomy, laparoscopy took a significantly longer time for diagnosis (41.27 ± 12.04 min *vs* 27.64 ± 13.32 min, $P = 0.02$), but the time for therapeutic laparoscopy was similar to that of open surgery. The length of incision was significantly reduced in the laparoscopy group compared with the laparotomy group (5.27 ± 1.86 cm *vs* 15.73 ± 1.06 cm, $P < 0.01$). In the final post-mortem examination 72 h after surgery, both laparotomy and laparoscopy offered a definitive diagnosis with no missed injuries. Postoperative complications occurred in four cases (three following laparotomy and one following laparoscopy, $P = 0.326$). The average recovery period for bowel function, vital appearance, and food re-intake after laparoscopy was 10.36 ± 4.72 h, 14.91 ± 3.14 h, and 15.00 ± 7.11 h, respectively. All of these were significantly shorter than after laparotomy (21.27 ± 10.17 h, $P = 0.004$; 27.82 ± 9.61 h, $P < 0.001$; and 24.55 ± 9.72 h, respectively, $P = 0.016$).

CONCLUSION: Compared with laparotomy, laparoscopy offers equivalent efficacy for diagnosing and treating SBIs, and reduces postoperative complications as well as recovery period.

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Key words: Laparoscopy; Laparotomy; Small bowel injury; Porcine model; Diagnosis; Treatment; Penetrating injury; Firearm injury

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INTRODUCTION

The vast anatomic space occupied by the gastrointestinal tract predisposes it to penetrating injuries. In penetrating trauma, the small bowel is most frequently injured, followed by the large intestine and stomach^[1]. Small bowel injury (SBI) is seldom diagnosed preoperatively especially when there are no frank signs of hemoperitoneum or peritonitis^[2,3].

Laparotomy is considered the gold standard for evaluation of intra-abdominal injuries sustained from trauma^[4]. However, complications following negative or nontherapeutic laparotomy can be as high as 20%-40%^[5-7]. Therefore, it is advantageous to avoid a negative laparotomy while providing a reliable and accurate alternative diagnostic procedure^[8]. While laparoscopy has become a standard component of diagnosis and therapy for many conditions in general surgery, its role in trauma remains controversial. Many concerns about the safety, sensitivity, and specificity of laparoscopy have limited its application in abdominal trauma^[9-12], particularly in detecting SBIs^[13-15]. It was reported that laparoscopy in trauma initially resulted in a high rate of missed injuries (41%-77%) and considerable criticism of laparoscopy as a diagnostic tool^[9,10]. Studies in 1993 and 2006 showed little statistical change in its reliability; only 20% of SBIs were correctly identified by laparoscopy, and sensitivity was 25% for diagnosis of hollow viscus and retroperitoneal injuries^[16,17]. The high proportion of missed occult SBIs with laparoscopy in trauma (LIT) is a major reason why some surgeons still preclude LIT use today^[18]. These considerations conflict with the considerable advances that have been made in LIT.

In this present study we mimicked injuries to the small intestines by firing bullets into the abdomen of anesthetized pigs. We tried to provide a reproducible and hemodynamically stable porcine model with multiple SBIs. We also aimed to evaluate the safety and effectiveness of diagnostic and therapeutic laparoscopy compared with the laparotomy for SBI in the porcine model.

MATERIALS AND METHODS

Experimental design

The study protocol was approved by the Institutional Review Board of the Research Institute of Surgery (RIS), which was affiliated to the Third Military Medical University in Chong Qing (People's Republic of China). Animal welfare and experimental procedures were carried out strictly in accordance with the guide for the Animal Care and Use Committee of RIS.

A prospective, randomized, comparative study was conducted between February and July 2010. Enrolled in this research were 28 consecutive healthy pigs native to Chong Qing (all females). The pigs were provided and fed by staff from the Medical Animal Research Center of Da Ping Hospital (Affiliated to the Third Military Medical University).

After mastering the skilled techniques gained from previous experience in modeling and treating SBIs, we carried out this study to ascertain if laparoscopy alone could replace laparotomy in diagnosing and treating SBIs in this porcine model.

Preoperative preparation

All 28 pigs had free access to food and tap water without oral intake of antibiotics. After premedication with intramuscular administration of azaperone 4 mg/kg, ketamine 10 mg/kg, and atropine 0.02 mg/kg, general anesthesia was induced by 3% pentobarbital (1 mL/kg) *via* the left ear vein; an additional 3-5 mL of 3% pentobarbital was given if the pigs became restless. A 6.5-F endotracheal tube was applied, and pigs allowed to breathe spontaneously. They were ventilated with room air using a standard ventilator if necessary. Most of the animals were in the anesthetic plane between medium and deep anesthesia [muscles relaxed; most reflexes (palpebral, corneal) absent; pupillary light reflex slow or absent].

SBI modeling procedures: After anesthesia, the pigs were transferred to the shooting cabin to establish the model. Pigs were laid in the left lateral recumbent position; the right forelimb and hindlimb were abducted and suspended on the shooting shelf whereas the left forelimb and hindlimb were fixed horizontally. The entry point was 2 cm medial and 3 cm cephalad from the point of the right hip (this position was determined according to our previously experience). Once the entry point was defined, the location of the predicted exit point was obtained by ensuring that both points were in the right quadrant of the abdomen and the line between the two points was horizontal. The predicted exit point was equivalent to the entry point moving medially, and was 4 cm lateral to the abdominal midline (Figure 1).

The injury was inflicted by a 56-type military firearm. It fired 7.62-mm steel-core bullets weighing 7.9 g at a shooting distance of 5 meters. The military firearm was used after fine adjustment of the ballistic trajectory according to the position of animal. All devices and the professional marksman were provided by the RIS.

Surgical procedures: After shooting at the abdomen, pigs were rapidly transferred to the operating room. Immediately before surgery, all pigs were randomized to one of the two surgical approaches (laparotomy or laparoscopy). The interval between shooting and surgery was similar in each pig; approximately 70-80 min.

Laparotomy group: Pigs were placed in the supine

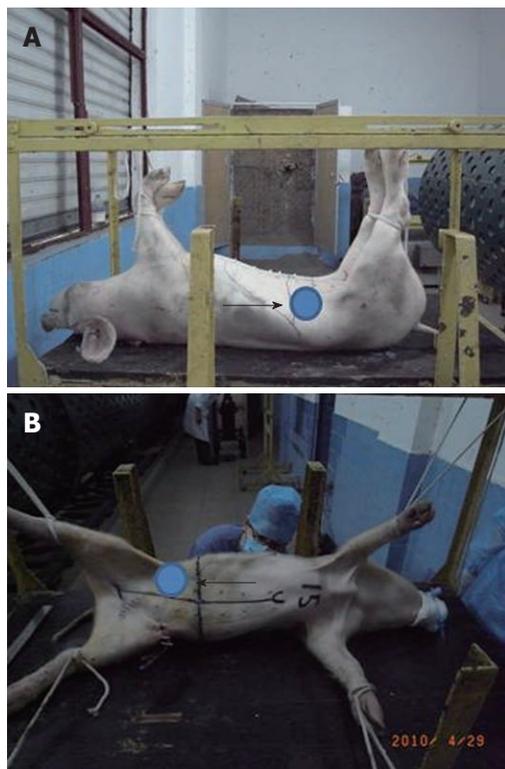


Figure 1 Animal model. The experimenters fixed the position of the anesthetized pig. The black arrows point at the entry and exit wounds of the abdomen.

position. Access to the abdominal cavity was gained through a midline incision ranging from approximately 7 cm above the umbilicus to approximately 9 cm below the umbilicus. The abdominal wall was drawn back with a metal retractor. Viscera (particularly the intestinal tract) were exposed manually. The sequence of exploration was from the major vessels, liver, spleen, the gyri centripetales and gyri centrifugales of the colon to the left kidney and left segment of the pancreas; this was continued from the sigmoid colon, rectum, bladder, and cecum to the ileum and jejunum; then continued from the stomach, duodenum, and the pancreatic head to the right segment of the pancreas and right kidney. After exploration, surgical treatment was performed for intra-abdominal injuries. Intestinal resection and anastomoses were undertaken if the diameter of the intestinal rupture was longer than 50% of the intestine circumference, otherwise simple repair with edge trimming was applied; end-to-end single-layer anastomoses were conducted with running number 0 silk sutures;

Laparoscopy group: A pneumoperitoneum of 13 mmHg with CO₂ insufflation was established after insertion of a Veress needle. A 10-mm camera access port was introduced 1 cm above the umbilicus. A 30° laparoscope connected to a camera allowed endoscopic visualization. Two additional 5-mm or 10-mm ports were inserted in the left lower quadrant of the abdomen in right-angled triangular fashion under laparoscopic guidance. Pigs were positioned in a Trendelenburg angle or

a reverse angle to facilitate free access to the intestinal tract and other viscera. The sequence of exploration and some of the specific rules of treatment during surgery were the same as those mentioned in the laparotomy. The small bowel was examined for traumatic injuries by two 5-mm atraumatic bowel graspers from the ileocecal valve to the ligament of Treitz (running the bowel). Once exploration was accomplished, effective therapeutic laparoscopic procedures were undertaken to treat the injuries. Larger bleedings were controlled through application of a laparoscopic clip or with bipolar forceps. If the intestinal injuries were not severe (injury scale lower than Grade II^[19]) with the exception of ruptures of the colon or rectum, single repair with single-layer interrupted sutures was undertaken entirely under the laparoscopic view (total laparoscopy). Otherwise, intestinal resection and anastomoses were performed through an extended 5-cm incision of the exit wound (video-assisted laparoscopy).

After anastomoses and debridement, the abdominal cavity was irrigated with approximately 2000 mL normal (0.9%) saline, and a drainage tube placed in the pelvic cavity. The exit wound was managed with sharp debridement and two layers of interrupted sutures for the deep fascia and skin and the surgical incision. Administration of anesthetics was stopped. Both laparoscopy and laparotomy were performed by the same surgeons in our study, all of whom trained at RIS and used similar techniques.

Postoperative management

Postoperatively, a clean dry dressing was sutured with the abdominal skin to prevent contamination of the incision. Each pig was transferred to the feeding room, kept in a single cage, and injected with 25 mg pethidine 3 h after surgery. All pigs received 500 mL 5% glucose solution containing 1 g cefradine and 0.5 g metronidazole per day through a peripheral vein. The eating habits and physical activities of the pigs were closely monitored three times daily to detect signs of peritonitis and generalized sepsis. The recovery of bowel function was recorded as the time of return of bowel sounds. At 72 h after surgery, all animals were sacrificed for a thorough exploration of the abdomen to check for missed injuries.

Statistical analysis

Statistical analysis were carried out with SPSS software (SPSS/PC + 16.0; SPSS, Chicago, IL, United States). Values are presented as mean \pm standard deviation; $P < 0.05$ was considered significant. Statistical comparisons of data were carried out by the Student *t*-test, the Chi-square test or repeated measures as appropriate. In addition, Fisher's exact test was applied if the sample was < 5 .

RESULTS

SBI model

The point of the entry wound was 2.1-2.9 cm medial (mean, 2.60 \pm 0.19 cm) and 1.2-1.7 cm cephalad (mean,

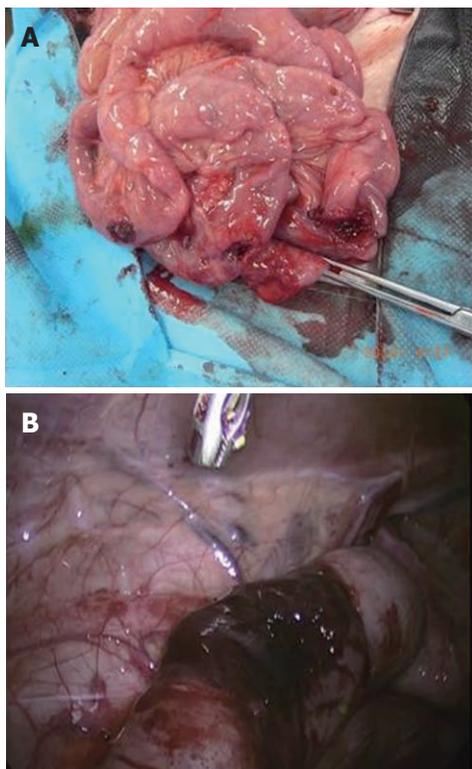


Figure 2 Porcine model with gastrointestinal injuries. A: Multiple ruptures of the small intestine; B: Injuries to the sigmoid colon.

1.5 ± 0.12 cm) from the point of the right hip. The exit wounds were located in the right lower quadrant of the abdomen, and 3.2-5.8 cm (mean, 4.53 ± 0.51 cm) lateral to the midline.

The vital signs of all pigs were stable within 1-2 h after shooting. None of the pigs died from gunshot wounds (GSW) to the abdomen. All 28 pigs underwent routine exploration of the abdominal cavity. All had single or multiple injuries to the small bowel, one (3.57%) with sigmoid ruptures (Figure 2), and one (3.57%) with cecum ruptures. Injuries to the liver, spleen, the gyri centripetales and gyri centrifugales of the colon, crucial vessels and retroperitoneal organs were not observed. The details of the SBIs are listed in Table 1; the organ injury scale was based on that of Moore *et al*^[19]. In addition, the severity of SBIs in the two groups was also compared. We considered injury numbers of Grade II SBIs < 3 as minor injuries, and injury numbers of Grade III or more SBIs ≥ 1 as severe injuries. In the laparoscopy group, 5 cases were found with minor injuries and 9 cases with severe injuries, while in the laparotomy group, 4 cases were diagnosed with minor injuries and 10 cases with severe injuries. There was no significant difference between the groups concerning the severity of SBIs.

Perioperative outcomes

All 28 pigs were randomized into either the laparotomy group or the laparoscopy group (14 in each group). According to the operative findings, both laparotomy and laparoscopy offered a definitive diagnosis with no

missed injuries (all confirmed by the final post-mortem examination). Compared with laparotomy, laparoscopy took a significantly longer time for diagnosis (41.27 ± 12.04 min *vs* 27.64 ± 13.32 min, $P = 0.02$), but the time of therapeutic laparoscopy was similar to that of open surgery (83.27 ± 23.43 min *vs* 79.00 ± 19.17 min, $P > 0.05$). Furthermore, according to our analysis, the overall operative (diagnostic plus therapeutic) time was slightly longer in the laparoscopy group, but the difference did not reach statistical significance ($P = 0.12$). In addition, the length of the incision was significantly reduced in the laparoscopy group compared with the laparotomy group (5.27 ± 1.86 cm *vs* 15.73 ± 1.06 cm, $P < 0.01$).

Although all 28 pigs survived during the 72 h of follow-up, the recovery was not uneventful. Three cases (3/14, 21.5%) in the laparotomy group developed post-operative complications (one small bowel volvulus, one gastric retention, and one abdominal cavity infection and abscess) while only one case (1/14, 7.14%) had a complication of incisional infection in the laparoscopy group. No complications related to the technique (e.g., leakage, obstruction due to tight anastomosis, bleeding) were found. Although the incidence of complications was slightly higher following laparotomy, the difference between groups was not significant ($P = 0.326$). The average recovery period for bowel function, vital appearance, and food re-intake after laparoscopy was 10.36 ± 4.72 h, 14.91 ± 3.14 h, and 15.00 ± 7.11 h, respectively, following laparoscopy. All of these were significantly shorter than after laparotomy (21.27 ± 10.17 h, $P = 0.004$; 27.82 ± 9.61 h, $P < 0.001$; and 24.55 ± 9.72 h, respectively, $P = 0.016$).

DISCUSSION

Few studies have been carried out focusing on the methodology of SBI in pigs arising from GSW^[20,21]. According to our experience, the entry and exit points were extremely critical in establishing a purely SBI model. Through our work on intra-abdominal anatomy, we found that the distribution pattern was considerably regular in pigs. The small bowel had a centralized distribution in the right abdomen, and the gyri centripetales and gyri centrifugales of the colon were located in the left upper quadrant in a circular fashion. The cecum was approximately 20 cm in length with no obvious mesentery; it was mainly located in the left lower quadrant with its end pointing towards the pelvic cavity. Therefore, we assumed that if both the entry and exit wounds were located on the right lower quadrant of the abdomen, avoiding bony structures such as right ribs, right hip and pubis, there would be injuries only to the small intestine. As predicted, in the present study, all 28 pigs were diagnosed with SBIs.

One of the greatest concerns about LIT has been its unreliability in detecting SBIs^[13,14], a major reason why some surgeons still preclude LIT today. However, according to the results of the final post-mortem ab-

Table 1 Summary of the details of small bowel injuries in all pigs after gunshots

Case	Injury Scale ¹ of SB						Treatment	
	IH	IL	II	III	IV	V		Sum
1	1		2	1			4	Laparotomy
2			1				1	Laparoscopy (total)
3	2	1	2				5	Laparoscopy (total)
4	3	1	4	2		1	11	Laparoscopy (video-assisted)
5			3				3	Laparotomy
6	2		2		6		10	Laparoscopy (video-assisted)
7	3		4				7	Laparotomy
8	1		2				3	Laparotomy
9	1		1	1			3	Laparoscopy (video-assisted)
10	4	2			1	1	8	Laparoscopy (video-assisted)
11	1		2				3	Laparotomy
12	1	1	3	2	1		8	Laparotomy
13	1		2	1	1		5	Laparotomy
14		1	1				2	Laparoscopy (total)
15		1		3	4		8	Laparoscopy (video-assisted)
16	1		2	1	1		5	Laparotomy
17	1	1	2				4	Laparotomy
18			2	1	1		4	Laparotomy
19	1		1				2	Laparoscopy (total)
20				2		1	3	Laparotomy
21	1		2	1	1		5	Laparoscopy (video-assisted)
22		1	1				2	Laparotomy
23	2		3	1			6	Laparoscopy (video-assisted)
24	1		2	1	1		5	Laparotomy
25			1	1	1		3	Laparoscopy (video-assisted)
26	2			4		1	7	Laparotomy
27	1	1	2				4	Laparoscopy (total)
28		1	2	1			4	Laparoscopy (video-assisted)

¹Based on the reference of Moore *et al*^[19]. IH: Hematoma, contusion or hematoma without devascularization; IL: Laceration, partial thickness, no perforation; II: Laceration, laceration < 50% of circumference; III: Laceration, laceration > 50% of circumference without transaction; IV: Laceration, transection of small bowel; V: Laceration, transection of small bowel with segmental tissue loss vascular, devascularized segment; SB: Small bowel.

dominal examination, the present study demonstrated that laparoscopy could also offer equivalent efficacy for diagnosing SBIs compared with laparotomy, with no missed injuries. According to our experience, the systematic approach for diagnostic laparoscopy exploration of the gastrointestinal tract was very important for avoiding missed SBIs^[15], and mainly consisted of all the principles of open exploratory laparotomy for trauma and the technique of running the small bowel. During the exploration, we initially lifted an 8-10-cm segment of the small bowel with two 5-mm atraumatic bowel graspers at the ileocecal valve, and one side of the bowel and mesentery was observed. The graspers were then turned 180 degrees, and the other side of the bowel was visualized. This sequence was repeated until the ligament of Treitz was reached. Therefore, after using this systematic approach, we could correctly identify SBIs, minimizing the potential of a missed injury. Although diagnostic laparoscopy was time-consuming, and took approximately 45 min to diagnose intestinal injuries in each case, which was significantly longer than the laparotomy exploration, the safety and sensitivity of diagnostic laparoscopy for

SBIs was good according to our research.

While surgeons disputed the disadvantages of laparoscopy for SBIs, the benefits of laparoscopy were evident^[22]. First, by directly visualizing the abdominal cavity, laparoscopy allowed the surgeon to exclude the presence of other associated intra-abdominal injuries, and was also able to eliminate the blind zone of visual fields when using a 30° laparoscope. Secondly, following the laparoscopic guidance, the SBIs could be treated directly using a laparoscopic technique, or using a notably shorter incision, which was near the injuries. In the current study, five pigs in the laparoscopy group were diagnosed with simple SBIs, and received total laparoscopy for treatment. In addition, video-assisted therapeutic laparoscopy, which utilized a 6-7 cm incision, was successfully carried out in nine pigs with relatively severe SBIs. Therefore, laparoscopy would also be a reliable and accurate alternative therapeutic procedure for severe SBIs^[23]. Thirdly, compared with the laparotomy group, the length of incision was significantly shortened in the laparoscopy group. The length of incision is an important factor affecting surgical stress^[24]. Thus, given the minimally invasive nature of laparoscopy, there is good reason to assume that laparoscopy is advantageous over conventional laparotomy in reducing surgical trauma. Another benefit of laparoscopy was the significantly reduced recovery period after surgery. The almost 50% reduction in recovery periods of bowel function, vital appearance, and food re-intake in the present research was not surprising considering the less invasive nature of the procedure. Therefore, laparoscopy could be a promising minimally invasive approach for treating SBIs^[25,26].

Limitation

One overwhelming limitation of this study was that a major proportion of patients with GSW to the abdomen would have significant injuries involving not only the small intestines, but also many other kinds of abdominal organs, which would make the conditions of the patients very complicated. Thus, therapeutic laparoscopy might not be the first choice for patients with GSW. However, we believe that, in the hemodynamically stable patient with abdominal penetrating injury and without extensive intra-abdominal adhesions, a thorough laparoscopic examination is possible and feasible. The results of diagnostic laparoscopy would be a useful guidance, and directly influence the modality of further treatment. Moreover, we aimed to evaluate the feasibility and validity of diagnostic and therapeutic laparoscopy for SBI, which was the chief objective of our study, and we did obtain convincing evidence supporting the use of laparoscopy for SBIs.

In conclusion, our research indicates that laparoscopy could be a minimally invasive approach for diagnosing and treating SBIs. Compared with laparotomy, laparoscopy could offer equivalent efficacy for SBIs, and reduces postoperative complications as well as recovery period.

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COMMENTS

Background

While laparoscopy has become a standard component of diagnosis and therapy for many conditions in general surgery, its role in abdominal trauma remains controversial. Many concerns about the safety, sensitivity, and specificity of the laparoscopy have limited its application in abdominal trauma, particularly in detecting small bowel injuries (SBIs).

Research frontiers

One of the greatest concerns about laparoscopy in trauma (LIT) has been its unreliability in detecting SBIs, a major reason why some surgeons still preclude LIT today. This study aimed to evaluate the safety and effectiveness of diagnostic and therapeutic laparoscopy compared with laparotomy for SBIs in a porcine model.

Innovations and breakthroughs

Few studies have been carried out focusing on the value of laparoscopy for SBIs due to gunshot wounds in pigs. To the best of the authors' knowledge, the present study was the most detailed and systematic study establishing a purely SBI porcine model. Furthermore, the study also provided a comprehensive picture of the safety and effectiveness of laparoscopy and laparotomy in trauma.

Applications

In the hemodynamically stable patient with abdominal penetrating injury, a thorough diagnostic and therapeutic laparoscopy was possible and feasible. Compared with laparotomy, laparoscopy offers equivalent efficacy for diagnosing and treating SBIs, and reduces postoperative complications as well as recovery period.

Peer review

The author compared the safety and efficacy of laparoscopy for diagnosing and treating small bowel injuries in a trauma model using pigs. The study is well designed, and the article is also important and interesting.

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Analysis of TLR4 and TLR2 polymorphisms in inflammatory bowel disease in a Guangxi Zhuang population

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Abstract

AIM: To study the polymorphisms of toll-like receptor 4 (*TLR4*) gene *Asp299Gly*, *Thr399Ile* and *TLR2* gene *Arg753Gln*, *Arg677Trp* and susceptibility to inflammatory bowel disease (IBD) in the Zhuang population from Guangxi, China.

METHODS: A case-control study was performed from February 2007 to October 2011 which included 146 Zhuang patients with IBD in the experimental group and 164 healthy Zhuang subjects who acted as the control group. All patients and healthy subjects were from the Guangxi Zhuang Autonomous Region of China. Genomic DNA was extracted from intestinal tissue by the phenol chloroform method. *TLR4* gene *Asp299Gly*, *Thr399Ile* and *TLR2* gene *Arg753Gln*, *Arg677Trp* were amplified by polymerase chain reaction (PCR), and then

detected by PCR-restriction fragment length polymorphism (RFLP).

RESULTS: The *TLR4* gene *Asp299Gly* was digested using *Nco* I restriction enzyme, and a single band of 249 bp was observed which showed that it was a wild type (AA). The *TLR4* gene *Thr399Ile* was digested using *Hinf* I restriction enzyme and only the wild type (CC) was detected. In addition, the *TLR2* gene *Arg677Trp* was digested using *Aci* I restriction enzyme and only the wild type (CC) was detected. The *TLR2* gene *Arg753Gln* was digested using *Pst* I restriction enzyme. Only the wild type (GG) as a single band of 254 bp was observed during RFLP. Overall, no heterozygous or homozygous single nucleotide polymorphism mutations were found in patients with Crohn's disease and ulcerative colitis both in the *TLR4* gene *Asp299Gly*, *Thr399Ile* and the *TLR2* gene *Arg677Trp*, *Arg753Gln* in the Zhuang population from the Guangxi Zhuang Autonomous Region of China.

CONCLUSION: The *TLR4* gene *Asp299Gly*, *Thr399Ile* and *TLR2* gene *Arg753Gln*, *Arg677Trp* polymorphisms may not be associated with IBD in the Zhuang population from the Guangxi Zhuang Autonomous Region of China.

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Key words: Toll-like receptor 2; Toll-like receptor 4; Inflammatory bowel disease; Gene polymorphism

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic disorder caused by multiple factors in genetically susceptible hosts, and includes ulcerative colitis (UC) and Crohn's disease (CD). The molecular basis of the pathogenesis is not completely clear, but involves a complex interaction of factors such as genetics, immunology, environment and infection. The incidence of IBD in Western populations has increased with an estimated incidence of 0.10%-1.00% for UC and 0.35%-1.00% for CD during the past few decades^[1]. NOD2/CARD15 was the first verified predisposing gene for CD, where three NOD2/CARD15 polymorphisms, *Arg702Trp*, *Gly908Arg* and *Leu1007fsinsC*, were found to be significantly associated with CD in Caucasian populations^[2,3]. Nevertheless, these single nucleotide polymorphisms (SNPs) were not reported to be associated with CD in Japanese, and Hubei, Zhejiang, or Hong Kong populations in China^[4-7], thus, their exact role in CD is controversial. Recently, genome wide association studies, provided evidence for several determinants including Toll-like receptor 2 (TLR2) and TLR4^[8]. TLR4 is upregulated in intestinal epithelial cells, macrophages, and dendritic cells in patients with UC and CD. In contrast, the expression of TLR2 is unchanged. The *Asp299Gly* and *Thr399Ile* polymorphisms of the *TLR4* gene were shown to be associated with lipopolysaccharide hyporesponsiveness and with both CD and UC in some studies^[9,10]. The *TLR2* gene *Arg753Gln* polymorphism frequency is approximately 1%-4% in the Caucasian population, significantly higher than that in Indian patients with IBD^[11].

In view of the discrepant data regarding the association between key regulatory genes and IBD susceptibility, the purpose of our study was to investigate whether the known gene polymorphisms in TLR2 and TLR4 determine susceptibility to IBD in the Guangxi Zhuang population from the Guangxi Zhuang Autonomous Region. Guangxi has a large Zhuang population, where genetic diseases and gene polymorphisms are unique. Therefore, research on the relation between TLR2 and TLR4 polymorphisms and IBD in Chinese Zhuang patients from the Guangxi Zhuang Autonomous Region is needed.

MATERIALS AND METHODS

Patients

The study group consisted of 146 IBD Zhuang patients without genetic kinship enrolled in the Gastroenterology Department, First Affiliated Hospital of Guangxi Medical University, from February 2007 to October 2011. The control group included 164 healthy Zhuang subjects without genetic kinship who were healthy individuals or patients with functional dyspepsia, and did not have liver or gastrointestinal diseases. All patients had a well-established diagnosis of UC or CD, according to standard clinical criteria based on endoscopic and histopathological examinations. There were no significant differences

in age and sex between the study group and the control group. All patients and healthy controls gave informed consent and the study was approved by the ethical committee of the institute.

DNA extraction and genotyping of the *TLR4* and *TLR2* polymorphisms

Genomic DNA was extracted according to the modified protocol of Taggart. The primers used to amplify the *TLR4* gene (*Asp299Gly*, *Thr399Ile*) were designed according to the National Center for Biotechnology Information gene database, NM_138554, and the *TLR2* gene (*Arg677Trp*, *Arg753Gln*) with NM_003264 shown in Table 1 (primers were synthesized in SHENGGONG Biological Technology Co., Ltd., Shanghai, China).

Amplification was performed using H_2O_2 10.5 μ L, 2 \times Taq polymerase chain reaction (PCR) Master-mix 11.5 μ L (TIANGEN), DNA 1 μ L, and 1 μ L in addition to 10 μ mol/L of each primer in a total volume of 25 μ L. For *Asp299Gly* and *Arg753Gln*, cycle conditions were an initial denaturation for 5 min at 95 $^{\circ}$ C, followed by 28 cycles of denaturing at 95 $^{\circ}$ C for 40 s, annealing at 58 $^{\circ}$ C for 30 s, primer extension at 72 $^{\circ}$ C for 50 s, followed by a final extension at 72 $^{\circ}$ C for 10 min. For *Thr399Ile* and *Arg677Trp*, cycle conditions were an initial denaturation for 5 min at 95 $^{\circ}$ C, followed by 28 cycles of denaturing at 95 $^{\circ}$ C for 40 s, annealing at 62 $^{\circ}$ C for 40 s, primer extension at 72 $^{\circ}$ C for 50 s, followed by a final extension at 72 $^{\circ}$ C for 10 min (Thermo Electron Corporation, Waltham, MA, United States). All the PCR products were electrophoresed on a 1.5% agarose gel, with 1 \times tris-borate-EDTA buffer, V = 100V for 30 min, and visualized under ultraviolet illumination (Bio-Rad Gel Doc-2000, United States).

Five μ L PCR products of *TLR4* gene (*Asp299Gly*, *Thr399Ile*) and *TLR2* gene (*Arg677Trp*, *Arg753Gln*) were digested at 37 $^{\circ}$ C overnight with 0.5 μ L (10 U) *Nco* I, *Hinf* I, *Aci* I, and *Pst* I restriction enzymes (Fermentas), respectively. After enzymatic digestion, the fragments were separated and visualized by gel electrophoresis (2% Yito Bio-Instrument Company Ltd., Shanghai, China).

PCR products of each SNP were purified with a PCR purification kit (QIAGEN, Hilden, Germany) and were sequenced using an ABI Prism 377 DNA sequencer (LIU HE HUA DA GENE Technology Co, Ltd., Beijing, China).

Statistical analysis

The genetic equilibrium was tested using Hardy-Weinberg. Allele and genotype frequencies in patients and in controls were compared using the χ^2 test with SPSS 13.0, and *P* values were considered significant at a level of < 0.05.

RESULTS

RFLP analysis was performed to assess the status of SNPs in our samples after digestion with restriction enzymes (Figure 1).

Table 1 Specific polymerase chain reaction primers and restriction enzymes for each single nucleotide polymorphism				
Gene	Amino acid substitution	Sequence of primers(5'→3')	Fragments (bp)	Enzymes
TLR4	<i>Asp299Gly</i>	F-GATTAGCATACTTAGACTACTACCTCCATG R-GATCAACTTCTGAAAAAGCATCCCCAC	249	<i>Nco</i> I
	<i>Thr399Ile</i>	F-GGTTGCTGTCTCAAAGTGATTTTGGGAGAA R-ACCTGAAGACTGGAGAGTGAGTTAAATGCT	406	<i>Hinf</i> I
TLR2	<i>Arg677Trp</i>	F-GCCTACTGGGTGGAGAACCCT R-CCAGTTCATACTTGCACCCTC	199	<i>Aci</i> I
	<i>Arg753Gln</i>	F-CCTGGCAAGTGGATCATTGAC R-GGCCACTCCAGGTAGGTCTT	254	<i>Pst</i> I

TLR: Toll-like receptor.

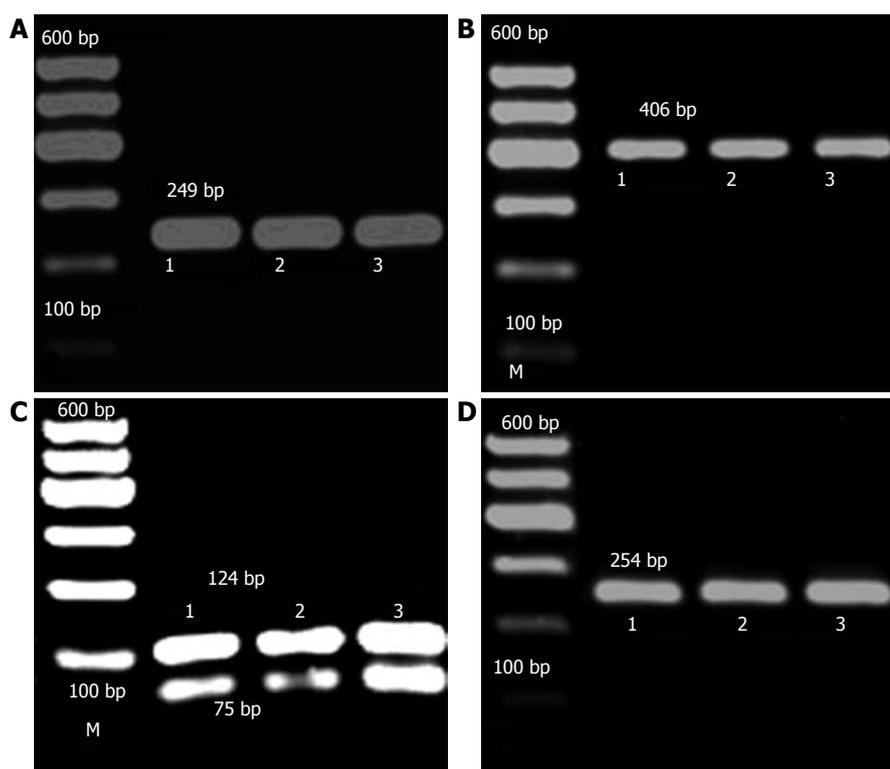


Figure 1 Polymerase chain reaction-restriction fragment length polymorphism analysis. A: Electrophoresis of *Asp299Gly* restriction fragment length polymorphism (AA type); B: Electrophoresis of *Thr399Ile* restriction fragment length polymorphism (CC type); C: Electrophoresis of *Arg677Trp* restriction fragment length polymorphism (CC type); D: Electrophoresis of *Arg753Gln* restriction fragment length polymorphism (GG type). M: Marker; 1: Ulcerative colitis; 2: Crohn's disease; 3: Control.

TLR4 gene *Asp299Gly* PCR-RFLP

Nco I restriction enzyme identified the sequence of CCATGG. For the heterozygous type (AG), this could be generated when digested with *Asp299Gly* and three bands of 249 bp, 223 bp and 26 bp, where one chain was cut, and the others were not. For the homozygous type (GG), a transite to G, *Nco* I identified the mutated site, and the mutated DNA was visible as a double band of 223 bp and 26 bp, whereas a single band of 249 bp was observed in the wild type (AA), as the site was not cut. In this study, only the wild type (AA) was detected, and no other types (Figure 1A).

TLR4 gene *Thr399Ile* PCR-RFLP

Hinf I restriction enzyme identified the sequence of GANTC. For the heterozygous type (CT), this could be

generated when digested with *Thr399Ile* and three bands of 406 bp, 377 bp and 29 bp, where only one chain was cut, and the others were not. For the homozygous type (TT), C transite to T, *Hinf* I identified the mutated site, and the mutated DNA was visible as a double band of 377 bp and 29 bp, whereas a single band of 406 bp was observed in the wild type (CC), as the site was not cut. In this study, only the wild type (CC) was detected, and no other types (Figure 1B).

TLR2 gene *Arg677Trp* PCR-RFLP

Aci I restriction enzyme identified the sequence of CCGC. For the heterozygous type (CT), this could be generated when digested with *Arg677Trp* and three bands of 199 bp, 124 bp and 75 bp, where one chain was cut, and the others were not. For the homozy-

gous type (TT), C transite to T, the site was not cut by *Aci* I restriction enzyme, and a single band of 199 bp was observed, for the wild type (CC), it identified the site, and the DNA was visible as a double band of 124 bp and 75 bp. In this study, only the wild type (CC) was detected, and no other types (Figure 1C).

TLR2 gene *Arg753Gln* PCR-RFLP

Pst I restriction enzyme identified the sequence of CTGCAG. For the heterozygous type (GA), this could be generated when digested with *Arg753Gln* and three bands of 254 bp, 214 bp and 40 bp, where one chain was cut, and the others were not. For the homozygous type (AA), G transite to A, *Pst* I identified the mutated site, and the mutated DNA was visible as a double band of 214 bp and 40 bp, whereas a single band of 254 bp was observed in the wild type (GG), as the site was not cut. In this study, only the wild type (GG) was detected, and no other types (Figure 1D).

DISCUSSION

UC and CD are multifactorial diseases of unknown etiology. Despite being disorders of the gastrointestinal tract, an abnormal immune response directed against the gut microflora has been postulated as a possible explanation in genetically susceptible hosts^[12].

Numerous studies have been performed on the frequency of NOD2/CARD15 mutations in IBD populations in Western Europe and Northern America. In CD patients from Northern America or Western Europe, allele frequencies ranged from 9.1%-12.9%, 6.6%-16.0% and 3.3%-6.0% for *Arg702Trp*, *1007insC*, and *Gly908Arg*, respectively^[13]. Interestingly, a recent study reported lower allele frequencies in Finnish IBD patients for all three NOD2/CARD15 variants in CD patients compared with the above-mentioned studies and similar frequencies in patients with UC^[14]. Additionally, in Asian IBD populations, NOD2/CARD15 variants were not detected at all^[4,5]. Thus, there is a need for more studies in different populations with IBD. Human TLRs participate in the innate immune response and signal the activation of adaptive immunity. Therefore, these TLRs may be important in autoimmune diseases such as IBD, rheumatoid arthritis and systemic lupus erythematosus^[15].

This is the first study to report the *TLR4* (*Asp299Gly*, *Thr399Ile*) and *TLR2* (*Arg677Trp*, *Arg753Gln*) gene mutations in patients with IBD from the Guangxi Zhuang population of China, where the ethnic background is heterogeneous, and includes Han, Zhuang and Rao. In this study, the mutation genotypes of the *TLR4* gene *Asp299Gly*, *Thr399Ile* and *TLR2* gene *Arg677Trp*, *Arg753Gln* were not found in the Zhuang population with IBD. Our results are in agreement with those from studies on Asian patients from Hong Kong, Hubei, Zhejiang, Shanghai and Japan^[4,7].

In recent years, several studies have reported divergent results^[16]. The allele frequency of the *Asp299Gly* variant ranges from 0%-10% in UC, from 8%-13% in

CD and from 3%-15% in healthy controls^[17]. Franchimont *et al*^[10] reported that the *TLR4* SNP was associated with UC and CD in a Belgian study. This association was in accordance with Dutch, Greek, Australian and German populations with CD, and an association with colonic disease has been described^[18-21]. In one German cohort, an association between the *TLR4* *Thr399Ile* SNP and UC was demonstrated^[22]. However, because of substantial heterogeneity between populations, no association was noted in Scottish CD patients^[23]. The *TLR2* gene *Arg753Gln* variant frequency was 9.4% in Germany, and 1% in Spain^[24], the *TLR2* gene *Arg677Trp* was 30.3% in Tunisia^[25], where the difference was significant. In addition, Pierik *et al*^[26] reported that an association was found between non-synonymous variants and extensive colonic disease with UC and CD in the *TLR2* genes.

In our study, we did not find mutations in *TLR4* gene *Asp299Gly*, *Thr399Ile* and *TLR2* gene *Arg677Trp*, *Arg753Gln* in the Zhuang population with IBD. These findings were different from those in Tunisia, Germany and Spain. Our results showed that the *TLR4* gene *Asp299Gly*, *Thr399Ile* and *TLR2* gene may not be associated with IBD patients from the Guangxi Zhuang Autonomous Region of China. This is possibly due to the existence of racial and geographic differences.

The present study supports the notion that genetics, immunology, environment and infection may be vital in the pathogenesis of IBD. However, the heterogeneity in the small number of available studies limited the ability to draw conclusions. Further studies using a larger cohort of patients with IBD are warranted to identify the risk factors and genetic susceptibility to IBD.

COMMENTS

Background

The pathogenesis of inflammatory bowel disease (IBD) is not completely clear, however, contributing factors may include immunology, genetics, environment and infection. It has been reported that the *NOD2/CARD15* polymorphisms (single nucleotide polymorphisms, SNPs) were found to be associated with Crohn's disease (CD) in Caucasian populations. However, the SNPs were not found to be associated with CD in Japan and China. In addition, the *TLR2* and *TLR4* were reported to provide evidence for several determinants. The study assessed whether the known gene polymorphisms in the toll-like receptor 2 (*TLR2*) and *TLR4* genes determined susceptibility for IBD in the Guangxi Zhuang population. Since Guangxi has a large Zhuang population, genetic diseases and gene polymorphisms are unique.

Research frontiers

TLR4 (*Asp299Gly*, *Thr399Ile*) and *TLR2* (*Arg677Trp*, *Arg753Gln*) were found to be associated with IBD in Tunisia, Germany and Spain, but not in Hong Kong, Hubei, Zhengjiang, Shanghai and Japan. This is possibly due to the existence of racial and geographic differences. This study determined whether the *TLR4* (*Asp299Gly*, *Thr399Ile*) and *TLR2* (*Arg677Trp*, *Arg753Gln*) were associated with IBD in the Guangxi Zhuang population from the Guangxi Zhuang Autonomous Region of China.

Innovations and breakthroughs

Only a few studies have investigated the *TLR2* and *TLR4* gene polymorphisms in China. Moreover, all the subjects in these studies were from the Han population. This is the first study to report that *TLR4* gene *Asp299Gly*, *Thr399Ile* and *TLR2* gene *Arg753Gln*, *Arg753Gln* polymorphisms may not be associated with IBD in the Zhuang population from the Guangxi Zhuang Autonomous Region of China.

Applications

TLR2 and TLR4 variants may be rare or non-existent in the Zhuang population from the Guangxi Zhuang Autonomous Region of China.

Terminology

TLRs play a key role in microbial recognition in innate immunity and control the adaptive immune responses. Among the TLRs, TLR2 recognizes bacterial components such as lipoprotein, lipoteichoic acids, peptido-glycan and zymozan. TLR4 requires CD14 to form the lipopolysaccharide (LPS) receptor. LPS, found in the outer membrane of Gram-negative bacteria, is opsonized by LPS-binding protein, and recognized by CD14. The LPS-LPS binding protein-CD14 complex activates TLR4, which results in the activation of NF- κ B. TLR2 and TLR4 gene mutations or deletions can induce abnormal immune responses.

Peer review

This study demonstrates no association of TLR2 and/or TLR4 polymorphism with IBD in a patient cohort within a restricted Chinese population. Although this is a "negative" result, it adds to the impact of genetic and/or ethnical predisposition to IBD.

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Puncture injection of para-toluenesulfonamide combined with chemoembolization for advanced hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is difficult to eradicate due to its resilient nature. Portal vein is often involved in tumors of large size, which exclude the patient from surgical resection and local ablative therapy, such as percutaneous ethanol injection (PEI) and radiofrequency ablation (RFA) because they were considered neither effective nor safe. Currently, there is almost no effective treatment for HCC of such condition. As a unique antitumor agent in form of lipophilic fluid for local injection, para-toluenesulfonamide (PTS) produces mild side effects while necrotizing the tumor tissues quickly and efficiently. Being largely different from both PEI and RFA therapies, PTS can disseminate itself in tumors more easily than other caustic agents, such as alcohol. So PTS may offer additional benefit to HCCs with vascular involvement. We herein describe a 70-year-old HCC patient who was treated with the combination of PTS injection and transcatheter arterial chemoembolization, resulting in a significantly improved clinical prognosis.

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INTRODUCTION

Transcatheter arterial chemoembolization (TACE) has become the standard treatment for unresectable hepatocellular carcinoma (HCC). Nonetheless, clinical outcomes are often unsatisfactory, especially for recurrent cases. As a novel anticancer agent, para-toluenesulfonamide (PTS) is completely different from genetic, classical chemical and molecular targeted drugs, and has shown amazing antitumor effect in animal HCC experiment^[1,2]. Primary pharmacological studies suggest that PTS inhibits tumor growth by acting as a tumor necrotizing agent^[1,2]. PTS may strengthen the effect of TACE in advanced HCC. We herein report a patient with refractory HCC who was treated with PTS injection after TACE, which resulted in a very good clinical prognosis.

CASE REPORT

The patient was a 70-year-old man. A mass was found in his right lobe of the liver in May 2004. He refused

to receive further diagnosis and treatment until January 2006 when he began to suffer from anorexia and dull abdominal pain. Computer tomography (CT) detected a large mass (about 9.5 cm × 8.5 cm × 8.5 cm) in the right lobe of the liver with significant enhancement (Figure 1A). Alpha-fetoprotein (AFP) level was > 1210 ng/mL. He was diagnosed with a histologically proven HCC. Due to the large tumor size and invasion to the portal vein, the patient underwent two rounds of TACE with an interval of one month. The drugs used were 5-fluoro-2-deoxyuridine (1.0 g), epirubicin (40 mg) and lipiodol (10 mL). In May 2006, three months after the second TACE, a routine postoperative CT scan found a minimal decrease in tumor size with necrosis < 25% (Figure 1B), while the AFP level was still > 1210 ng/mL. The second angiography revealed hepatic artery-portal vein fistula and hepatic arteriovenous fistula. Although additional gelfoam pieces were used and the fistula disappeared after embolization, a poor prognosis was still predicted. Consequently, PTS (Beijing Vision Drugs Development Ltd., Beijing, China) was used to enhance the effect of TACE after informed consent was obtained from the patient.

The mass was punctured percutaneously with a fine needle under CT guidance. After the tip of the needle was manipulated into the mass, about 10 mL of PTS was injected intratumorally in a multi-point fashion. This injection was repeated four times. The interval between each procedure was between three and seven days according to the patient's clinical condition. When all five injections were completed, the AFP level dropped to 1106 ng/mL. Two months later, a routine follow-up CT showed moderate improvement, no blood being supplied to the tumor (Figure 2A), and the AFP level dropped further down to 185 ng/mL. In an attempt to investigate the increased efficacy, PTS injections were repeated three more times. Following a routine check two months later, a CT examination of the abdomen demonstrated complete deposit of oil and no signs of recurrence or tumor embolism (Figure 2B). Furthermore, the AFP was within the normal reference range (4.44 ng/mL).

The only side effect of this therapy was abdominal pain, which occurred after the first two procedures, but subsided shortly thereafter in approximately 10 min. No other discomfort was noted post-procedurally. Four years following the last PTS treatment, the patient exhibited no evidence of recurrence and no other abnormal liver function serum values, and he had a normal serum AFP level. The AFP level changes in the course of the treatment are summarized in Table 1.

DISCUSSION

Despite the fact that TACE has become the standard treatment for unresectable HCC, it is frequently unsuccessful. Rate of local recurrence following tumor resection is also unacceptably high^[3-6]. In addition, TACE alone fails to control the tumor completely, often leading

Table 1 Changes of alpha-fetoprotein in the course of therapy

Time (yr-mo)	APF (ng/mL)	TACE	PTS injections
2005-4	533.8		
2006-1	> 1210	Yes	
2006-2	> 1210	Yes	
2006-5	> 1210	No	5 times
2006-6	1106	No	
2006-8	185	No	3 times
2006-10	4.44	No	
2008-6	2.69	No	
2009-3	3.84	No	
2010-4	3.86	No	

A sharp decrease in alpha-fetoprotein (AFP) levels occurred right after para-toluenesulfonamide (PTS) injections. The initial series of transcatheter arterial chemoembolization (TACE) failed to control the tumor (although the TACE's effect on fistula embolization was irreplaceable). PTS treatment appeared to be effective and well-tolerated. A 4-year follow-up showed no sign of recurrence.

to a poor prognosis. In this report, the patient had received two sessions of TACE and embolized the fistula successfully before admission. Although little effect was got on HCC, the TACE did restrict the tumor's growth and metastasis that made it possible for PTS to eradicate the tumor later. Due to the large tumor size and involvement of the portal vein, this lesion was inoperable, and other local ablative therapies, such as percutaneous ethanol injection, microwave coagulation therapy and radiofrequency ablation (RFA) were considered neither effective nor safe. Complications caused by these modalities may result in a high mortality rate. Therefore, we attempted to treat the patient with the combined administration of PTS and TACE. Consequently, the patient's condition was significantly improved with satisfactory tumor control and without severe complications.

Para-toluenesulfonamide (P-TSA) is the active ingredient in PTS. The P-TSA is a white, odorless, crystalline substance that has a very low solubility in water. The molecular formula is C₇H₉NO₂S. The injection solution is a clear, colorless, oil liquid with a characteristic odor and contains 330 mg/mL P-TSA. PTS has been approved for clinical trial injection in both 3 mL ampoule and 5 mL ampoule. Recommended storage temperature is 25 °C (77°F), although a range of 10-35 °C (50-90°F) is acceptable. Long-term exposure to light should be avoided.

PTS produces mild side effects while necrotizing the tumor tissues effectively and thoroughly. However, PTS is still in the phase of clinical trial, and the mechanism of the antitumor activity of PTS is still unclear. Primary pharmacological studies suggest that PTS inhibits tumor growth by acting as a tumor necrotizing agent^[1,2].

It has been shown that PTS does not cause serious side effects that have been observed frequently in conventional chemotherapy and locoregional therapy (e.g., RFA, alcohol injection, *etc.*), such as fever, bone marrow suppression, stomach discomfort, hemorrhage, needle-track seeding, lesion abscess, liver failure, biloma, biliary

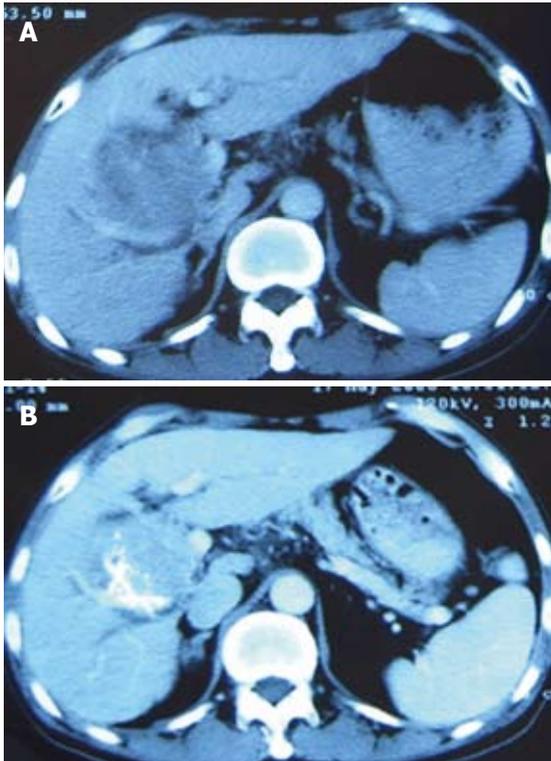


Figure 1 Transcatheter arterial chemoembolization had little effect on the hepatocellular carcinoma, but restrict the size of tumor mass. A: Contrast computer tomography (CT) before the procedure showed a huge hypodense mass with remarkable enhancement and portal vein embolus; B: Two months after the initial 2 series of transcatheter arterial chemoembolization, contrast CT still found a hypodense nodule with partial contrast enhancement and portal vein invasion.

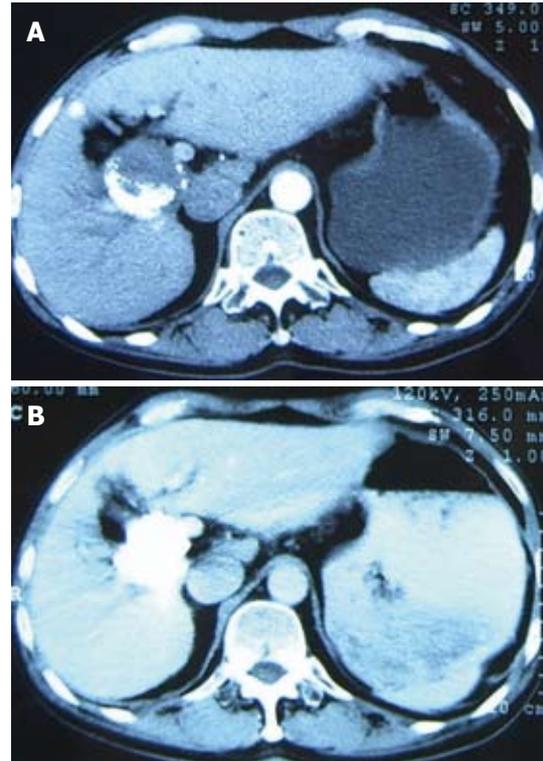


Figure 2 The following para-toluenesulfonamide injections resulted in a significantly improved clinical prognosis. A: Contrast computer tomography (CT) after the first 5 para-toluenesulfonamide (PTS) injections demonstrated a decreased nodule with partial retention of lipiodol. Remarkable necrosis and no blood supply were found in the rest part of the tumor; B: Two months after the last 3 PTS injections, contrast CT revealed homogeneous dense retention of lipiodol within the entire tumoral mass and no recurrent lesion was identified.

stricture, portal vein thrombosis, and hemothorax^[7-9]. Because most anticancer drugs are corrosive and extremely toxic, they will destroy both cancer cells and normal cells when given locally at high concentrations. PTS is a local therapeutic drug that is injected directly into the tumors and has been shown to cause selectively necrosis in a variety of cancers with minimal damage to normal tissues^[2,10].

Local ablative therapies share similar difficulties with surgical resection. The size, site and number of tumors, vascular and extrahepatic involvement as well as liver function of the patient pose a relatively minor effect on the usage of PTS^[8,9]. PTS is a more readily available alternative to the local ablative therapies.

PTS, in form of lipophilic fluid, kills tumor cells by a rodent mode. Local and intratumoral injection is the optimal route of PTS delivery. Being largely different from both alcohol and RFA therapies, PTS can disseminate itself in tumors more easily than other caustic agents, such as alcohol. Therefore, a successful PTS administration is to approach to the anatomically dangerous or hard-to-reach areas and diffuse to the target area and induce injury to the tumor tissues. This might be the mechanism as to why PTS combined with TACE could effectively treat the HCC with vascular invasion. As a locoregional antitumor agent, PTS is safe^[11,2,10]. But up to date, PTS

is still only a locally-used antitumor agent. It is intended mainly for the treatment of a limited number of detectable tumors. PTS is not suitable to be used alone for the treatment of multifocal HCCs.

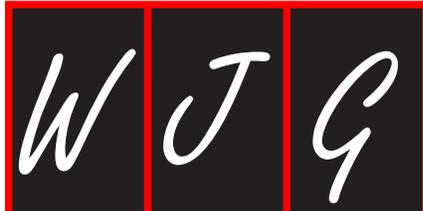
This case report demonstrated that PTS is effective in treating liver cancers by intratumoral injection, which was hypothesized to enhance the effect of TACE. This combined therapy may prove to be useful in the treatment of patients with refractory and recurrent HCC. Therefore, subsequent large, multi-center, randomized controlled studies are needed to facilitate the introduction of PTS as a novel modality for the treatment of cancers.

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GENERAL INFORMATION

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- Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

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- Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 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]

No author given

- 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]

Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

- 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]

No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS A Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 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

Author(s) and editor(s)

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

- 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>

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean \pm SD or mean \pm SE.

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Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as ν (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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APPENDIX I Meetings

ABOUT COVER Dr. Jing-Yun Ma, a renowned expert in biomedical editing and publishing, worked as the editorial director and senior editor of *World Journal of Gastroenterology* (1996-2005) and other major national and international medical journals (1976-1995). She is now the Editor-in-Chief of Jing-Yun Ma Expert Office for Biomedical Editing and Publishing.

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Role of nuclear receptor NR4A2 in gastrointestinal inflammation and cancers

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Abstract

NR4A2 is a transcription factor belonging to the steroid orphan nuclear receptor superfamily. It was originally considered to be essential in the generation and maintenance of dopaminergic neurons, and associated with neurological disorders such as Parkinson's disease. Recently, NR4A2 has been found to play a critical role in some inflammatory diseases and cancer. NR4A2 can be efficiently trans-activated by some proinflammatory cytokines, such as tumor necrosis factor- α , interleukin-1 β , and vascular endothelial growth factor (VEGF). The nuclear factor- κ B signaling pathway serves as a principal regulator of inducible NR4A expression in immune cells. NR4A2 can trans-activate Foxp3, a hallmark specifically expressed in regulatory T (Treg) cells, and plays a critical role in the differentiation, maintenance, and function of Treg cells. NR4A2 in T lymphocytes is pivotal for Treg cell induction and suppression of aberrant induction of Th1 under physiological and pathological conditions. High density of Foxp3⁺ Treg cells is significantly associated with gastrointestinal inflammation, tumor immune escape, and disease progression. NR4A2 is produced at high levels in CD133⁺ colorectal carcinoma (CRC) cells and significantly upregulated by cyclooxygenase-2-de-

rived prostaglandin E₂ in a cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA)-dependent manner in CRC cells. The cAMP/PKA signaling pathway is the common pathway of NR4A2-related inflammation and cancer. NR4A2 trans-activates osteopontin, a direct target of the Wnt/ β -catenin pathway associated with CRC invasion, metastasis, and poor prognosis. Knockdown of endogenous NR4A2 expression attenuates VEGF-induced endothelial cell proliferation, migration and *in vivo* angiogenesis. Taken together, NR4A2 emerges as an important nuclear factor linking gastrointestinal inflammation and cancer, especially CRC, and should serve as a candidate therapeutic target for inflammation-related gastrointestinal cancer.

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Key words: NR4A2; Inflammation; Immune cells; Signaling pathway; Gastrointestinal carcinoma

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INTRODUCTION

A quarter of cancer-related deaths worldwide are currently associated with chronic inflammation^[1]. Most malignancies in the digestive system such as colorectal carcinoma (CRC), gastric cancer and hepatocellular carcinoma belong to the inflammation-related cancers^[2-5]. The type of inflammation related to these cancers is chronic inflammation, or more precisely, nonresolving inflammation. The development and progression of these tumors

in the digestive system are accompanied by angiogenesis, release of inflammatory cytokines and/or chemokines, and recruitment of immune cells in the tumor microenvironment. Apart from some infectious agents such as hepatitis B virus and *Helicobacter pylori*, inflammation-related molecules and their related inflammatory networks play important roles in the development of this group of cancers. However, it is clear that a single or even a few molecules are unable to represent the inflammatory predisposition of a given cancer. In the inflammation-related cancers, inflammatory molecules do not function in isolation, but interact with one another to form inflammatory networks. A small number of highly connected protein nodes (known as hubs) in the inflammatory network may have high probabilities of engaging in essential biological functions. In our previous work on screening hub genes associated with liver metastasis of gastric cancer, we identified NR4A2, one of the hub genes related to gastric cancer malignant phenotype^[6]. Here, we summarize the role of NR4A2 in inflammation and cancer.

NR4A2 (also known as NOT, TINUR, RNR-1, HZF-3 and Nurr1) maps to chromosome 2q22-23 and is a transcription factor belonging to the steroid nuclear hormone receptor superfamily. This superfamily also includes two other members, NR4A1 (also known as HMR, N10, TR3, NP10, GFRP1, NAK-1, NGFI β and Nur77) and NR4A3 (also known as CHN, TEC, CSMF, MINOR and NOR1)^[7]. Their ligand-binding pockets are hidden by bulky amino acids, therefore, the three nuclear factors belong to an orphan receptor subfamily. All three subfamily members bind to a consensus sequence AAAGGTCA as monomers or to palindromic DNA binding motif sequence TDATATTTX₆AAATGCCA as homodimers. Expression of each family member alone is sufficient to activate the above sequence-directed transcriptional activities, indicating that the family members are constitutive orphan steroid receptors that do not require ligands for activation^[8]. The three nuclear receptors have also been implicated in cell cycle regulation, energy metabolism, apoptosis, inflammation and carcinogenesis. They are important for apoptosis in lymphocytes and other cell types^[9-12] and for the differentiation of dopaminergic neurons^[13]. NR4A1 and NR4A3 have been shown to be involved in apoptosis-related pathways. NR4A1 has been reported to play either an antiapoptotic or proapoptotic function depending on cell types. NR4A3 has been reported to play a partly redundant functional role with NR4A1 in inducing T-cell apoptosis^[9]. Knocking out both NR4A1 and NR4A3 in mice leads to rapid postnatal development of acute myeloid leukemia (AML), indicating that they function as potent tumor suppressors^[14,15]. Meanwhile, the three NR4A receptors are transcriptional regulators of hepatic glucose metabolism and lipid metabolism. NR4As function as novel branches of cyclic adenosine monophosphate (cAMP)-dependent regulators of hepatic glucose production under healthy and diabetic conditions. Activated NR4A1 and NR4A3 elevate the production of blood glucose^[16]. NR4As inhibits adipo-

cyte differentiation, leading to repressed adipogenesis^[17]. NR4A1 and NR4A2 can form heterodimers with retinoic acid receptor and influence retinoid signaling and Th17 cell differentiation^[18,19].

NR4A2 has been previously linked to neurological diseases because of its essential role in the generation and maintenance of dopaminergic neurons in the brain. Gene encoding NR4A2 may be susceptibility gene for neurological diseases such as Parkinson's disease, schizophrenia, and manic depression. Homozygous NR4A2 knockout mice have severe impairments in midbrain neuronal development and dopamine expression, and die soon after birth^[20]. However, the horizontal and vertical movement of the heterozygous NR4A2 knockout mice is impaired by the reduction in brain dopamine^[21]. The most important event in downregulating NR4A2 expression in brain tissues is mutations, such as missense mutations in exon 3, and point mutations in exon 1 of NR4A2 have been reported in disorders related to dopaminergic dysfunction such as schizophrenia, manic depression, and familial Parkinson's disease^[22-24]. In recent years, however, more researches have focused on the relationship between NR4A2 and inflammation.

NR4A2 AND INFLAMMATION

NR4A2 and immune cells

Immune cells are essential in controlling the inflammatory response. T and B lymphocytes, macrophages, neutrophils, and mast cells are all important in the maintenance of chronic inflammation and play active roles in the initiation and progression of inflammation-related cancers. Cytotoxic CD8⁺ T lymphocytes and CD4⁺ T helper lymphocyte subpopulations [Th1, Th2, Th17, and regulatory T (Treg) cells] play key roles in balancing cancer-promoting inflammation and antitumor immunity in the tumor microenvironment. Imbalances in Th1/Th2, neutrophil/CD8⁺ T cells, and CD8⁺ T cell/Treg cell in tumors or adjacent tissues, high density of intratumoral macrophage infiltration, as well as high circulating neutrophil-to-lymphocyte ratio are associated with the prognosis of cancer patients^[25]. Recently, NR4A2 has been found to function as a critical regulator in T lymphocytes, macrophages, mast cells and even fibroblasts.

Treg cells (CD4⁺CD25⁺Foxp3⁺) inhibit immune responses by suppressing CD8⁺ T cell or Th1 cell function, playing an important role in maintaining immune homeostasis. Foxp3, a hallmark of Treg cells, is specifically expressed in Treg cells and plays a critical role in the differentiation, maintenance and effector functions of these cells. Foxp3 has recently been identified as a direct target of NR4A2. The -209 to +12 promoter region of Foxp3 encoding region has been confirmed to be the minimal responsive region to NR4A2. NR4A2 regulate CD4⁺ T cells by inducing Foxp3 and strongly repressing Th1 cytokine production, such as interleukin (IL)-2 and interferon (IFN)- γ . NR4A2 in T cell is pivotal for Treg cell induction and suppression of aberrant induction of

Th1 under physiological and pathological conditions. Deletion of NR4A2 in T cells attenuates the induction of Treg cells and causes aberrant induction of Th1, leading to exacerbation of colitis. Both IFN- γ and IL-17 are apparently repressed under Th1 and Th17 conditions, respectively, and Foxp3 can be induced under Th1 and Th17 conditions by NR4A2^[26]. Thus, NR4A2 is crucial in lineage maintenance and effector functions of Treg cells and in regulation of the Th1/Treg balance, contributing to immune homeostasis (Figure 1A).

Macrophages can be divided into two main classes: M1 and M2. M1 macrophages (classically activated) originate upon encounter with IFN- γ and microbial stimuli and are characterized by IL-12 production and consequent activation of a polarized type I T cell response, fighting against tumors, producing high amounts of inflammatory cytokines, and activating adoptive immunity. M2 macrophages are responsible for angiogenesis, remodeling and repair of wounded tissues, and promote carcinogenesis and downregulate M1 function. NR4As are highly inducible in macrophages by diverse inflammatory stimuli. Treatment of macrophages with lipopolysaccharide, cytokines, or oxidized lipids triggers the transcriptional induction of NR4A expression. NR4A2 is an intermediate maker for macrophage activation^[27]. The nuclear factor (NF)- κ B signaling pathway is a principal mediator of inducible NR4A expression in macrophages^[28]. NR4A2 is a target of macrophage migration inhibitory factor (MIF) signaling and plays an active role in regulating mitogen-activated protein kinase (MAPK) phosphatase 1, a critical MAPK signaling inhibitor^[29]. NR4A2 may serve as a potential transcriptional mediator of inflammatory signals in activated macrophages. However, it is necessary to define the difference in NR4A2 expression between M1 and M2; the two functionally different macrophages.

NR4A2 and autoimmune diseases

NR4A2 plays either a proinflammatory or anti-inflammatory role. The conflicting roles of NR4A2 depend on the type of immune disorders. Multiple sclerosis (MS) is an autoimmune disease mediated by Th17 and Th1 cells in the central nervous system (CNS). In an animal model of MS, NR4A2 is selectively upregulated in T cells isolated from the CNS, while forced expression of NR4A2 augments promoter activities of IL-17 and IFN- γ genes, leading to excess production of these cytokines^[30]. In contrast, NR4A2 can also exert anti-inflammatory and neuroprotective effects by docking to NF- κ B/p65 on target inflammatory gene promoters in an NR4A2/CoREST transrepression pathway in microglia and astrocytes, leading to protection of dopaminergic neurons from inflammation-induced death^[31]. Furthermore, NR4A2 is a key downstream mediator of cAMP response element-binding protein (CREB)-induced neuroprotection after insults leading to excitotoxic and oxidative stress^[32].

Chronic inflammation is a hallmark of rheumatoid arthritis and osteoarthritis. The arthritis is a well-established

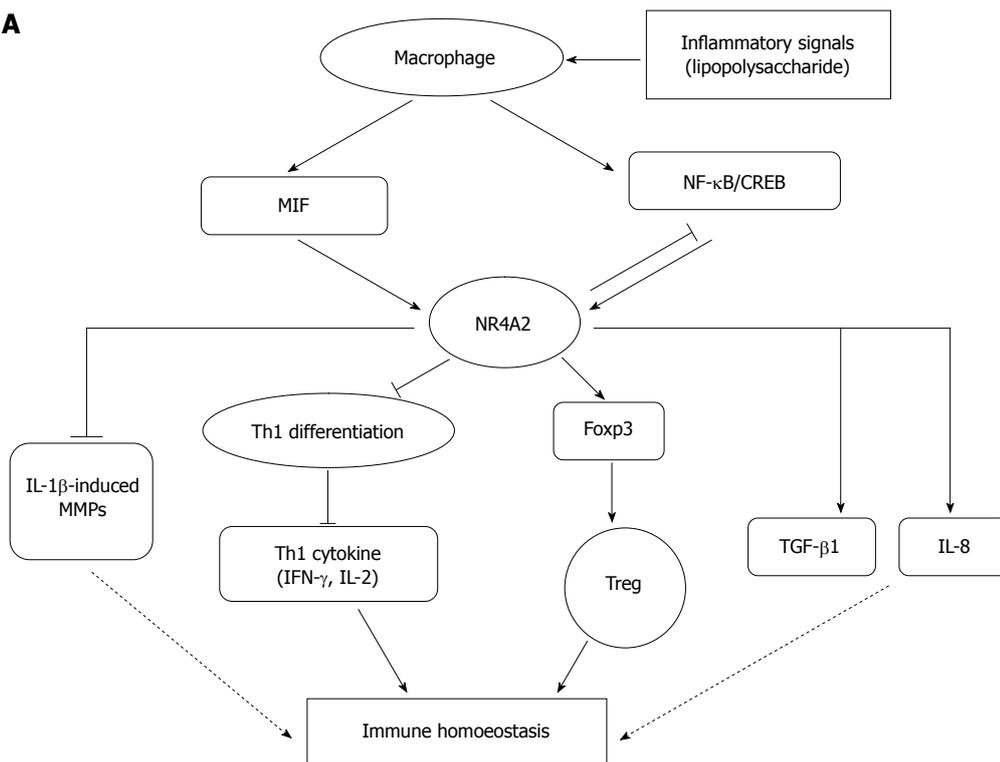
disease model to study the mechanism by which NR4A2 is involved in the process of chronic inflammation. NR4A2 expression is markedly higher in synovial tissue of patients with rheumatoid arthritis compared with normal subjects. The high expression of NR4A2 is caused by the stimulation of some proinflammatory cytokines, such as tumor necrosis factor (TNF)- α , IL-1 β and prostaglandin E₂ (PGE₂). Ectopic expression of NR4A2 induced by TNF- α in normal synoviocytes significantly increases cell proliferation and survival, promotes anchorage-independent growth, and induces migration and invasion^[33]. The trans-activation of NR4A2 by these cytokines and molecules depends on distinctive subunit binding to the NR4A2 promoter. IL-1 β and TNF- α trans-activate the NR4A2 *via* a proximal promoter region that contains a consensus NF- κ B DNA-binding motif. IL-1 β - and TNF- α -induced NF- κ B binding to this site is due predominantly to p65-p50 heterodimer and p50 homodimer complexes^[34]. NR4A2 robustly promotes IL-8 expression *via* co-operating with the NF- κ B/p65 subunit in the presence of TNF- α in human inflammatory disease^[35]. NR4A2 and TNF- α also synergistically induce matrix metalloproteinase (MMP)-13 protein which is a critical enzyme for the degradation of type II collagen molecules^[36]. NR4A2 trans-activates MMP-13 by directly targeting the proximal region of the MMP-13 promoter^[33]. PGE₂ signaling leads to the phosphorylation of CREB transcription factors, which can, in turn, bind to the NR4A2 promoter region -171/-163 and activate transcription. PGE₂ can repress IL-1 β -induced MMP-1 and activate NR4A2 expression. Meanwhile, NR4A2 antagonizes the progression of IL-1 β -induced MMP-1 in osteoarthritic cartilage^[37]. In human synovial tissue, corticotropin-releasing hormone (CRH) also can induce the expression of NR4A2, which in turn upregulates CRH gene expression. NR4A2 is a downstream effector molecule in the modulation of endothelial function by CRH signaling^[38]. Thus, NR4A2 as a mediator of an autocrine inflammatory cascade to amplify the inflammatory response participate in the CRH-receptor-mediated signaling in synovial tissue *via* the cAMP/CREB pathway. Figure 1B depicts the potential role of NR4A2 on the development of autoimmune diseases (MS and rheumatoid arthritis) and neurodegenerative diseases like Parkinson's disease.

NR4A2 AND GASTROINTESTINAL CANCER

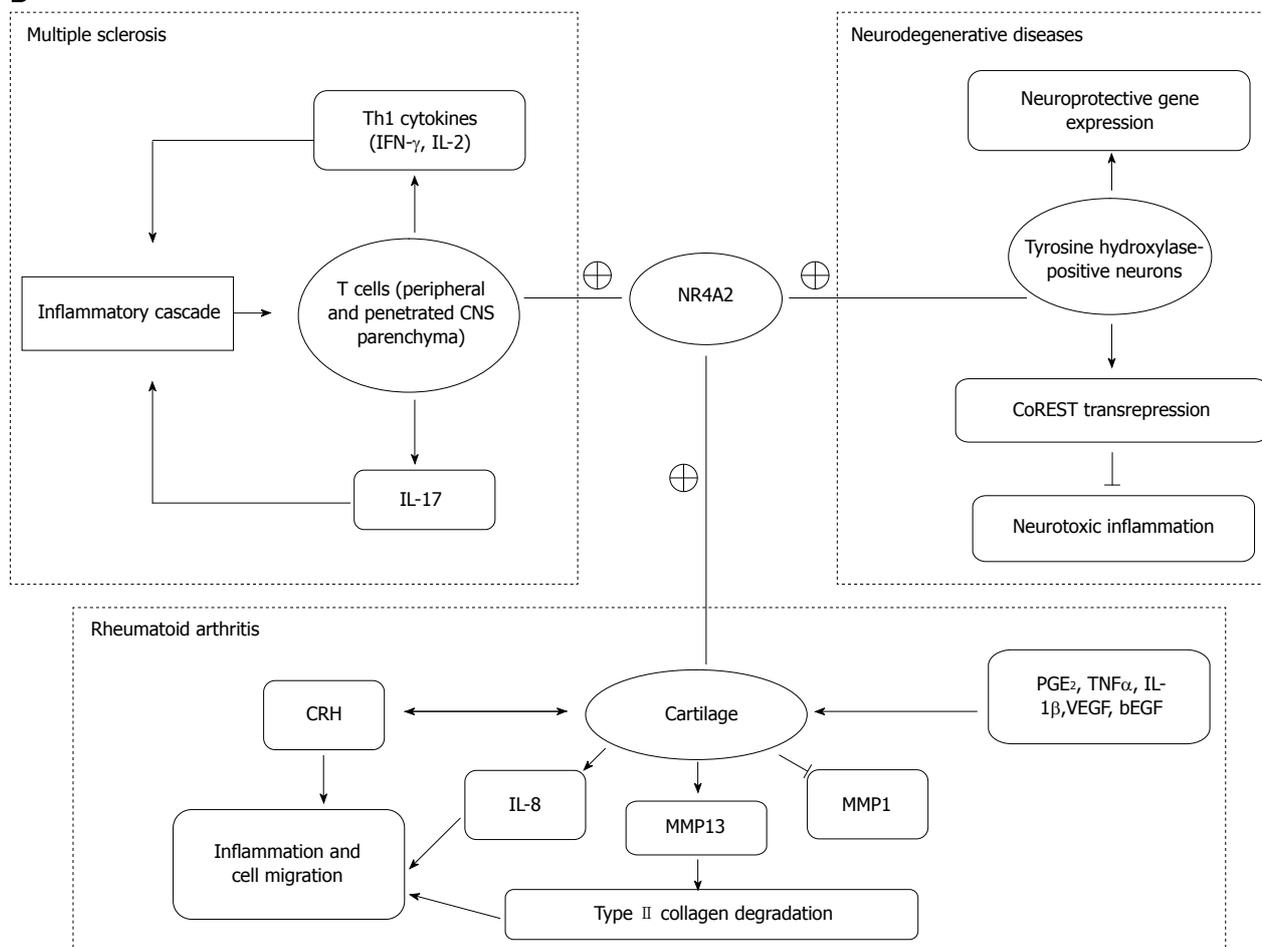
NR4A2 promotes proliferation of cancer cells

NR4A2 and endothelin-1 are expressed at a significantly higher level in the CD133⁺ fraction than the CD133⁻ fraction of colon cancer cells. The overexpression of CD133, NR4A2, and endothelin-1 is also evident in human colon cancer specimens compared to normal tissues. Furthermore, effective knockdown of CD133 protein is associated with a parallel reduction of NR4A2 and endothelin-1 protein, indicating the existence of a functional

A



B



C

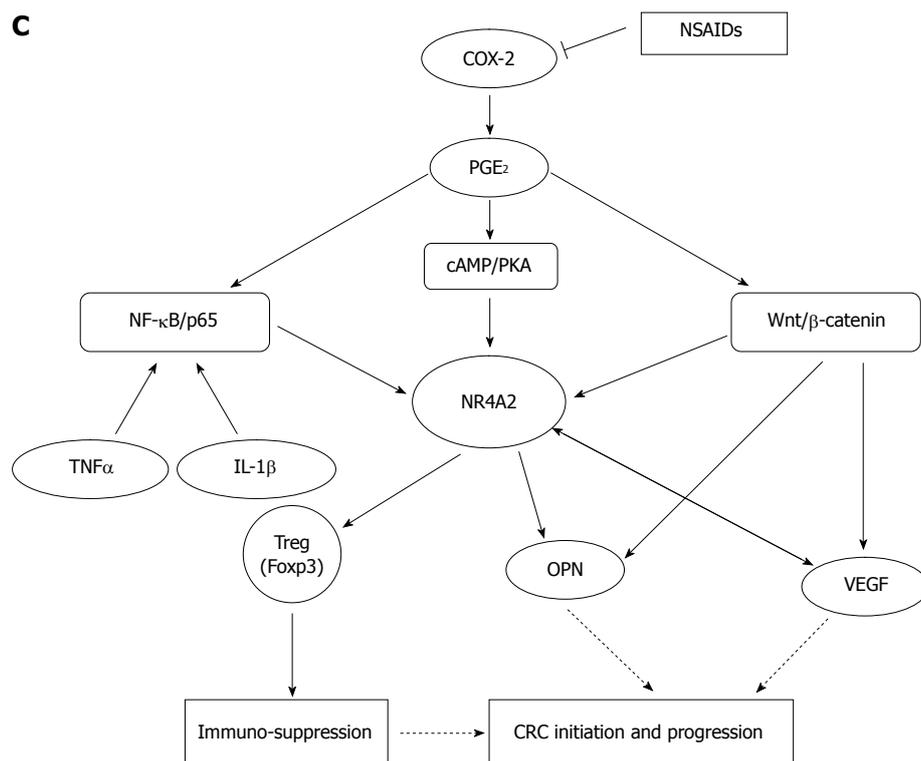


Figure 1 Potential role of NR4A2. A: Potential role of NR4A2 in immune homeostasis; B: Potential role of NR4A2 in development of autoimmune diseases (MS and rheumatoid arthritis) and neurodegenerative diseases; C: Potential role of NR4A2 in development of colorectal carcinoma. →: Promote; ⊥: Inhibit; +: NR4A2 up-regulated in these tissues/cells. MIF: Migration inhibitory factor; NF: Nuclear factor; cAMP: Cyclic adenosine monophosphate; CREB: cAMP response element-binding protein; IL: Interleukin; TGF: Transforming growth factor; IFN: Interferon; CNS: Central nervous system; PGE₂: Prostaglandin E₂; TNF: Tumor necrosis factor; VEGF: Vascular endothelial growth factor; bEGF: Basic epidermal growth factor; CRH: Corticotropin-releasing hormone; MMP: Matrix metalloproteinase; COX: Cyclooxygenase; NSAID: Nonsteroidal anti-inflammatory drug; PKA: Protein kinase A; OPN: Osteopontin; CRC: Colorectal carcinoma.

relationship between CD133, NR4A2 and endothelin-1 expression in colon cancer cells^[39]. NR4A2 is involved in anchorage-independent growth of cancer cells and plays a significant role in mediating thromboxane A₂ receptor induced cell proliferation and transformation in several cancer cell lines^[11,40]. NR4A2 also plays a key role as a transcriptional integration point between the eicosanoid and fatty acid metabolic pathways involving in energy utilization *via* fatty acid oxidation, thus facilitating CRC cell survival and growth^[41]. These results indicate that NR4A2 expressed in cancer cells might play an active role in promoting the initiation and progression of CRC.

NR4A2-related inflammatory pathways in gastrointestinal cancers

CRC is closely related to inflammation, because regular use of nonsteroidal anti-inflammatory drugs (NSAIDs) reduces relative risk of developing CRC by 30%^[42]. NSAIDs inhibit cyclooxygenase (COX) enzymes and prostaglandin synthases, which are key components in the arachidonic acid metabolism pathway. The main mechanism of action of NSAIDs on CRC is to inhibit COX-2, the inducible isoform of the COX enzyme. COX-2 is upregulated in inflammation and cancer, resulting in the production of PGE₂ which binds to and activates G-protein-coupled prostaglandin E receptor 1 (EP1)^[43]. COX-2-derived PGE₂ is produced at high levels

in CRC. Some evidence indicates that PGE₂ plays a pro-oncogenic role in CRC progression. PGE₂ stimulation of the human EP1 receptor upregulates the expression of NR4A2 by a mechanism involving the sequential activation of the Rho, protein kinase A (PKA), CREB and NF-κB signaling pathways^[44]. NR4A2 is an immediate early gene induced by PGE₂ in a cAMP/PKA-dependent manner in CRC cells. NR4A2 can stimulate progression of CRC downstream from COX-2-derived PGE₂^[45]. The common pathway of NR4A2-related inflammation and cancer is the cAMP/PKA signaling pathway. The cAMP/PKA signaling pathway plays a critical role not only in inflammation but also in carcinogenesis^[46-48]. It has been proven that cAMP/PKA can activate CREB-dependent reporter gene expression in gastric cancer cell lines^[49]. Through cAMP/PKA activation, PGE₂ stabilizes the complex of PI3K/Ras to inhibit cell apoptosis in colon cancer cell lines^[50]. The proximal promoter region of NR4A2 contains a CREB-binding site by which this transcriptional factor participates in cAMP-mediated induction^[32]. However, the cAMP/PKA pathway is not the only way by which NR4A2 participate in the progression of CRC. PGE₂ either modulates the β-catenin signaling axis, a key pathway for colorectal tumorigenesis, or act via NR4A2^[51]. PGE₂ can induce the expression of NR4A2 and two target genes of Wnt/β-catenin: vascular endothelial growth factor (VEGF) and cyclin D1. NR4A2

protein can be regulated by Wnt signaling^[52]. Meanwhile, NR4A2 and Wnt signaling are sequentially repressed after blockade of COX-2 activity, with NR4A2 inhibition occurring in the first few hours of the treatment, whereas repression of the Wnt/ β -catenin pathway happens a few days later. This change might be associated with COX-2 inhibitor-induced downregulation of osteopontin (OPN) whose high expression level significantly correlates with advancing tumor stage in human CRC^[53]. OPN, a marker of colon cancer progression, is a direct target of Wnt/ β -catenin pathway. Furthermore, NR4A2 trans-activates OPN by directly binding to the NR4A2 response element at the OPN promoter^[54]. OPN is involved in the development of various inflammatory conditions^[55] and also play a pivotal role in carcinogenesis and metastasis of digestive system cancer, such as gastric cancer and hepatocellular carcinoma. High expression of OPN in tumors is associated with tumor invasion, metastasis, and poor prognosis in gastric cancer and CRC^[53,56]. Downregulation of OPN, probably through blockade of NR4A2 and Wnt signaling, is an important component of the antitumor activity of COX-2 inhibitors^[57]. VEGF potently and rapidly induces expression of NR4A2 mRNA, protein and its promoter activity in endothelial cells. Deletion of the putative CREB site in the proximal region of the NR4A2 promoter markedly reduces VEGF-induced promoter activity. VEGF also stimulates the binding of nuclear CREB protein to its site in the NR4A2 promoter. Knockdown of endogenous NR4A2 expression attenuates VEGF-induced endothelial cell proliferation, migration and *in vivo* angiogenesis^[58]. The growth and metastasis of cancer cells rely on angiogenesis, and VEGF is an important angiogenic factor. It has been reported that VEGF is highly expressed in gastrointestinal cancers and plays a pivotal role in tumor angiogenesis, tumor growth, and metastasis^[59-61]. The correlation among the expression levels of OPN, COX-2 and VEGF in gastric cancer indicates that OPN, COX-2, and VEGF synergistically promote angiogenesis and metastasis^[62]. The three molecules are all closely related to NR4A2. The NF- κ B signaling pathway serves as a principal regulator of inducible NR4A expression in some chronic inflammation, while NF- κ B pathway plays a major function in CRC development and progress^[63]. These results indicate that aberrant expression of NR4A2 provokes several inflammation-related signaling pathways and promotes the development and progression of gastrointestinal cancers. Figure 1C depicts the potential role of NR4A2 on the development of CRC.

NR4A2-related lymphocytes in gastrointestinal inflammation and cancers

Foxp3⁺ Treg cells are significantly increased in gastric mucosa of patients with gastritis, peptic ulcer, and those with gastric cancer, as compared with healthy controls^[64]. An imbalance of colitogenic Th1 cells and Treg cells facilitates the development of aggravated chronic enterocolitis^[65]. Although NR4A2 has not been implicated in

predicting CRC prognosis, the NR4A2-related Th lymphocytes are closely linked to adverse outcome of CRC patients. Foxp3⁺ and CD3⁺ T-cell densities are increased in CRC tissues compared with autologous normal mucosa. Furthermore, a low CD3⁺/Foxp3⁺ cell ratio and low numbers of CD3⁺ T cells in tumors predict shorter disease-free survival and are stronger prognostic variables than tumor stage or number of lymph node metastases^[66]. The density of Foxp3⁺ Treg cells in tumor draining lymph nodes (TDLNs) is dramatically higher than that in peripheral blood lymphocytes, but significantly lower than that in tumor-infiltrating lymphocytes. Foxp3⁺ Treg cells in TDLNs are more correlated with disease progression and potentially influence CD8⁺ T-cell functions^[67]. A new Treg cell population, CD8⁺CD25⁺Foxp3⁺ cells, has been found in CRC tissues. IL-6 and transforming growth factor- β 1 can synergistically induce the generation of these new Treg cells that may contribute to tumor immune escape and disease progression^[68]. The CRC patients with high expression of the Th17 cluster have a poor prognosis, whereas patients with high expression of the Th1 cluster have prolonged disease-free survival, thus functional Th1 and Th17 clusters yield opposite effects on patient survival in CRC^[69]. Th17 and Treg cells accumulate in the tumor microenvironment of early gastric cancer and then infiltration of Treg cells gradually increases according to disease progression, in contrast to Th17 cells^[70]. Significantly more Foxp3⁺ Treg cells accumulate in gastric tumors. The elevated Foxp3 expression in tumor-infiltrating Treg cells correlates with expression of COX-2 and PGE₂ and is associated with the TNM stage in gastric cancer patients. Tumor-infiltrating Treg cells with increased Foxp3 expression can mediate immune suppression *via* COX-2/PGE₂ production in the gastric cancer microenvironment^[71]. NR4A2 trans-activates the transcription factor Foxp3, while Foxp3 plays a key role in Treg cell function. Furthermore, NR4A2 is highly expressed in the CD133⁺ CRC cells and also pivotal for Treg cell induction and suppression of aberrant induction of Th1 cells^[26,39]. NR4A2 is a target of MIF/MAPK signaling, while MIF expression in tumors is inversely associated with the prognosis of hepatocellular carcinoma^[25,29]. Thus, it can be inferred that NR4A2 expression in lymphocytes and/or tumor cells might promote gastrointestinal inflammation and carcinogenesis and also indicate poor prognosis of CRC and gastric cancer.

QUESTIONS CONCERNING NR4A2 FUNCTIONS IN HUMAN CANCER

There are conflicting data concerning the oncogenic or tumor suppressive function of the three NR4A family members. Several lines of evidence mentioned above indicate that NR4A2 plays an important role in promoting inflammation and gastrointestinal cancers. Downregulation of NR4A2 results in reduced anchorage-independent growth that is largely attributable to increased

anoikis, furthermore, downregulation of NR4A2 as well as NR4A1 promotes intrinsic apoptosis in several other experimental cancer cells such as cervical cancer^[11]. Thus, NR4A family members exhibit oncogenic functions with regard to cell proliferation and anti-apoptosis. However, knocking out both NR4A1 and NR4A3 in mice leads to rapid postnatal development of AML, indicating they function as critical tumor suppressors^[14]. NR4A2 also exhibits tumor suppressor function in bladder cancer. Chemical-induced activation of NR4A2 results in bladder cancer cell apoptosis and suppresses bladder cancer growth, as reported by the research group of Kamat^[72]. However, this group also reported that cytoplasmic dislocation of NR4A2 in bladder cancer was associated with poor prognosis, and silencing of endogenous NR4A2 attenuated the migration of bladder cancer cells, indicating that NR4A2 functions as a tumor-promoting factor^[73]. NR4A2 is a nuclear factor, therefore, cytoplasmic dislocation might indicate loss of function of NR4A2. Thus, NR4A2 is more likely to function as a tumor suppressor in bladder cancer. These conflicting data can be explained by the hypothesis that NR4A2 functions differently in different tissues. More studies using cancer of different origins are necessary to elucidate the functions of NR4A2 in human cancers.

In summary, NR4A2 plays a pivotal role in some inflammatory diseases and cancers. NR4A2 functions as an inflammatory mediator primarily *via* at least two distinct signaling pathways, cAMP/PKA and NF- κ B, suggesting an important common role for this transcription factor in mediating multiple inflammatory signals. NR4A2 trans-activates the transcription factor Foxp3, while Foxp3 plays a key role in Treg cell function. Treg cells contribute significantly to tumoral immune escape and disease progression. NR4A2 trans-activates OPN while OPN is a direct target of Wnt/ β -catenin pathway. There is a crosstalk between NR4A2 and Wnt/ β -catenin signaling pathways in human gastrointestinal cancers such as CRC. Taken together, the aberrant expression of NR4A2 in the tumors and tumor-infiltrating lymphocytes might facilitate gastrointestinal inflammation, carcinogenesis and cancer metastasis. NR4A2 emerges as an important nuclear factor linking gastrointestinal inflammation and cancers, especially CRC, and might serve as a candidate therapeutic target for the inflammation-related gastrointestinal cancers.

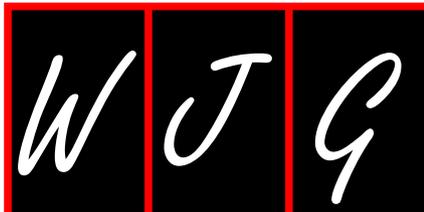
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Regenerative medicine technology applied to gastroenterology: Current status and future perspectives

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INTRODUCTION

In quality of Guest Editor, I am delighted to introduce this special issue of *World Journal of Gastroenterology (WJG)* focusing on regenerative medicine and gastroenterology.

The term “regenerative medicine” (RM) was coined only in 1999 and refers to the field of health sciences which aims to replace, regenerate or bioengineer *ex novo* human cells, tissues, or organs to restore or establish normal function^[1]. Its main trait is the multidisciplinary approach required to reach its goals. As a corollary, RM investigations are made possible by synergistic contributions of researchers with different backgrounds, namely physicians, veterinarians, cell biologists, extracellular matrix experts, bioengineers, chemists and biochemists, physicists, molecular biologists, biomaterial scientists, mathematicians and statisticians, immunologists, physiologists, geneticists, and others. For this reason, in 2006, the United Nations Educational, Scientific and Cultural Organization defined RM as a super discipline whose contours are still being defined^[2]. It should be noted that the term “(bio)engineering” is often, yet erroneously, used as synonymous to RM. In fact, the process of regenerating cells, tissues and organs can occur *in vivo* or *ex vivo*, and may require cells, natural or artificial scaffolding materials, growth factors, or combinations of all three elements, whereas the term bioengineering is narrower in scope and strictly defined as manufacturing body parts *ex vivo* by seeding cells on or into a supporting scaffold.

The past decade has been marked by numerous ground-breaking achievements in the field of RM. Researchers have developed sophisticated, cutting-edge technologies to manufacture functional substitutes of relatively simple organs such as vessels, bladders, segments

Abstract

This special issue of *World Journal of Gastroenterology* has been conceived to illustrate to gastroenterology operators the role that regenerative medicine (RM) will have in the progress of gastrointestinal (GI) medicine. RM is a multidisciplinary field aiming to replace, regenerate or repair diseased tissues or organs. The past decade has been marked by numerous ground-breaking achievements that led experts in the field to manufacture functional substitutes of relatively simple organs. This progress is paving the ground for investigations that aims to the bioengineering and regeneration of more complex organs like livers, pancreas and intestine. In this special issue, the reader will be introduced, hand-in-hand, to explore the field of RM and will be educated on the progress, pitfalls and promise of RM technologies as applied to GI medicine.

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Key words: Regenerative medicine; Gastroenterology

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of upper airways and urethras^[1,3]. These organs were engineered from patients' own cells and successfully implanted to replace diseased or damaged tissue. At the same time, stem cells treatments are being developed and tested in seminal clinical trials for treatment of various diseases^[4-6].

This special issue of *WJG* has been conceived to illustrate to gastroenterology operators how RM may impact gastrointestinal (GI) medicine. The manuscript by Carbone *et al*^[7] stigmatizes the unmet clinical needs in gastroenterology and paves the ground for the following papers which show the fields and clinical settings where RM has the potential to meet those needs. In particular, Domínguez-Bendala *et al*^[8] from the Diabetes Research Institute of the University of Miami, FL, review the most promising avenues of research aimed at generating a potentially inexhaustible supply of insulin-producing cells for islet regeneration. These avenues include the differentiation of pluripotent and multipotent stem cells of embryonic and adult origin along the beta cell lineage, and the direct reprogramming of non-endocrine tissues into insulin-producing cells. Dufrane *et al*^[9] from the Pôle de Chirurgie Expérimentale et Transplantation, Université Catholique de Louvain, Brussels, Belgium, complete the session on RM applied to diabetes sciences by drawing state of the art of investigations aiming to produce a bioartificial pancreas to treat diabetes, with emphasis on islet encapsulation technology and immunoisolation.

Four papers are focused on the bioengineering and regeneration of different segments of the gastrointestinal tract. Koch *et al*^[10] from the Wake Forest Institute of Regenerative Medicine, Wake Forest University, Winston Salem, United States, report on the most recent developments in the bioengineering of sphincteric units of the GI tract, while Londono *et al*^[11] from the McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, United States, focus on the esophagus. The group at the Child Health and Great Ormond Street Hospital, University College London, London, lead by Totonelli *et al*^[12] complements the view on the bioengineering and regeneration of the esophagus by presenting it from the perspective of the pediatric surgeon. This session is completed by a seminal review from the Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan, lead by one of the pioneers of the field, Takagi *et al*^[13]. This manuscript^[13] reports on the most recent progress and clinical translation of one of the most cutting-edge bioengineering technologies, namely cell-sheet, which will certainly fascinate readers. Finally, the special issue is completed by a review paper on the state of the art liver bioengineering by our group at Wake Forest University, Winston Salem, United States^[14].

I do believe that readers will enjoy to be introduced, hand-in-hand, to this field, RM, which has the potential to change the way we approach diseases and how we

strategize treatments. I am very grateful to the publisher and the Editor-in-Chief for granting the great opportunity to guest edit the special issue in question. Importantly, the success of this issue has been made possible by the outstanding contribution of the above mentioned authors who are among the biggest names in the field.

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Present and future cell therapies for pancreatic beta cell replenishment

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Abstract

If only at a small scale, islet transplantation has successfully addressed what ought to be the primary endpoint of any cell therapy: the functional replenishment of damaged tissue in patients. After years of less-than-optimal approaches to immunosuppression, recent advances consistently yield long-term graft survival rates comparable to those of whole pancreas transplantation. Limited organ availability is the main hurdle that stands in the way of the widespread clinical utilization of this pioneering intervention. Progress in stem cell research over the past decade, coupled with our decades-long experience with islet transplantation, is shaping the future of cell therapies for the treatment of diabetes. Here we review the most promising avenues of research aimed at generating an inexhaustible supply of insulin-producing cells for islet regeneration, including the differentiation of pluripotent and multipotent stem cells of embryonic and adult origin along the beta cell

lineage and the direct reprogramming of non-endocrine tissues into insulin-producing cells.

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Key words: Human embryonic stem cells; Induced pluripotent stem cells; Mesenchymal stem cells; Beta cell differentiation; Reprogramming; Islet transplantation

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INTRODUCTION

Type 1 diabetes is an autoimmune disease characterized by the targeted destruction of the insulin-producing beta cells within the pancreatic islets. This is a chronic condition that requires daily insulin administration to maintain blood glucose levels within acceptable limits. However, because of the impossibility of exogenous insulin to accurately mimic islet function in the long run, diabetes often progresses with the development of debilitating complications, including kidney failure, blindness and vascular degeneration. Whole pancreas transplantation is an effective means to permanently correct hyperglycemia, but because of the risks inherent to any major surgery is rarely indicated as a treatment for diabetes. Islet transplantation is a less invasive procedure based on

the isolation of islets from their surrounding tissue and subsequent implantation in the recipient's liver^[1-8]. The method entails the enzymatic and mechanical separation of the islets from the rest of the organ. Since islets have a different density than acinar tissue, centrifugation can be used to enrich for layers of high purity that are infused intraportally into the liver of the patient, where they lodge and revascularize in a matter of weeks^[9-13]. The evolution of this technology has followed a typical pattern of innovation^[14] in which the hype elicited by an early milestone (the invention of the isolation method^[9]) ballooned with a technical achievement (the development of an steroid-free immunosuppression protocol that allowed for long-term graft survival^[15]), only to burst with the realization that the long-term outcome was not nearly as good as expected (only 20% of the patients remained insulin-independent five years after the procedure^[16]). As it is also commonly seen in most innovations, a dry "trough of disillusionment" ensued, during which researchers had to struggle with a hostile scientific and financial environment fed by the perception that islet transplantation was a therapeutic *cul-de-sac*. This has been so until very recently, when new developments (such as reports of novel T-cell depleting strategies that prolong graft survival and support function at rates that stand comparison to those seen when transplanting the entire organ^[17]) have shifted the perception again. Of course, the expectations have now been adjusted to the reality, and few would contend now that this therapy represents the future treatment of choice for diabetes. However, the advent of stem cells in the clinical arena has helped refocus the goals of islet transplantation, which is now seen as an invaluable testing ground for the next generation of cell therapeutics rather than as the next breakthrough in the fight against diabetes. Indeed, it is expected that stem cell-derived insulin-producing cells will take over islets in the near future, making this therapy available to millions (as opposed to hundreds) of patients. It is reasonable to expect that the transition to stem cells, if not seamless, will be easier and faster than for other conditions for which there is no cell therapy today. From adult and embryonic stem cells to somatic tissue engineering and islet regeneration, this review will describe the best positioned candidates to lead this transition in the next decade.

MESENCHYMAL STEM CELLS

Mesenchymal stem cells (MSCs) are ubiquitously found throughout the entire body. Tissues from which they can be commonly isolated and expanded include the stroma of the bone marrow, the adipose tissue and the umbilical cord^[18]. Whether or not MSCs from disparate sources are one and the same is still subject to debate. Indeed, to be considered MSCs they must share a number of characteristics such as adherence to plastic, capacity to differentiate along the bone, cartilage and fat lineages, presence of some surface markers (CD73, CD90 and CD105) and absence of others (chiefly those of the hematopoietic

lineage). However, some MSCs express markers of pluripotency (Oct4, Nanog) whereas others do not. Proliferation rates vary greatly between clones even if obtained from the same tissue, as evidenced by a recent example in which division rates of umbilical cord blood-derived MSCs ranged anywhere from 36 h to nearly 9 d^[19]. To complicate things even more, clones not only from the same tissue, but also from the same donor, also exhibit substantial variability^[20]. While these lines of evidence would suggest that MSCs comprehend a far too diverse collection of cell types to make common assumptions about their true identity and therapeutic potential, these differences ultimately reside in epigenetic signatures that -as shown time and again after the advent of reprogramming techniques- are anything but irreversible. From this perspective, even if some subsets of MSCs (such as those residing in the umbilical cord blood) would appear to be more adept than others at acquiring beta cell characteristics, considerations such as the ease of procurement and expansion or the ability to derive them from the prospective recipient (as would likely be the case when considering fat-derived MSCs) may ultimately weigh more than their apparent developmental potential at the time of isolation. This same consideration applies to what many perceive as an ontological limitation of MSCs to become pancreatic islets, since beta cells and MSCs arise from different embryonic germ layers. While this is true, the epigenetic landscape has proven in recent years to be much more pliable than previously thought. Be it by direct reprogramming into pancreatic endocrine tissues or by de-differentiation followed by re-education into insulin-producing cells, there is a growing consensus in the field of diabetes that MSCs may after all be of more use than that of providing a beneficial immunomodulatory^[21] and pro-angiogenic^[22] microenvironment for other cells.

Most approaches described until now to coax MSCs into beta-like cells are based on the sequential addition to their culture medium of soluble factors known to have an effect on the progression of pancreatic development. Partial success has been reported in recent years with MSCs derived from a wealth of different sources, including pancreatic ductal and acinar tissues^[23-25], fat^[26,27], amniotic fluid^[28], umbilical cord blood and placenta^[19,29-33], bone marrow^[34-37], endometrium^[38,39], and even from the very islets of Langerhans^[40-44]. However, unlike for human embryonic stem (hES) cells^[45-47], researchers in the field of MSCs still have failed to define a "gold standard" method of differentiation that results in bona fide, mature or otherwise, beta cells. Efforts may have been dispersed by the parallel pursuit of many different MSC sources, each one purportedly superior to the others, with an overall lack of focus on the description of robust protocols that may result in successful beta cell differentiation from all of them. Only recently have steps been taken to adopt the strategies developed for hES cells to the differentiation of primitive populations of MSCs^[19]. These caveats aside, the use of MSCs seems to be solidly ingrained in the pipeline of cell therapies diabetes. As

mentioned earlier, even in the unlikely case that all our efforts at converting them into cells with the ability to regulate glucose *in vivo* were to fail, MSCs are known for their ability to foster a favorable microenvironment for other cells to engraft. They do this by secreting a plethora of trophic agents such as nerve growth factor, basic fibroblast growth factor, vascular endothelial growth factor, brain-derived neurotrophic factor, insulin-like growth factor-1, and hepatocyte growth factor^[48]. There is no reason why the favorable results observed when co-transplanting islets with MSCs^[49-53] could not be extended to stem cell-derived beta-like cells.

NON-MESENCHYMAL ADULT STEM CELLS

Because of their shared provenance, hematopoietic cells from the bone marrow and umbilical cord blood cell are often mistaken with MSCs. Although some studies have shown that there are multipotent stem cells in the hematopoietic compartment of these tissues, in most cases the use of stromal (or mesenchymal) cells would be for non immune-related regeneration (see above), whereas hematopoietic cells would be primarily used to treat immune-related disorders. Examples of the latter are the decades-old practice of bone marrow transplantation or the recent attempts to reset the immunological clock of diabetes by autologous transplantation of bone marrow-derived stem cells^[54,55]. More recently, some groups have tested the administration of bone marrow-derived hematopoietic cells directly into the pancreas of the subject, an approach that has yielded promising results in type 2 diabetic patients when combined with hyperbaric oxygen therapy^[56]. Local injection of hematopoietic stem cells has also been tested clinically for the treatment of limb ischemia and diabetic neuropathy^[57]. In general, the mechanism by which these cells might exert their action is likely related to their ability to stimulate vascular regeneration (which may in turn result in enhanced islet function when injected in the pancreas) rather than to their direct differentiation into beta cells.

As for potential stem/progenitor cells residing in the pancreas, the ongoing debate about their existence is beyond the scope of this manuscript and has been already reviewed in^[58]. Whether or not they exist and have an active role on the physiological regeneration of the organ, to this date the only evidence that true pancreatic progenitors can be isolated and expanded *in vitro*^[59,60] is very preliminary and needs independent confirmation. The findings by Cardinale *et al.*^[61] on stem cell populations in the adult extrahepatic biliary tree can be propagated *ex vivo* and give rise to hepatocytes, cholangiocytes or pancreatic islets are also very promising but warrant similar caution.

EMBRYONIC STEM AND INDUCED PLURIPOTENT STEM CELLS

Mouse embryonic stem (ES) cells have been a staple of

developmental biology laboratories for the most part of the thirty years since they were first isolated^[62,63]. Their human counterparts, however, are a much more recent addition^[64]. When cultured according to very precise specifications, these unique cells proliferate at high speeds (typical population doubling times are in the range of 24-48 h) and in an indefinite manner, while retaining the potential to differentiate into derivatives of all three embryonic layers (endoderm, ectoderm and mesoderm). It is not difficult to appreciate in the evolution of this technology, as applied to human therapy, the same pattern previously described for islet transplantation: (1) a technology trigger, namely the initial characterization of ES cells by the team of James Thomson in 1998: A field whose main pursuit for more than two decades had been to perfect ES cell isolation techniques from non-murine species with the goal of generating higher animal models of human disease, suddenly became the new, potentially most powerful weapon to combat it; (2) The peak of inflated expectations, coincident with the description of what appeared to be a most simple method, easy to translate from mouse to human cells, to obtain insulin-producing beta cells^[65]; (3) The trough of disillusionment, which came about with the realization that such method was in reality generating not beta but neuroectodermal cells and that their purported insulin staining obeyed to an artifactual uptake of insulin from the culture medium rather than to its synthesis and secretion^[66]; (4) The slope of enlightenment, a methodical path of research that ended up with the unraveling of the perfect combination of factors leading to the specification of definitive endoderm, the first and critical step along the beta cell lineage^[67,68]; and (5) The current plateau of productivity, in which protocols that elaborate upon that initial milestone^[45,46,69,70] have reached the pre-clinical level^[47]. Normoglycemia is now routinely attained when transplanting hES cell-derived beta cell progenitors into immunodeficient diabetic mice. The approach of transplanting cells halfway their differentiation course is based on the observation that beta cells differentiate better and acquire full functionality when allowed to mature *in vivo*, due to factors that remain largely unknown. The well documented risk of tumorigenesis posed by hES cells (heightened when transplanting non-terminally mature derivatives) is currently being addressed as part of the strict controls required by regulatory agencies prior to the approval of their use in clinical trials. These include the separation of pancreatic progenitors from the rest of the population^[47] and the also antibody-based selective ablation of tumorigenic cells^[71]. The leading company pushing for hES cell-based clinical trials for diabetes (Viacyte, Inc.) complements the above strategies with that of encapsulating the cells inside an immunoisolation device that would double as a physical barrier to contain potential tumor-forming escapees. In fact, if this barrier allowed for the total elimination of immunosuppression in the recipient, in theory such escapees would be recognized as allografts by the host and thus promptly rejected.

Induced pluripotent stem (iPS) cells are a much newer

addition to the regenerative medicine armamentarium - one that has been saluted by many as an ethically sounder alternative to hES cells^[72-79]. The incredible pace at which this field of research progresses is evidenced by the fact that, merely four years after the first report on the simple reprogramming technology that allowed us to make cells “go back in time” to pluripotency without the need of destroying human embryos^[72-77,79-82], iPS cells have had time not only to rise to the heights of the Pantheon of therapeutic candidates, but also to start falling^[80,83]. Seen at first as a combination of the best features of adult and embryonic stem cells (pluripotency coupled to the potential for autologous isolation), it is now that we are starting to see some worrisome signs that even the most drastic reprogramming may leave traces of the original epigenetic memory of the cell^[84] or cause the iPS derivatives to age faster^[85]. Much worse than that, reprogramming can also induce mutations or activate oncogenes^[86-88] regardless of the method used, i.e., the effect is not necessarily associated to viral insertional mutagenesis. In this context, the successful adaptation to iPS cells of a beta cell differentiation protocol originally designed for hES cells^[89] represents only a preliminary proof of concept that it does not in any way get us closer than we are with hES cells to clinical therapies. Moreover, the potential advantages of using patient-derived iPS cells for type 1 diabetes is dubious inasmuch as, this being an autoimmune disorder, the recipient's immune system would be expected to swiftly reject any neogenic beta cells derived through this means.

LATERAL REPROGRAMMING

While “vertical” reprogramming would cause a terminally differentiated cell to go back in time and re-acquire full pluripotency, the notion of lateral reprogramming is based on the observation that the epigenetic signature of any differentiated cell can also be overridden in a manner that ultimately leads to the adoption of another terminally differentiated phenotype. In this sense, the older term “transdifferentiation” might be more appropriate to describe this type of horizontal reprogramming. As with iPS cell reprogramming, this young field started using DNA-integrating approaches (chiefly by means of viral vehicles), but is expected to follow the steps of the former in the use of non-integrative methods such as protein transduction^[90,91], episomal constructs^[78], DNA minicircles^[92], synthetic mRNAs^[93] and even small molecules identified through high-throughput screening^[94].

Earlier attempts at using reprogramming to obtain beta cells used the liver as a substrate. The choice of this organ is hardly surprising, since numerous studies confirm that liver and pancreas are susceptible of inter-conversion under multiple experimental, physiological or pathophysiological circumstances, including dietary depletion of copper^[95-97], spontaneous tumoral processes or administration of chemicals such as diethylnitrosamine^[98] or dexamethasone^[99]. In fact, fully differentiated hepatocytes and pancreatic beta cells share common molecular

mechanisms for glucose sensing^[100,101], with glucokinase being a prominent marker or both. From an evolutionary perspective, the existence of a single hepatopancreas in many invertebrates indicates that the two organs started as one. In vertebrates, liver and pancreas arise from the same progenitor cell pool^[102-104], and their separation is dictated by signals secreted by the developing heart^[103,104,111,112]. More to the point, in adult mice and humans alike, the extrahepatic biliary tree harbors progenitor cells with the ability to differentiate into hepatic and pancreatic tissues^[61,113].

The systemic delivery of the master pancreatic regulator Pdx1^[114,115] to mice was arguably the experiment that inaugurated the field of lateral reprogramming at the beginning of the century^[116,117]. The authors of those pioneering studies reported the appearance of insulin-positive cells in the liver of the animals, in sufficient amounts as to restore normoglycemia when the recipients were diabetic. Of even higher significance was the ulterior finding that reprogramming was not contingent to the ectopic expression of the gene, but persistent in time long after the expected clearance of the adenovirus used to deliver it^[118]. Similar results were independently confirmed by other groups, using either Pdx1 alone or in combination with other genes, such as NeuroD or MafA, known to act synergistically with the former^[119-126]. These reports were received with a mixture of expectation (the total liver-to-pancreas transdifferentiation described in transgenic frogs that expressed Pdx1 under the control of an early liver promoter^[127] was nothing short of extraordinary) and vague disappointment. This is because with the exception of the above transgenic setting (which obviously did not have any clinical applicability), none of the other strategies had yielded cells that could unequivocally be defined as true beta cells. When conducted *in vitro*, transdifferentiation resulted in cells that, at best, had a hybrid hepatocyte-beta cell phenotype and were incapable of regulating insulin secretion according to changes in glucose concentration. And when done *in vivo*, the observation that the inflammation elicited by the use of adenoviruses could be as important to induce reprogramming as the actual genes -if not more-, was a troubling discovery that caused legitimate concerns about how “clean” the strategy actually was^[120].

After an impasse of a few years, during which the excitement that first welcomed the advent of this new technology had started to wane, the impact of a recent report^[128] renewed the enthusiasm of the scientific community. The choice of genes (Pdx1, Ngn3 and MafA) was not particularly novel, as comparable combinations of factors had already been assayed on liver^[101,103,121,129-131] with all the aforementioned limitations. What was novel was the use of pancreatic acinar tissue as the substrate for reprogramming. Perhaps because of the even closer developmental ties between the beta cells and the surrounding acinar parenchyma that engulfs the islets in the native organ, the observed transdifferentiation to beta cells upon direct injection of these genes in the pancreas

of recipient mice was convincing enough to reawaken excitement about the clinical prospects of reprogramming.

CONCLUSION

Upon reviewing the diversity of options potentially available to us in the near future, one may get the impression that current efforts are somewhat dispersed. Clinicians and patient advocates alike often voice their concern that such dispersion may stand in the way of an effective allocation of manpower and resources to the strategy that has the highest translational potential. But who is to say which strategy this is at this early stage of the game? Indeed, based on the trends observed in the field, it is plausible that the approach with the highest chance of success will be a multi-pronged one, possibly involving more than one cell type and addressing both replacement and immunomodulation.

hES cells are well positioned from the standpoint of the robustness of the described differentiation methods and the heavy private and public investment bestowed upon the team that leads these efforts. ViaCyte received last year more than \$20 million from the California Institute for Regenerative Medicine to speed up pre-clinical studies that could lead in the very near future to an Investigational New Drug application to the FDA. However, unlike other prospective therapies based on the use of adult stem cells (heavily represented in clinical trials), hES cell-based ones still have to break ground from a regulatory perspective. The recent withdrawal of Geron's phase I clinical trials for spinal cord injury (which pioneered the use of hES cells in the clinical arena) represents an unfortunate turn of events that is not likely to help foster a more favorable climate for new trials. Fortunately, recent advances at immunoisolation^[132,133], coupled with refinements at enriching for the fraction of cells with therapeutic potential^[47,71] are likely to surmount the main concerns posed by regulatory agencies. Despite the initial impression that iPS cell-based protocols could be largely swapped with those designed for hES cells, developments already discussed in the relevant section urge caution about their clinical use, which, if ever realized, will undoubtedly lag behind that of hES cells. The potential advantage of using autologous cells for type 1 diabetes is dubious at best and logistically impractical. In fact, even if autoimmunity was not a consideration and we still wanted to obtain pluripotent cells as closely matched to the recipient as possible, an alternative is human leukocyte antigen (HLA)-homozygous hES cells, which have been derived by parthenogenesis. One of these cell lines is reported to carry the most widespread HLA haplotype in North America^[134,135].

Adult stem cells, especially those with known ontogenic potential to become definitive endoderm, have been remarkably difficult to identify - let alone isolate and expand. The recent discovery of such potential populations in the adult extrahepatic biliary tree^[61,113] or the islets themselves^[60] may open promising avenues of research,

but until these early results are validated, MSCs are likely to keep the lion's share of attention and funding. Easy to derive, characterize and expand, their apparent genealogic disconnect with the endoderm from which organs such as the liver or the pancreas arise has not been an obstacle for those who champion them in the context of diabetes. Progress at developing methods to coax these cells into insulin-producing cells, albeit slow, is starting to pick up speed with the description of more primitive MSC populations and better differentiation methods. The burgeoning field of reprogramming is also likely to have a say on whether the developmental potential of MSCs is intrinsically limited to mesodermal lineages. Although none of the 11 currently listed MSC-based clinical trials for the treatment of either type 1 or type 2 diabetes makes use of MSCs previously differentiated into beta-like cells *in vitro* (www.clinicaltrials.gov), their immunomodulatory and niche-forming properties are now widely acknowledged and could be the basis of both combination (such as islets and MSCs^[49]) and stand-alone therapies in the short term.

Lateral transdifferentiation, finally, may not necessarily take a backseat to the above approaches, provided that non DNA-based reprogramming techniques are refined to the extent required for the process to be efficient. Indeed, progress in this direction may effectively leapfrog years of research on stem cell differentiation, and end up in contention to become the therapy of choice for type 1 diabetes. While *ex vivo* reprogramming of acinar tissue may not represent a viable strategy in the long run (pancreatic biopsies from the prospective patients would be risky, and the use of acinar tissue from deceased donors would present the same scarcity problems that afflict organ donation in general), recent advances in "DNA-free gene therapy"^[136,137] may soon change the perception that *in vivo* reprogramming of cell fate is a clinical implausibility. More so, because one commonly cited shortcoming of these approaches -i.e., the fact that reprogramming is seldom complete, invariably leaving traces of the original fate- might in fact work in our benefit if the autoimmune response seen in type 1 diabetes is elicited only by true beta cells, but not the reprogrammed ones. While this is only a speculation at this point, the potential therapeutic implications of such finding would be incommensurable. Finally, it is necessary to emphasize that most of the approaches herein listed could also be potentially applied not only to type 1 diabetes, but also to all types of insulin-dependent conditions resulting in glycemic dysregulation, including type 2 diabetes.

In the next few years, we expect to see a progressive shift from islet transplantation to the transplantation of hES cells in an immunoisolation setting. Parallel efforts will be made at using MSCs and hematopoietic stem cells for immunomodulation, which will be accompanied by an emphasis on the exploration of endogenous regeneration pathways. A cure is likely to ultimately arise from a combination of many, if not all, of the above approaches.

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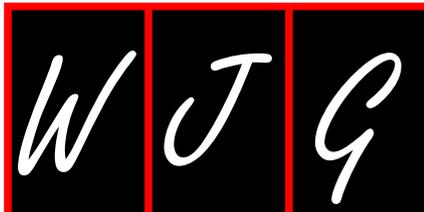
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Macro- or microencapsulation of pig islets to cure type 1 diabetes

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Abstract

Although allogeneic islet transplantation can successfully cure type 1 diabetes, it has limited applicability. For example, organs are in short supply; several human pancreas donors are often needed to treat one diabetic recipient; the intrahepatic site may not be the most appropriate site for islet implantation; and immunosuppressive regimens, which are associated with side effects, are often required to prolong survival of the islet graft. An alternative source of insulin-producing cells would therefore be of major interest. Pigs represent a possible alternative source of beta cells. Grafting of pig islets may appear difficult because of the immunologic species barrier, but pig islets have been shown to function in primates for at least 6 mo with clinically incompatible immunosuppression. Therefore, a bioartificial pancreas made of encapsulated pig islets may resolve issues associated with islet allotransplantation. Although several groups have shown that encapsulated pig islets are functional in small-animal

models, less is known about the use of bioartificial pancreases in large-animal models. In this review, we summarize current knowledge of encapsulated pig islets, to determine obstacles to implantation in humans and possible solutions to overcome these obstacles.

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Key words: Cell transplantation; Diabetes mellitus type 1; Islets of Langerhans; Porcine; Xenografts

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INTRODUCTION

Type 1 diabetes has been treated successfully by transplanting islets of Langerhans, the endocrine tissue that releases insulin^[1]. Despite the clinical efficacy of islet transplantation, serious issues preclude its broad clinical application, including the side effects of chronic immunosuppressive regimens and a shortage of human donors. For example, it was recently estimated that only one pancreas is available per 333 patients with type 1 diabetes in the United States^[2]. This situation is aggravated by the need of each recipient undergoing transplantation

for 2-4 pancreases^[1,3]. This shortage of pancreas donors therefore justifies the search for alternative sources of insulin-producing cells. Swine appear to be the major candidates for islet procurement because: (1) humans have been treated with porcine insulin for > 40 years (pig and human insulin differ by only one amino acid); (2) pigs have large litters with offspring that rapidly attain adult size and are therefore amenable to genetic engineering; (3) pig pancreases contain large islets that respond to glucose stimulation; and (4) since pigs are widely bred and slaughtered for food, the use of their islets to restore human health may be an option that could satisfy sociocultural and ethical concerns^[4-6].

Unlike primarily vascularized organs, pancreatic islets are implanted without direct connection to the host vascular network, with 7 to 14 d needed to re-establish blood flow^[7-12]. Thus, it was thought that pig islet xenografts could escape typical hyperacute rejection and acute vascular rejection^[13]. *In vivo*, however, pig islets in non-immunosuppressed nonhuman primates are rejected by both humoral and cellular immune reactions^[14-16]. Diffuse, presumably nonspecific IgG deposits were observed within islet-associated accumulations of platelets 12 h after transplantation. Deposits of large amounts of IgM and moderate to large amounts of C3, C5, and C9 were present on islet surfaces 2 to 3 d after xenografting^[15-17]. Anti-galactosyl (anti-Gal) and non-Gal^[18,19] IgM antibodies bind to islet surfaces soon after transplantation and activate the classical complement pathway, as well as promoting neutrophil infiltration. These humoral immune responses to pig islets are consistent with early T-cell-independent immune system activation and are reminiscent of mechanisms that operate during the hyperacute rejection of solid-organ xenografts^[20]. Humans and nonhuman primates have preformed anti-pig antibodies that rapidly recognize the Gal epitope on islet endothelial cells. During pig islet-to-primate xenotransplantation, however, the expression of Gal epitopes is influenced by the age of the pig. Gal residues are present on 20% of fetal, but on only 5.1% of adult, islet β cells^[21-23]. Since Gal expression persists after islet isolation^[24,25], Gal remains a target for humoral xenorejection.

Islet xenografts that survive immediate blood-mediated inflammatory reactions and additional humoral damage will be subject to acute cellular xenograft rejection. Following transplantation of fetal pig islets under the kidney capsule, the cellular infiltrate in primates has been found to consist mainly of CD8 T cells (implicating the indirect pathway), whereas the cellular infiltrate in rodents was dominated by macrophages. T-cell infiltration precedes macrophage influx, with small numbers of CD3⁺ T cells observed 12 h after transplantation^[14]. After 24 h, equal numbers of CD3⁺ T cells and neutrophils were observed, and after 72 h, CD3⁺ T cells dominated, representing 50% to 80% of all infiltrating cells. After 72 h, large numbers of macrophages were observed, with T cells localized at the periphery of and within transplanted islets. In addition, increased E-selectin expres-

sion on portal vein endothelial cells correlated with the infiltration of neutrophils, which caused tissue damage by releasing enzymes, active oxygen intermediates, and proinflammatory cytokines, and produced chemokines that attracted dendritic cells and T lymphocytes. Pig islet xenorejection seems theoretically easier to overcome, but because hyperacute and acute vascular rejections do not occur, the rapid destruction of pig islets within 72 h of transplantation into nonhuman primates demonstrates the strength of xenorejection.

Thus, an immunosuppressive regimen is mandatory for the long-term survival of pig islets in primates^[26,27]. Although several immunosuppressive strategies have successfully suppressed alloimmune responses, T-cell-mediated xenimmune responses have proven more resistant to immunosuppressive therapy^[28,29]. This may be due to the greater molecular incompatibility between donor and recipient, which activates particularly the innate immune response^[30].

Until recently, the maximum reported duration of pig islet survival (insulin-positive cells, no function) following transplantation under the kidney capsule in nondiabetic cynomolgus monkeys and immunosuppression with anti-thymocyte globulin, anti-interleukin-2R mAb, cyclosporine, and steroids was 53 d^[31]. In March 2006, however, two studies reported that neonate or adult pig islets xenotransplanted into primates survived and functioned for > 180 d^[26,27]. More recently, the transgenic expression of a human complement-regulatory protein (hCD46) on porcine islets was shown to enhance the survival of islets xenotransplanted into cynomolgus monkeys with streptozotocin (STZ)-induced diabetes for > 12 mo^[32]. In addition, transplantation of galactosyl knock-out neonate pig islets was found to significantly enhance normoglycemia rates in diabetic primates, likely due to the decreased susceptibility of these xenografts to innate immunity mediated by complement and preformed xenoantibodies^[33]. These results, however, required treatment with a heavy immunosuppressive regimen, in particular an anti-CD154-specific mAb, an antibody that induced thromboembolic events precluding its clinical use^[34]. Despite the unacceptability of these immunosuppressive regimens in humans, these results are very encouraging since an alternative, nontoxic regimen combined with xenotransplantation of pig islets may induce normoglycemia in diabetic patients.

A bioartificial pancreas, in which islets of Langerhans are encapsulated within a semipermeable membrane, may be an alternative therapeutic device for patients with insulin-dependent (type 1) diabetes mellitus. It may constitute a safe and simple method of transplanting islets without the need for immunosuppressive therapy. Since the semipermeable membrane protects the islets from the host immune system, the islets are likely to survive and release insulin for a long period of time, thereby controlling glucose metabolism in the absence of immunosuppressive medication. Nevertheless, several important questions are associated with the transplanta-

Table 1 Bioartificial device configurations for encapsulation of pig islets

	Macroencapsulation	Microencapsulation	Conformal coating
Type of pig islets tested	Adult/neonate	Adult/neonate	Adult
Suitable material with biocompatibility	Alginate	Alginate with/ without PLL/PLO over-layer	PEG
Implantation site	Intraperitoneal	Intraperitoneal	Into the liver <i>via</i> the portal vein
Proof of concept in preclinical studies in diabetic primates	Subcutaneous +	Kidney subcapsular space +	-
Maximum survival of pig islets and diabetes correction	Mean 24 wk correction of glycated hemoglobin < 7% after transplantation of the MCD into subcutaneous tissues	Max 804 d but never reproduced; Biocompatibility confirmed under the kidney capsule up to 6 mo after transplantation	ND
Clinical study	+	+	ND
Clinical efficacy	No insulin-independence Glycated hemoglobin improvement; porcine insulin detected in recipient sera up to 4 yr after transplantation with insulin positive cells into hollow-fiber devices.	No insulin independence Survival up to 9.5 yr with insulin-positive cells after transplantation into the peritoneum and detection of urinary porcine C-peptide up to 11 mo after transplantation	ND
Advantages for large-scale clinical application	Easy procedure for transplantation into subcutaneous tissue Simple procedure to remove the graft from subcutaneous tissue	Easy procedure for transplantation by simple injection into the peritoneum Large scale encapsulation of large number of islets	- Capability of transplantation into the liver - Reduction of graft size
Limitations for large-scale clinical application	Limited islet oxygenation Difficulty to transplant into the peritoneal cavity	Large volume of encapsulated islets limiting transplantation into the peritoneum Islet survival limited by absence of biologic interaction with encapsulation material No ability to remove the graft after transplantation	- No stability for long-term islet immunoprotection

PLL: Poly-lysine; PLO: Poly-ornithine; MCD: Monolayer cellular device; ND: Not determined; PEG: Polyethylene glycol.

tion of immunoisolated adult pig islets as a “bioartificial pancreas”^[35] (Table 1).

VARIATIONS IN CAPSULE SIZE: MACROENCAPSULATION OR MICROENCAPSULATION

Macroencapsulation

In the first reports of encapsulation, a large number of islets were immunoisolated between flat-sheet double membranes^[36,37]. This type of single macroencapsulation device could be implanted with minimal surgery at different sites, including the peritoneal cavity, subcutaneously, or under the renal capsule. Although several types of biomaterial have been used to produce microcapsules, including nitrocellulose, alginate, acrylonitrile, and agarose, these devices usually had some toxicity and activated nonspecific foreign body reactions, resulting in fibrotic overgrowth with subsequent necrosis of the encapsulated tissue^[35]. A subcutaneously transplanted microdevice (TheraCyte device, Baxter Healthcare), 4 cm in length, shaped like a teabag, and made of a bilayered polytetrafluoroethylene membrane, was recently found to be biocompatible^[38,39]. Neonatal pig cells inside the graft (i.e., cells immunohistochemically positive for insulin and glucagon) remained viable for up to 8 wk after xenotransplantation into nondiabetic cynomolgus

monkeys, with no evidence of reaction with adjacent subcutaneous tissue^[40]. Moreover, one of 12 non-immunosuppressed adolescents became insulin independent and 5 children had reduced insulin requirement after transplantation of porcine islets encapsulated in hollow-fibers with porcine Sertoli cells, which likely have immunomodulating properties^[41,42].

A “monolayer” configuration of macroencapsulated pig islets (monolayer cellular device) implanted subcutaneously (see below) has been found to significantly improve diabetes control (glycated hemoglobin < 7%) in primates for 6 mo without any immunosuppression^[43]. In this encapsulation system, islets were seeded as a monolayer on an acellular collagen matrix, enhancing their interactions with a biologic membrane and increasing islet concentration per unit surface area. In addition, diabetes was controlled for up to 1 year in 2 diabetic primates after retransplantation with new monolayer cellular devices. Unfortunately, the lifespan of adult pig islets limited long-term graft function. Diabetic control was completely maintained for > 32 wk after the cotransplantation of adult pig islets and adipose mesenchymal stem cells^[44]. A phase 1 clinical study is currently ongoing to assess the safety and efficacy of this device for allotransplantation of encapsulated islets into humans.

Microencapsulation

Another approach consists of the microencapsulation

of 1-3 islets per semipermeable immunoprotective capsule. The spherical configuration of these microcapsules resulted in a higher surface-to-volume ratio than did the tube or disk geometry of microcapsules, resulting in a higher diffusion rate^[45]. Furthermore, microcapsules can be injected in large numbers, are durable and are difficult to disrupt mechanically^[46,47].

Two recent studies describing transplantation of microencapsulated neonatal pig islets in an alginate matrix confirmed their biocompatibility in nondiabetic monkeys as well as their capacity to partially regulate diabetes^[39,40]. Several protocols must be followed to increase the survival of alginate microencapsulated pig islets for up to 6 mo without immunosuppression in nondiabetic primates^[25]: (1) Before transplantation, the islets should be cultured in medium containing 1.8 mmol CaCl₂; (2) Animal serum should be omitted from the culture medium; and (3) The graft should be composed of > 90% well-shaped capsules. Some of these islets survived for > 6 mo and were able to respond *in vitro* to glucose challenge 135 and 180 d after implantation. In addition, the implantation site (peritoneum, kidney capsule, or subcutaneous space) must be suitable^[48,49], with the subcutaneous space considered a good choice for clinical applications^[50].

Following transplantation of microencapsulated adult pig islets into spontaneously diabetic cynomolgus monkeys, blood glucose became normalized and the monkeys became insulin independent for periods ranging from 120 to 804 d^[51]. Although these results were encouraging for the clinical application, they may have been dependent on the diabetic status of the recipient, the exact formulation of the capsules, and the immune response against pig islets (see below). To date, these results have been confirmed by only two casuistic manuscripts describing xenotransplantation in primates of microencapsulated neonatal pig islets^[39,40]. One study confirmed the biocompatibility, for up to 8 wk, after transplantation of encapsulated pig islets in nondiabetic animals, and the second demonstrated that these microcapsules could regulate the diabetic state of diabetic recipients. Although the latter showed that daily exogenous insulin requirements were reduced by a mean of 43% compared with control animals transplanted with empty capsules, neither group showed changes in weekly blood glucose levels^[39]. The absence of solid consistent data on glucose metabolism (e.g., changes in glycosylated hemoglobin concentration, glycosuria, intravenous glucose tolerance testing) renders this casuistic study difficult to interpret.

Living cell technologies (LCT) showed that porcine islet cells had survived and insulin production was maintained in a human patient 10 years after transplant of pig islet cells^[52]. These findings demonstrated the long-term safety, viability, and functionality of encapsulated porcine islets in a human patient, without the use of immunosuppression. In 1996, a 41-year-old patient with diabetes was injected with pig islet cells to help regulate his blood glucose levels and control his diabetes. This

patient's insulin requirement was reduced by 34% for over one year. Ten years later, the patient was still obtaining benefit from the transplant, and laparoscopic examination revealed living and functioning pig islet cells in his abdomen.

Two phase 1 trials have shown that intraperitoneally infused microencapsulated human islets can be considered safe for up to 3 years^[53,54]. Although insulin independence was not achieved, glycemic control was improved, with a reduction in insulin daily requirement. In 2007, LCT launched a phase 1/2a study in Moscow of encapsulated neonatal insulin-producing porcine pancreatic islet cells (commercially called DIABECCELL[®]). Seven patients with insulin-dependent diabetes have received between one and three implants of DIABECCELL[®] (5000 and 10 000 IEQ/kg), with none showing marked adverse events 18 to 96 wk after transplantation. At last follow-up, the blood glucose concentrations in 5 patients were within the normal range (5.8-8.2 mmol/L). Two patients have shown excellent responses and do not require exogenously administered insulin. All recipients showed improvements in diabetes control, with lower glycated hemoglobin (% HbA1c) concentrations.

Following the successful completion of this phase 1/2a clinical trial in Russia, LCT launched phase 2b clinical trials, which are currently underway in New Zealand and Argentina. In contrast, although diabetes control was achieved by repeated injections of encapsulated islets (up to 4 infusions and up to 779 000 islets equivalent), a humoral response was induced, with cytotoxic antibodies found in the recipient sera 4 to 8 wk after transplantation, and necrosis of the islets at 16 mo^[54].

Conformal coating of cell surfaces

A serious issue remains in using microcapsules as bioartificial pancreases, namely, the increase in total volume of the implant after microencapsulation. The average diameter of islets is roughly 150 μ m, making the average diameter of capsules about three times as large and the total volume of microcapsules about 27 times as large. In clinical settings, the volume of islet suspensions is 10 mL, making it > 270 mL after microencapsulation. A site to implant such a large volume is difficult to find.

Much effort has been made to reduce the size of the capsules. For example, smaller microcapsules, about 300 μ m in diameter, could result in a reasonable total volume for clinical application^[55]. In clinical allotransplantation programs, islets are transplanted into the liver through the portal veins, and capsules of diameter larger than the islets themselves may plug larger blood vessels, resulting in severe thrombosis of the liver. The diameter of the encapsulated islets should therefore be much smaller than at present, to allow for their transplantation through the portal veins. A technique to coat islets with a very thin membrane or with conformal coating may reduce the diameter of these microcapsules, allowing their transplantation into the liver through the portal veins.

Several types of coating have been tested to im-

munoisolate islets from the host immune systems. For example, the surface of islets has been modified with thin membranes made of amphiphilic polymers, such as polyethylene glycol (PEG)-conjugated phospholipid (PEG-lipid) and polyvinyl alcohol carrying long alkyl chains^[56-59]. The thickness of the PEG layer formed on the cell surface was several nanometers, but depended on the molecular weight of the PEG.

Surface modification did not change the morphology or viability of the islets^[60]. Transplantation of 5000 porcine islets modified with PEG-N-hydroxysuccinimide (NHS) ester into the livers of NOD-severe combined immunodeficiency mice through the portal vein resulted in the transient normalization of blood glucose concentrations, but these concentrations later increased. The surface of islets covered with PEG reacted with the amino groups of the collagen layer remaining on the islet surface^[61]. Although transplantation of islets covered with a PEG-NHS-modified surface into recipient rats treated with low-dose cyclosporine resulted in the maintenance of normoglycemia for 1 year, normoglycemia was maintained for only 11 d in the absence of cyclosporine despite the surface modification with PEG.

Although a conformal PEG layer may form on the cells or islet surfaces at the nanometer level and this method enables a drastic reduction in total graft volume compared with conventional microcapsules, the PEG layer on the islets was not stable and disappeared from the cell surface over 3 d.

Use of a layer-by-layer method may enhance the stability of PEG-lipid membranes on the cell surface. Various functional groups, such as maleimide and biotin, can be easily introduced to the end of the PEG chain of PEG-lipids^[58] and can be used as reaction points to form multilayer membranes on the cell surface. A layer-by-layer membrane can also be formed by the reaction between biotin and streptavidin. Biotin-PEG-lipids are anchored to the cell membranes of islets and are further covered by streptavidin. The modified islets can be alternatively exposed to a biotin-bovine serum albumin conjugate solution and a streptavidin solution to form 20 layers. The thickness of the membrane is approximately 30 nm. A glucose stimulation test demonstrates the ability of the modified islets to control insulin release in response to changes in glucose concentration. After intraportal transplantation of modified islets with PEG-lipid into STZ-induced diabetic mice^[59], most islets were not damaged and remained intact in the blood vessels of the liver for 1 h to 1 d after transplantation.

VARIATION OF MATERIALS

The choice of material to use for cellular encapsulation is a crucial parameter because failure of microencapsulated islet grafts is usually regarded as a consequence of insufficient biocompatibility, inducing a nonspecific foreign body immune reaction against the microcapsules and resulting in progressively fibrotic overgrowth of the

capsules. This overgrowth interferes with adequate nutrition of the islets and consequently causes islet cell death. There are major distinctions between water-soluble polymers, such as alginate, and water-insoluble polymers, such as poly (hydroxyethyl methacrylate-methyl methacrylate)^[35]. However, a major obstacle in using water-insoluble polymers for encapsulation of cells is the requirement for an organic solvent, which usually interferes with cellular function^[62]. Despite their solubility in aqueous solutions, alginate-based capsules have been shown to remain stable for several years in small and large animals as well as humans^[51,63-67]. The method of alginate capsule formation is based on the entrapment of islets in alginate droplets, which are transformed into rigid beads by gelification in a divalent cation solution, mostly Ca^{2+} . In most studies to date, alginate beads were coated with a second layer to reduce the porosity of the capsule membrane^[35,68]. In the pig-to-primate model, alternating layers of poly-L-lysine and a polyornithine were used to surround the alginate core^[40,51]. The latter type of layer, however, has been associated with polyamino acid cytotoxicity and mechanical instability of the microcapsules, limiting their application^[35,69,70]. Several groups have recently reported that encapsulation in simple alginate microbeads can protect pig pancreatic cells against xenorejections in diabetic mice^[46,47,71]. Although several chemical formulations of alginate (e.g., high-mannuronic/guluronic; high/low viscosity, with or without additional peptide sequences) have been proposed for islet immuno-isolation, we found that high-mannuronic alginate was the most suitable to obtain selective impermeability for molecules over 150 kDa (as an IgG) before and after transplantation, and optimal biocompatibility to avoid nonspecific inflammatory response associated with surrounding angiogenesis, resulting in sufficient oxygen tension (about 40 mmHg) for the survival and function of encapsulated islets^[72]. This type of alginate was biocompatible not only in a small-animal model (Wistar rat recipients) but also in a pig-to-primate model of xenotransplantation under the kidney capsule and skin for up to 6 mo (see below)^[25,43,48].

VARIATION OF IMPLANTATION SITES

The lack of revascularization of the encapsulated islets interferes with both the functional performance and the longevity of the grafts. Apparently, a site in which encapsulated islets are in close contact with the bloodstream is obligatory for clinical application. Unfortunately, it is difficult to find such a site because it must be of sufficient size to bear a large graft volume and be near blood vessels. Sites reported to allow successful nonencapsulated islet transplantation, such as the liver and spleen, do not meet these requirements because these sites are unable to tolerate the large volumes (> 16 mL) of capsules (of diameter > 600 μm) required for transplantation into primates. Therefore, most transplantations of encapsulated pig islets into primates were

intraperitoneal^[39,39,51]. Although this technique seemed relatively easy, the peritoneal site was not optimal. Indeed, recent studies in mice found that macrophages and lymphocytes are involved in the rapid degradation of encapsulated pig islets after their transplantation into the peritoneum^[71,73-76]. The peritoneum is, indeed, a preferential site for inflammation and immunologic reactions^[77] and peritoneal mesothelial cells facilitate the action of powerful innate immune mechanisms^[77]. Studies in mice showed that immunosuppression had beneficial effects, improving the biocompatibility and prolonging the survival of encapsulated pig islets transplanted into the peritoneum^[73,74,78]. This method of combining encapsulation and immunosuppression, however, remains incompatible with clinical applications. The biocompatibility of alginate-encapsulated pig islets depends on the implantation site. Encapsulated pig islets transplanted under the kidney capsule and under the skin demonstrated better biocompatibility than capsules transplanted into the peritoneum^[48]. Indeed, a cellular reaction essentially composed of macrophages was observed 7 d after transplantation into the peritoneum. This finding is in good agreement with results showing that macrophages are recruited 7 d after transplantation of encapsulated pig islets into the peritoneum of mice and rats^[73,74]. In addition, severe fibrosis surrounding intraperitoneally implanted capsules was observed 30 d after transplantation and was correlated with the loss of porcine C-peptide 7 d after implantation. In contrast, subcutaneous and kidney capsule implantation resulted in very weak cellular immune reactions against encapsulated pig islets, along with improved porcine islet viability; porcine C-peptide was detected in the sera of rats for 30 d after transplantation of encapsulated pig islets at both sites. These findings indicate that implantation into the subcapsular kidney and subcutaneous spaces improves the biocompatibility and *in vivo* survival of encapsulated pig islets, as well as enhancing pig islet function during the first 7 d after transplantation. The loss of the *in vivo* function activity of encapsulated pig islets transplanted into the peritoneum correlated with significant alterations in islet viability, a loss of insulin content, and significant reductions in insulin secretion after glucose stimulation. These findings may be associated with macrophage overgrowth of the area surrounding the capsules, creating a micro-environment of stress, with low oxygen tension, for pig islets^[75,76]. Indeed, macrophage activation, as shown by NO production and the release of the cytokines L-1 β and tumor necrosis factor- α , had a deleterious effect on islet function and viability^[73,75,76]. We found that implantation into kidney subcapsular and subcutaneous spaces improved the biocompatibility of encapsulated pig islets and significantly reduced macrophage recruitment. This reduction in pig islet stress and improved islet viability maintained insulin level per islet and insulin secretion after glucose stimulation^[48]. Subcutaneous tissue was recently shown to provide oxygen tension (20-40 mmHg) compatible with the function and survival of

encapsulated islets^[72]. Among the sites being tested for islet transplantation, with or without encapsulation, to improve the survival, engraftment and function of islets, are the brachioradialis muscle^[79], striated muscle^[80], the greater omentum^[81], and the anterior chamber of the eye^[82].

CONCLUSION

Immunosuppression remains the major limitation of allotransplantation or xenotransplantation of islets for type 1 diabetes. Extended survival of transplanted pig islets has recently been observed in primate models, but several questions and problems associated with immunosuppression remain to be resolved in terms of adjustment before clinical trials. A bioartificial pancreas made of encapsulated pig islets may overcome the two major hurdles to islet transplantation: the shortage of human organ donors and the requirement for immunosuppressive regimens.

The development of a bioartificial pancreas for preclinical/clinical studies requires the conjunction of integrated parameters such as the choice of a biocompatible material for encapsulation to maintain selective permeability. The encapsulation device should be designed to maintain mechanical properties and stability at an implantation site compatible with the viability and physiology of the encapsulated islets to control glycemic homeostasis.

Of the three major types of bioartificial pancreases (macroencapsulation, microencapsulation, and conformal encapsulation), the macroencapsulation system is the only method that has demonstrated the capacity to control diabetes in large animals and in preliminary clinical studies.

Several improvements must be made to reduce the size of the implant (by increasing islet concentration relative to the surface or volume of the implant), to improve oxygenation of islets (to limit islet death), and to develop a simple clinical procedure for bioartificial transplantation and easy access to a device allowing "re-alimentation" of the islets.

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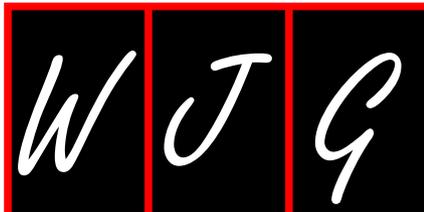
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Esophagus and regenerative medicine

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to be effective for the reconstruction of small patch defects, anastomosis reinforcement, and the prevention of stricture formation after endomucosal resection (EMR). More so, esophageal cancer patients treated with ECM scaffolds have shown complete restoration of a normal, functional, and disease-free epithelium after EMR. These studies provide evidence that a regenerative medicine approach may enable aggressive resection of neoplastic tissue without the need for radical esophagectomy and its associated complications.

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Key words: Esophageal repair; Biomaterial mediated esophageal repair; Extracellular matrix; Extracellular matrix scaffold

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Abstract

In addition to squamous cell carcinoma, the incidence of Barrett's esophagus with high-grade dysplasia and esophageal adenocarcinoma is rapidly increasing worldwide. Unfortunately, the current standard of care for esophageal pathology involves resection of the affected tissue, sometimes involving radical esophagectomy. Without exception, these procedures are associated with a high morbidity, compromised quality of life, and unacceptable mortality rates. Regenerative medicine approaches to functional tissue replacement include the use of biological and synthetic scaffolds to promote tissue remodeling and growth. In the case of esophageal repair, extracellular matrix (ECM) scaffolds have proven

INTRODUCTION

The default tissue response to injury in adult mammals is characterized by hemostasis, inflammation, and subsequent deposition of dense collagenous connective tissue (i.e., scar tissue)^[1-5]. The deposited scar tissue serves as a partial volume replacement for the missing native tissue and maintains the structural integrity of the tissue, albeit at a loss of normal function in many instances. This mechanism is adequate in most, but not all, tissues.

Some tissues in adults retain the ability to regenerate either as part of normal physiologic events or in response to injury. For example, the epidermis is com-

pletely replaced approximately every 40 d^[6-9]. The bone marrow sustains a regenerating population of cells to continuously replenish the hematopoietic cell population^[10-13], and the intestinal epithelium regenerates from a well described crypt stem cell population^[14-16]. The liver can respond to injury by a nonblastemal epimorphic regenerative mechanism^[17] and can replace most if not all of its lost hepatocellular mass if the native stroma remains intact^[18-20]. Skeletal muscle has limited regenerative potential and can respond to mild or repetitive injury with full return to structure and function^[21,22]. However, volumetric muscle loss (i.e., loss of greater the 20% of the muscle mass) results in deposition of scar tissue^[23]. Therefore regenerative potential is encoded into the genome of adult mammals but only functionally expressed in selected tissues or to a limited extent. It should also be noted that all of these examples of tissue/organ regeneration involve the participation of a reserve stem/progenitor cell population.

Those tissues with the inability to regenerate functional mass following injury are the cause of significant morbidity, aesthetic deformity, mortality, and are causally associated with a large fraction of the health care burden worldwide. For example, the inability to regenerate functional myocardium following ischemic coronary artery disease^[24-27], the dysfunctional central nervous system tissue following ischemic stroke or spinal cord injury^[28-35], and the lack of functional pancreatic beta cells following immune mediated destruction^[36-39] are the cause for a group of diseases that affects a large percentage of the aging population. Esophageal pathology, especially neoplasia, affects a rapidly increasing number of individuals in North America^[40,41] and worldwide^[42]. The lack of regenerative ability in the esophagus relegates this tubular structure to an inflammation/scarring response following injury, which in turn results in stricture and loss of function. Therefore, the standard of care for many esophageal diseases, especially overt cancer and its' precursor Barrett's disease with high grade dysplasia (HGD) involves esophagectomy; a procedure associated with a complication incidence approaching 50%^[43-46]. A regenerative medicine approach which can recreate functional esophageal tissue, preserve the integrity of the esophagus, and avoid the necessity for esophagectomy would offer a significant advancement in the arsenal of treatment methods available to affected patients.

PROBLEM

There are 5000 to 10 000 patients identified annually with non-neoplastic esophageal disease^[47] including congenital anomalies such as esophageal atresia, tracheoesophageal fistulas^[48], and corrosive injuries^[49,50]. The incidence of Barrett's esophagus (BE) and esophageal adenocarcinoma has increased dramatically and esophageal cancer now represents the world's sixth leading cause of cancer death with 300 000 new cases each year^[41,42,51]. The management of Barrett's disease with HGD and intramucosal

adenocarcinoma remains controversial. Esophagectomy has been the standard of care for HGD based on the high incidence of progression to subsequent neoplasia^[52,53]. However, the majority of patients in which esophageal neoplasia is diagnosed have disease limited to the mucosa and the involvement of regional lymph nodes is unlikely. Because esophagectomy is associated with high morbidity rates and a marked compromise in quality of life, there has been a great deal of interest and success in minimally invasive endoscopic approaches which involve esophageal preserving techniques in patients with superficial malignancy^[54].

STANDARD OF CARE

There are several endoscopic ablation techniques for BE with HGD and for superficial adenocarcinoma. Radiofrequency ablation (RFA) has become accepted as a viable treatment for BE, especially flat BE, in light of a recent sham controlled randomized trial^[55]. RFA has been shown to effectively ablate BE with very low rate of stricture formation. For BE with nodularity, endomucosal resection (EMR) with or without ablation therapy has been shown to be safe and effective to eradicate BE and prevent the recurrence of BE with minimal complications^[56]. Excellent survival has been found in long term follow up studies in which endoscopic approaches were used to treat HGD and intramucosal adenocarcinoma^[57,58].

However, the development of metachronous lesions is common (21.5%) with risk factors that include piecemeal resection, no ablation therapy of flat BE after EMR, long-segment BE, multifocal neoplasia, and the prolonged time required for complete eradication of the lesions^[59]. Currently used techniques invariably include one or more of the stated risk factors. These risk factors are compounded by the inability to remove all affected tissue as an *en bloc* specimen by endoscopic techniques; thus less than optimal specimens are available for histopathologic examination of the removed tissue.

A stepwise radical endoscopic resection (SRER) has been proposed to treat BE refractory to RFA and/or EMR. A recent multicenter randomized trial^[60] has demonstrated encouraging results of SRER but the technique involved a greater number of therapeutic sessions and complications such as esophageal stenosis requiring dilation in up to 50% of cases.

In summary, the limitations of currently used endoscopic techniques include the necessity for numerous interventions, the high incidence of metachronous lesions, the absence of a suitable tissue specimen for histologic assessment, and the unavoidable sampling error that occurs especially in patients with long segment Barrett's. Ideally, *en bloc* resection of the entire abnormal epithelium in a single procedure without any compromise of tissue specimens collected for histopathologic examination would be possible. A regenerative medicine strategy that would facilitate restitution of the resected esophageal tissue without concomitant stenosis would represent a significant

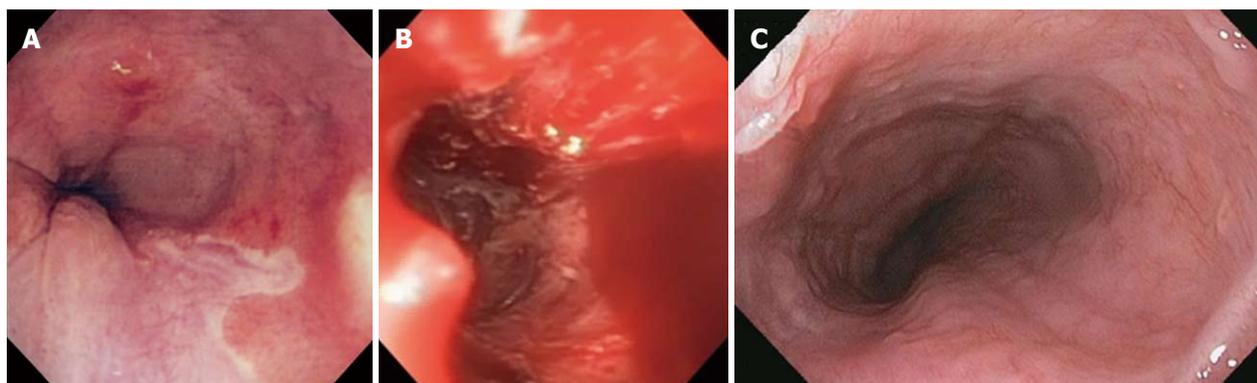


Figure 1 Replacement of esophageal mucosa with extracellular matrix device after endoscopic resection for treatment of high grade dysplasia. A: High grade dysplasia before treatment; B: Esophagus after circumferential resection; C: Regenerated neoesophagus without stricture 3 mo post operatively.

advancement in the treatment of esophageal disease.

REGENERATIVE MEDICINE STRATEGIES FOR THE TREATMENT OF ESOPHAGEAL DISEASE

Classic tissue engineering and regenerative medicine approaches involve either cell based therapies, utilization of a scaffold material, and/or use of bioactive molecules such as growth factors, cytokines and chemokines. In reality, the goal of all approaches is to alter or avoid the default inflammatory/scar tissue response to esophageal injury, and either replace the missing tissue with engineered normal tissue or stimulate the endogenous formation of new, site appropriate functional tissue.

Although an esophageal epithelial stem cell population located in the basal layer of the esophagus has been identified^[61-65], their use in a cell based approach to functional esophageal reconstruction has not been described. Sheets of esophageal epithelial cells can be cultured^[66-68], but practical application of such cell sheet technology to resurface the esophageal lumen following ablative procedures has not been successful. An approach which involves the placement of xenogeneic extracellular matrix (ECM) showed that full thickness defects that included approximately 40%-50% of the circumference and 5 cm of length could facilitate a constructive, non-stenotic healing response with formation of all layers of the esophageal wall in a preclinical dog model^[47]. However, when reconstruction of complete circumferential full thickness defects was attempted with the same ECM scaffold approach, there was the uniform occurrence of severe stricture^[69]. Of note however, if the complete circumferential defects were not full thickness in nature and lesions were limited to the mucosa, then placement of the ECM scaffold upon the subjacent muscularis externa supported the endogenous regeneration of a functional mucosa without clinical stricture^[47,69-71].

These results suggested that a combination of the biologic scaffold material in contact with a native esophageal cell population (i.e., skeletal and smooth muscle plus

adventitial cells) was required for a constructive remodeling response to occur. Further studies showed that as little as 30% of the normal esophageal muscle tissue was required to support the constructive type of esophageal remodeling outcome which allowed for normal dietary habits and absence of any signs of esophageal disease^[69].

The promising results of these preclinical studies were the basis of successful endoscopic treatment for five patients with esophageal adenocarcinoma^[72]. All patients had long segment disease limited to the mucosa. Complete circumferential *en bloc* mucosal resection, ranging from 8 cm to 14 cm in length, was performed on these patients with subsequent placement of a xenogeneic ECM scaffold (SurgiSis™, Cook Biotech, Lafayette, IN) held in place by an expandable stent. The stent was removed within 9-17 d during which time the ECM scaffold integrated with the underlying muscular wall of the esophagus and supported complete epithelialization and formation of a new submucosal layer. All patients required transient post operative dilation for mild stricture but were able to then eat a normal diet without recurrence of disease. Several of these patients have had subsequent reflux surgery and require no further treatment (unreported data). In the context of classic approaches to regenerative medicine, one could consider the successful approach in these patients as a combination of scaffold plus the bioactive factors inherent in the ECM, plus the required endogenous host cells in contact with the scaffold.

Using a similar approach, three additional patients recently were subjected to endoscopic, circumferential *en bloc* resection of Barrett's with HGD, followed by fundoplication (Figure 1). The results support the findings from the previous study and provide further evidence for the use of this procedure as a feasible alternative to surgery for the treatment of HGD and intramucosal adenocarcinoma.

Alternative regenerative medicine approaches to creating esophageal tissue have been explored. Grikscheit *et al*^[73] adapted a technique previously used in intestinal engineering whereby organoid units, mesenchymal cores surrounded by epithelial cells, were isolated from neonatal

and adult rats, labeled with green fluorescent protein (GFP), and paratopically transplanted on biodegradable polyglycolic acid tubes before implantation within the omentum of syngeneic hosts. Four weeks later, the engineered esophageal tissue was either harvested or anastomosed as an onlay patch or total interposition graft^[73]. Histologic examination of these organoids showed a complete esophageal wall including mucosa, submucosa, and muscularis propria. These findings were confirmed with immunohistochemical staining for actin smooth muscle. Furthermore, the tissue-engineered esophagus architecture was maintained after interposition or use as a patch, and animals gained weight on a normal diet. GFP-labeled tissue-engineered esophagus preserved its fluorescent label, proving the donor origin of the tissue-engineered esophagus. The maximal amount of esophageal tissue that could be replaced by this method remains to be explored and the application of this technique to full circumferential lesions has not been investigated.

Similar cell based and/or scaffold based approaches to construct functional esophageal tissue have been investigated by others. In 2006, Marzaro *et al.*^[74] used esophageal ECM seeded with smooth muscle cells (SMCs) to repair a 2 cm defect in the tunica muscularis in a porcine model. They reported the ingrowth of SMCs with early organization into small fascicles. Two years later, Nakase *et al.*^[75] explored replacement of a full circumference esophageal defect with polyglycolic acid scaffolds seeded with epithelial cells. Good distensibility of the construct following implantation was reported although peristaltic activity of the new tissue was absent. The thickness of both the squamous epithelial layer and the smooth muscle layer of the engineered esophagus were similar to that of the native esophagus. These results confirmed the concept of biomaterials seeded with cells, either differentiated cells or stem/progenitor cells, as a potentially viable approach for the repair of damaged esophageal tissue.

The mechanisms by which ECM bioscaffolds alter the default proinflammatory esophageal healing response and instead promote a more constructive remodeling response are only partially understood. However it is known that degradation of the ECM releases a variety of growth factors including vascular endothelial growth factor and basic fibroblast growth factor, among others^[76]. The critical amounts of active growth factor and the specific factors required to support constructive tissue remodeling are unknown. ECM scaffold degradation *in vivo* occurs rapidly based upon results of preclinical studies in non-esophageal sites^[77-80] and the endoscopic procedures to remove the temporary stents in the patients treated for esophageal cancer suggests that degradation is also very rapid in this location. Scaffold degradation is considered important because it removes a persistent foreign material against which the host can mount a chronic inflammatory reaction and, perhaps more importantly, scaffold degradation results in the generation of bioactive cryptic peptides from component structural

molecules of the ECM such as collagen^[81]. These cryptic peptides, typically no larger than 10-12 amino acids in length, have been shown to have potent chemotactic and mitogenic activity for selected stem and progenitor cells *in vitro*^[81-84]. The role of this chemotactic phenomenon in constructive remodeling is not fully understood but logically it provides a method for supporting a regenerative type of response.

It is also known that the host response to the presence of a xenogenic ECM scaffold includes local modulation of the innate immune response from proinflammatory M1 macrophage mediated events toward more dominant constructive tissue remodeling M2 macrophage mediated processes^[77,85-87]. However, it is unknown which, if any, of these mechanisms occur or are important in the esophageal location.

CONCLUSION

Esophageal disease is an increasingly important problem and has very limited satisfactory treatment options. The default inflammatory and scarring response of the non-regenerating esophageal tissue not only creates severe morbidity from the disease process itself, but also limits the therapeutic options since manipulation and tissue injury are unavoidable sequelae of either invasive or minimally invasive endoscopic techniques. Regenerative medicine strategies that utilize cell based, scaffold based, and bioactive molecule based approaches potentially provide a viable alternative for both physicians and the affected patients. Preliminary early results of a bioactive ECM scaffold based approach have been promising.

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Esophageal tissue engineering: A new approach for esophageal replacement

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Abstract

A number of congenital and acquired disorders require esophageal tissue replacement. Various surgical techniques, such as gastric and colonic interposition, are standards of treatment, but frequently complicated by stenosis and other problems. Regenerative medicine approaches facilitate the use of biological constructs to replace or regenerate normal tissue function. We review the literature of esophageal tissue engineering, discuss its implications, compare the methodologies that have

been employed and suggest possible directions for the future. Medline, Embase, the Cochrane Library, National Research Register and ClinicalTrials.gov databases were searched with the following search terms: stem cell and esophagus, esophageal replacement, esophageal tissue engineering, esophageal substitution. Reference lists of papers identified were also examined and experts in this field contacted for further information. All full-text articles in English of all potentially relevant abstracts were reviewed. Tissue engineering has involved acellular scaffolds that were either transplanted with the aim of being repopulated by host cells or seeded prior to transplantation. When acellular scaffolds were used to replace patch and short tubular defects they allowed epithelial and partial muscular migration whereas when employed for long tubular defects the results were poor leading to an increased rate of stenosis and mortality. Stenting has been shown as an effective means to reduce stenotic changes and promote cell migration, whilst omental wrapping to induce vascularization of the construct has an uncertain benefit. Decellularized matrices have been recently suggested as the optimal choice for scaffolds, but smart polymers that will incorporate signalling to promote cell-scaffold interaction may provide a more reproducible and available solution. Results in animal models that have used seeded scaffolds strongly suggest that seeding of both muscle and epithelial cells on scaffolds prior to implantation is a prerequisite for complete esophageal replacement. Novel approaches need to be designed to allow for peristalsis and vascularization in the engineered esophagus. Although esophageal tissue engineering potentially offers a real alternative to conventional treatments for severe esophageal disease, important barriers remain that need to be addressed.

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Key words: Esophagus; Regenerative medicine; Tissue engineering; Scaffolds; Transplantation

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INTRODUCTION

Recent years have witnessed great interest in regenerative medicine, the replacement, repair and regeneration of tissues and organs^[1,2]. Particular interest has focused on the potential for this new field to offer new solutions for failing tissues and organs, and alternatives to transplantation, implants and reconstructive surgery, all of which have limitations.

Several conditions, both congenital and acquired, may require esophageal tissue replacement. In the pediatric population the primary indication for esophageal replacement is long-gap esophageal atresia (EA) with insufficient length for primary anastomosis. Patients with long-gap EA, which fail a primary repair, receive a denervated gastric pull-up or interposition graft using either jejunum or colon, with many associated early and late post-operative complications, such as stricture formation and the potentially carcinogenic effect of acid reflux^[3,4]. In children, gastric transposition and intestinal interposition can also be used in esophageal strictures not responsive to dilatation following failed EA repair or caustic ingestion, or for rare neoplastic conditions such as inflammatory pseudotumor, leiomyosarcoma and teratoma^[5]. By contrast, the commonest indication for esophageal replacement in adults is cancer, a condition whose incidence is escalating^[6], whilst colon interposition is sometimes indicated for diffuse Barrett's esophagus, a premalignant condition. Unfortunately, all of these methods of esophageal replacement severely impair the quality of life of recipient adults and children^[7,8] and present problems related to donor site morbidity. Even recent developments in endoluminal resection, which removes the diseased inner layers of the esophagus through an endoscope, whilst reducing morbidity, still results in a high rate of stenosis and consequent dysphagia^[9]. Despite its 60-year history, conventional organ transplantation is not a solution for the failure of every organ, due to technical and ethical issues, and is specifically unable to address the unmet needs of esophageal replacement. Thus, regenerative medicine techniques, which extend the boundaries of reconstruction and do not, in most applications, require immunosuppression, present attractive alternatives^[10].

Regenerative medicine has been used to describe the use of natural human substances, such as genes, proteins, cells, and biomaterials to regenerate diseased or damaged human tissue^[11,12] in order to restore normal function^[2].

Tissue engineering with the end-point of organogenesis has been successful through a combination of appropriate cells with a scaffold^[13-17] as well as the use of only one of these two components, for example in the repair of urethra^[18] and skin^[19] (Figure 1).

We review the literature relating to esophageal tissue engineering and suggest areas where research may lead to the most rapid clinical gains.

INFORMATION COLLECTION

We searched Medline, Embase, the Cochrane Library, National Research Register and ClinicalTrials.gov databases, using the search terms stem cell and esophagus, esophageal replacement, esophageal tissue engineering, esophageal substitution. The reference lists of papers identified in this way were searched and further papers identified. All full-text articles in English of potentially relevant abstracts were reviewed. Finally, acknowledged experts in this field were contacted for information on gaps in our review and information on unpublished studies.

TWO BROAD CATEGORIES OF INTERVENTION

Seventy-four papers were identified and are reviewed in this manuscript. Two broad categories of intervention were identified: the use of scaffolds alone, and a combination of cells and scaffolds.

Acellular scaffolds

The majority of identified studies transplanted acellular scaffolds with the aim that host epithelial and smooth muscle cells will migrate to repopulate the new conduit. Acellular scaffolds studied to date conform to one of three categories: synthetic, collagen alone and decellularized matrix.

Synthetic scaffolds: Acellular synthetic scaffolds such as polyethylene plastic^[20-22] and silicon^[23,24] have been used for esophageal replacement, but the nature of the materials did not allow cellular migration and led to poor results in animal models. When polyvinylidene fluoride (PVDF) and polyglactin-910 (Vicryl[®]) were compared for the regeneration of patch defects in rabbits, PVDF was shown to lead to improved results with an absence of strictures and neopithelialization^[25]. However, in a different study, the combination of Vicryl[®] and collagen brought about positive results both for patch and tubular defects in dogs, with a low mortality of 8.3%^[26]. The successful use of synthetic polymers in other organs such as the trachea^[27], suggests that this approach may appear attractive and further development of appropriate materials is needed.

Collagen scaffolds: In a series of experiments performed by a research group in Japan, porcine dermal collagen scaffolds were used to produce porous tubular structures (Table 1)^[28-33]. The general methodology involved the use of these

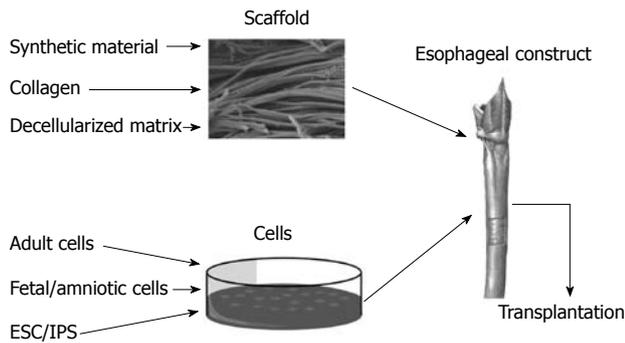


Figure 1 Esophageal tissue engineering. A tissue-engineered esophageal construct may be created by the combination of a scaffold and cells, grown in a bioreactor and transplanted in patients. A three-dimensional scaffold may be created from synthetic material, collagen or a decellularized matrix. Cells for the use of tissue engineering are derived from a number of sources such as the adult, fetus and the embryo. Additionally, non-seeded scaffolds may be transplanted with the aim of being repopulated by host cells. ESC: Embryonic stem cells; IPS: Induced pluripotent stem cells.

scaffolds to replace 5-10 cm tubular defects in the cervical or intra-thoracic portion of the esophagus in dogs. A silicon tube was used as a stent to support the scaffold until repopulation occurred. Aiming to avoid complications such as stenosis^[28,29], the research group compared whether this was related to the time for which the scaffold was supported by the stent. In an experiment where three groups of dogs had a 5-cm cervical surgically created defect, the stent was removed at either 2, 3 or 4 wk. With increasing stent duration, it was observed that greater epithelial and muscle cell densities were achieved in the collagen scaffold, and this correlated with decreased stenosis and mortality^[31]. However, when the collagen scaffold replaced 10-cm portions of the esophagus there was poor cellular migration in the muscular layer, suggesting that there are limitations to the size of defect that may be replaced by this methodology^[30]. Moreover, when the same methods were used to replace intra-thoracic portions of the esophagus in dogs, muscular regeneration was completely absent, something the authors attributed to the lack of a vascular supply in the thorax^[32]. In an attempt to address this, the scaffold was wrapped in omentum^[33], as has been successfully applied to tracheal tissue engineering^[34,35]. However, muscular regeneration remained absent, whilst an increase in mid-portion stenosis and mortality was observed^[33].

Decellularized matrix: Decellularized matrices are derived from human and animal organs and tissues that have been treated to remove cells and immunogenic material^[36]. Importantly, however, they retain the macro- and micro-architecture of the tissue of origin, and the molecular components of its natural extracellular matrix^[37-40]. They have the added hypothetical advantages over synthetic scaffolds of not producing potentially toxic degradation products or inducing inflammation characteristics that may be important in the prevention of stenosis^[20,41,42]. Decellularized scaffolds that have been used for esophageal organs originated from the esophagus

as well as from other tissues such as the small intestinal submucosa (SIS)^[28-31,43-47].

Significant heterogeneity exists among studies, both with respect to the type of scaffold, extent of surgery and species used, which partly explains the range of results reported. Thus, regeneration of the muscularis propria layer is seen to take place in some studies^[43,44,48], but not others^[49]. Studies that have attempted tube-interposition with SIS report the development of esophageal stenosis and increased mortality^[44,50,51]. By contrast, studies applying SIS as a patch repair demonstrated encouraging results^[44,50-53]. Badylak *et al.*^[45] laid sheets of SIS onto the raw internal surface of esophagus following endoscopic submucosal resection in five patients with superficial cancers. With a follow-up of 4 to 24 mo, the scaffold promoted physiological remodelling as evident by endoscopy and histological characterisation following biopsy. Strictures still formed, but only at areas outside those lined by SIS, suggesting that possible technical improvements in scaffold delivery could ameliorate this. In fact, when SIS was used to completely cover a 3 cm × 5 cm mucosal defect in the cervical esophagus, there was no stenosis and endoscopy at 4 wk demonstrated good integration of the scaffold^[46].

Hypothetically, decellularized esophageal tissue should retain the signals, both chemical and structural, that will direct the appropriate migration and differentiation of host cells, in a way unlikely to occur with scaffolds originating outside the esophagus, such as SIS. Ozeki *et al.*^[54] compared two methods of decellularization of adult rat esophagus based on deoxycholate and Triton X-100 respectively and assessed the resulting scaffolds using routine histology and biocompatibility. Those treated with deoxycholate showed superior mechanical properties, maintenance of the extracellular matrix and a lower DNA content than those treated with Triton X-100. Bhrany *et al.*^[55] found a combination of 0.5% sodium dodecyl sulphate and Triton X-100 to be effective in decellularization, albeit with a loss of tensile strength as measured by burst pressure studies. Our experience with the detergent-enzymatic treatment in the decellularization of the intestine^[39] allowed us to use the same methodology in the esophagus (Figure 2), leading to an improved preservation in microarchitecture^[56].

Cell-seeded scaffolds

To reduce complications arising from acellular approaches, some authors have seeded the scaffolds prior to transplantation. As mentioned, the two main cell types that are important for esophageal tissue engineering are those that will reconstitute the epithelium and the muscle layer on the luminal and extra-luminal sides respectively. Also important in the formation of a functional esophagus are the vascular and neuronal cell components but we could locate no studies that have studied these in engineered esophagus.

A number of *in vitro* experiments have examined the seeding and culture of esophageal epithelial cells and different scaffolds to assess the optimal combination. When a matrix composed of decellularized human skin was

Table 1 Overview of *in vivo* transplantation of acellular matrices

Animal model (n)	Scaffold type	Size	Scaffold regeneration	Results		Ref.
				Clinical course		
Canine (19)	Collagen with silicon stent (not removed)	5 cm circumferential gap, cervical esophagus	Partial epithelial regeneration	26% mortality		[28]
Canine (26)	Collagen with silicon stent (removed between 2 and 8 wk)	5 cm circumferential gap, cervical esophagus	Epithelial regeneration, no stenosis	0% mortality when stent dislodged after 4 wk (n = 4)		[29]
Canine (7)	Collagen with silicon stent (removed at 6 wk)	10 cm circumferential gap, cervical esophagus	Epithelial and partial muscular regeneration, no stenosis	29% mortality		[30]
Canine (43)	Collagen with silicon stent (removed either at 2, 3 or 4 wk)	5 cm circumferential gap, cervical esophagus	Epithelial and muscular regeneration, no stenosis	0% mortality when stent was removed at 4 wk (n = 16)		[31]
Canine (9)	Collagen with silicon stent (removed at 4 wk)	5 cm circumferential gap, thoracic esophagus	Epithelial but no muscular regeneration, mid-portion stenosis	11% mortality		[32]
Canine (14)	Collagen with silicon stent (removed at 4-8 wk) +/- OMPx	5 cm circumferential gap, thoracic esophagus	Epithelial regeneration, mid-portion stenosis	11% mortality in control group, 80% in OMPx group		[33]
Canine (15)	Extracellular matrix scaffold from either small intestine (n = 12) or urinary bladder submucosa (n = 3)	5 cm circumferential, cervical esophagus	Mucosal and muscular regeneration. Stenosis in case of complete circumferential defects	0% mortality		[44]
Pigs (10)	Elastin based acellular biomaterial patch (from porcine aorta)	2-cm circular defect, abdominal esophagus	Mucosal and muscular regeneration	0% mortality. No complications reported in treatment groups		[43]
Canine (12)	Urinary bladder matrix scaffold	Complete transection with replacement of endomucosa with matrix	Mucosal and muscular regeneration	0% mortality. No complications reported in treatment groups		[48]
Rats (67)	Small intestinal submucosa patch graft	Semi-circumferential defect, cervical or abdominal esophagus	Mucosal and muscular regeneration at 150 d	94% survival at 150 d		[50]
Rats (85)	Small intestinal submucosa patch graft, or tube interposition	Semi-circumferential defect or segmental esophageal excision	Tube interposition unsuccessful. Mucosal and muscular regeneration at 150 d in patch-group	100% survival for patch-group (and no complications reported), 0% survival for tube interposition group at 28 d		[52]
Rats (27)	Gastric acellular matrix scaffold	Patch defects, abdominal esophagus	Mucosal regeneration seen at 2 wk. No muscular regeneration seen up to 18 mo	11% complication rate		[49]
Pigs (14)	Small intestinal submucosa (tubular)	4-cm defect, cervical esophagus	Prosthesis not found either macroscopically or histologically	Only 1 pig survived the full 4 wk study. The other pigs have to be sacrificed prematurely due to severe stenosis		[51]
Human (5)	Porcine small intestinal mucosa	8-cm to 13-cm en-bloc resection of mucosa and submucosa for superficial carcinoma	Restoration of normal mucosa as early as 4 mo	Strictures; perforation in one patient		[45]
Human (1)	Porcine small intestinal mucosa	5 cm x 3 cm defect cervical esophagus	Intact esophagus with normal calibre	No complications encountered		[46]

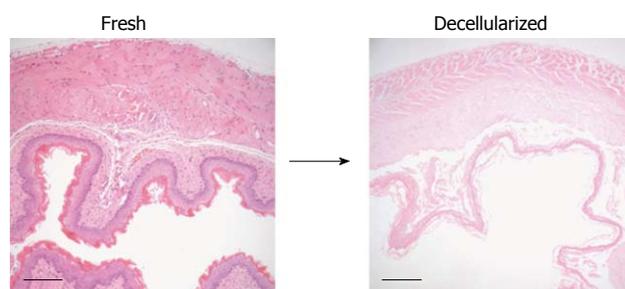


Figure 2 Production of esophageal natural acellular matrices. Decellularization involves treatment of fresh esophageal tissue with a combination of solutions that will remove the cells but maintain the structural characteristics of the native extracellular matrix. The optimal methodology of esophageal decellularization is currently under investigation. Our experience with the detergent enzymatic treatment is illustrated here with hematoxylin and eosin staining of a representative decellularized esophagus demonstrating preservation of the native architecture (Scale bar 100 μ m).

compared to synthetic scaffolds *in vitro* for the capacity to support cultured epithelial cells, the decellularized scaffold exhibited cell differentiation and surface confluence similar to native esophagus, whereas synthetic scaffolds demonstrated a discontinuous epithelial lining^[57]. Another study compared the growth of human esophageal squamous cells on human decellularized esophagus, porcine decellularized esophagus, human decellularized dermis, and collagen^[58]. Interestingly the porcine matrix and collagen gave better results leading to the formation of a mature stratified epithelium. When rat esophageal epithelial cells (EEC) were seeded onto 3-dimensional (3-D) collagen scaffolds they were shown to be viable for up to 8 wk *in vitro* but did not fully integrate within the scaffold, remaining on the surface as individual cells or small clusters^[59]. Seeding of sheep EEC on the same 3-D collagen scaffold resulted in the absence of epithelium sheet for-

mation, which was attributed to cellular penetration into the scaffold and loss of cell-to-cell contact^[60]. However, when the same cells were seeded on the 2-D collagen scaffolds a single layer of epithelium was evident following 3 wk of *in vitro* culture that remained viable up to 6 wk. The same group has also performed *in vivo* studies of vascularization of the EEC-scaffold construct by omental transplantation in lambs for 8-12 wk^[61]. Positive selection of the epithelial population could increase proliferative capacity as demonstrated by Kofler *et al.*^[62], who selected ovine EEC for expression of pancytokeratin (PCK) using fluorescence activated cell sorting. The PCK-negative subpopulation had minimal cell attachment on the collagen scaffolds, whereas the PCK-positive cells had a uniform distribution.

In vivo experiments using EEC-scaffold constructs, similarly to results in acellular approaches, have shown more promise for regeneration of partial rather than circumferential defects in rats and dogs^[63-65]. An innovative approach recently described seeded cells on a temperature-responsive dish that became hydrophilic at 20 °C and allowed harvesting of a single-cell sheet^[63]. When the cell sheets were transplanted in dogs that had undergone endoscopic submucosal resection, complete wound healing was observed at 4 wk with no signs of stricture and an intact epithelium. Wei *et al.*^[64] obtained mucosal epithelial cells from oral biopsy, or esophageal organoid units created following digestion of rat esophagi^[65]. These were seeded onto scaffolds and implanted as complete esophageal substitutes, but histology of the resultant muscle layers showed poor architecture.

To overcome the limitations of using EEC in isolation, esophageal constructs prepared using EEC-seeded collagen scaffolds were placed on the latissimus dorsi muscle of athymic mice with the intention to harvest and tubularize the muscle once the epithelial side has matured^[66,67]. Miki *et al.*^[68] found an increase in the number of epithelial layers from 2 when EEC seeded alone, to 18 when co-seeded with fibroblasts. A more recent study by Hayashi *et al.*^[69] cultured both epithelial and fibroblast cells on a bed of smooth muscle cells (SMC) embedded in a collagen gel *in vitro*, prior to transplanting them on the latissimus dorsi of athymic rats. Nakase *et al.*^[70] also aimed to combine different cell lines and scaffolds into one tubular structure in dogs. They used oral keratinocytes and fibroblasts cultured on human amniotic membrane and SMC seeded on poly (glycolic acid). These two scaffolds were then rolled together and implanted into the omentum for 3 wk, following which they were transplanted into a 3-cm intrathoracic esophageal defect. Both muscular and epithelial layers were present at 420 d of follow-up, although no peristaltic activity was observed.

FUTURE PERSPECTIVES

Based on the above literature, it is clear that although tissue engineering has been proposed as a solution for the current treatments of esophageal defects, currently, there

is no clear strategy for recreating all the portions of the esophagus in man^[71,72]. The problems that need to be solved are related to the optimal scaffold, the cell sources for the epithelial and muscular components, peristalsis and vascularization (Table 1). The stenotic changes that are the main complication encountered with esophageal constructs are likely related to poor regeneration of natural architecture.

The recent trend in organ tissue engineering has been to use decellularized scaffolds. It has been suggested that they would be an advantageous choice due to their enhancement of cellular proliferation, migration and differentiation. However, the lack of positive results when trying to replace a tubular defect, confirms that the use of biomaterials alone as a means of esophageal repair is unsuccessful. We envisage a point where “smart polymers” may replace scaffolds of biological origin and facilitate an “off-the-shelf” approach to esophageal tissue-engineering. Our group and collaborators in Sweden have used poly-hedral oligomeric silsesquioxane-poly (carbonate-urea) urethane, a synthetic material used in clinical trials of vascular grafting, as an alternative to biologic scaffolds in the generation of tracheal scaffolds^[73]. These have the added advantages of being tailor-made and retain biomechanical properties indefinitely, whilst there is no need for an organ donor, with all the attendant convenience, infection and ethical issues of the latter. However, early experience shows that these scaffolds do not epithelialize or vascularize easily^[27]. The study of cell-scaffold interactions is likely to substantially inform the development of better biomaterials for organ and tissue regeneration. Ritchie *et al.*^[74] found that esophageal muscle cells seeded onto collagen membranes required mechanical stimulation to retain normal contractile properties in a bioreactor, showing the importance of a multidisciplinary engineering approach to this problem, but we could find no other references to the application of bioreactors to esophageal tissue-engineering. *Ex vivo* models, such as bioreactors and microfluidic organotypic chambers, are urgently required in order to explore the effects of varying stem cell/cell-scaffold-signaling combinations in the generation of functional esophageal tissue pre-implantation.

The general consensus indicates a significant advantage in repopulating scaffolds with cells prior to implantation. Studies that have seeded EEC have had positive results in repopulating the epithelial layer, both as an onlay patch^[63,64] and as a total interposition graft^[65]. Nevertheless, as with cell-free approaches, in cases in which only the lumen was seeded, there was a poor regeneration of the muscular layer, indicating a need for co-seeding with SMC. This is not a surprise, since esophageal strictures can be managed clinically easily with an intestinal patch (free graft) as partial substitution while they have very high chance of recurrence when such material is used to repair the whole circumference. Studies are required to identify the optimal cell types and sources to repopulate esophageal scaffolds. Ideally, cell sources should be autologous, easy to harvest, highly proliferative, and should have the

ability to differentiate into many specialized cell types.

Equally important to muscular regeneration is the challenge of replicating peristaltic contractility and a vascular supply in an artificial esophagus. Watanabe *et al*^[75] developed nickel-titanium, shaped-memory, alloy coils, which were placed in an annular manner on a Gore-Tex vascular graft for esophageal replacement. Interestingly, low-voltage electrical current passing through the coils generated peristaltic movements in the artificial esophagus implanted in a goat model, suggesting that re-provision of appropriate muscular stimuli, either by enhanced neural regeneration or by electrical means, may be a profitable route for investigation if functionally normal swallowing is to be achieved. What is more, we propose that the physiological contribution of neural crest cells is a prerequisite for functional peristalsis.

Regarding the vascular component, the esophagus holds an additional challenge due to the tenuous intrinsic vascular anatomy of the esophagus in man and the association of stenosis with poor vascularization. Wrapping the engineered esophagus in the omentum prior to thoracic transplantation is one potential solution, as proposed by Nakase *et al*^[70]. However, results in this instance as well as in our use of omental wrapping for transplantation of tissue-engineered tracheas in humans^[35] were sub-optimal. More preclinical work on revascularization strategies is required. The use of intraluminal stents is another solution to avoid stenosis. Where collagen scaffolds were used in the above studies, the stenosis and mortality was inversely correlated to the length of stay of the intraluminal stent^[29,31]. The use of stents allows time for epithelial and muscular migration onto the cell-free scaffolds. In our recent pediatric tissue engineered trachea transplant^[35] we also used bio-absorbable stents, which were engineered using large mesh that allows epithelial ingrowth and persists for about 6 wk before complete degradation^[76,77].

CONCLUSION

In the near future, tissue engineering may represent a valid therapeutic alternative to treat severe congenital or acquired esophageal disorders. We present possible lines for investigation that could indicate what such products will look like, but propose that, in the short- to medium-term, a combination of decellularized scaffolds with muscle and epithelial cells of autologous (including autologous stem cell) origin are likely to be the most expeditious route. Major questions of vascularity, cell-cell and cell-scaffold interaction, and motility remain outstanding, however, before the bioengineered neo-esophagus becomes an established, effective treatment for complex congenital and acquired malformations in adults and children.

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How regenerative medicine and tissue engineering may complement the available armamentarium in gastroenterology?

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Abstract

The increasing shortage of donors and the adverse effects of immunosuppression have restricted the impact of solid organ transplantation. Despite the initial promising developments in xenotransplantation, roadblocks still need to be overcome and this form of organ support remains a long way from clinical practice. While hepatocyte transplantation may be effectively correct metabolic defects, it is far less effective in restoring liver function than liver transplantation. Tissue engineering, using extracellular matrix scaffolds with an intact but decellularized vascular network that is repopulated with autologous or allogeneic stem cells and/or adult cells, holds great promise for the treatment of failure of organs within gastrointestinal tract, such as end-stage liver disease, pancreatic insufficiency, bowel failure and type 1 diabetes. Particularly in the liver field, where there is a significant mortality of patients

awaiting transplant, human bioengineering may offer a source of readily available organs for transplantation. The use of autologous cells will mitigate the need for long term immunosuppression thus removing a major hurdle in transplantation.

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INTRODUCTION

Failure of abdominal organs is a significant cause of morbidity and mortality (Table 1). Patients with organ failure may benefit from non surgical therapies, such as insulin for endocrine pancreatic failure, parenteral nutrition for bowel failure and renal replacement therapy for renal failure. Until now, solid organ transplantation, and less convincingly cellular transplantation, represent the only way to provide a definitive treatment for organ fail-

ure, although the long-term impact is limited.

SOLID ORGAN TRANSPLANTATION

Over the last half a century, there have been major advances in the field of transplantation because of improved surgical techniques, anaesthesia, immunosuppression and peri-operative care. All these elements substantially improved patient and graft survival. Five-years patient and graft survival rates of 73% and 68.4% can nowadays be achieved in liver transplantation (LT), but still relatively poor long-term results are obtained for intestinal and pancreas transplantation with five-year patient and graft survival rates of 47% and 38%, and 82% and 52%, respectively^[1].

The major limitation to the widespread use of transplantation is the scarcity of organs. The gap between available organs and potential recipients increases every year, giving rise to serious ethical and practical dilemmas of equity and utility when allocating the organs. In March 2011, the United Network for Organ Sharing reported a solid organ transplant waiting list of 110 600; 16 133 patients were listed for liver, 1389 for pancreas and 262 for intestine transplants. The absolute numbers of transplants in 2010, in contrast, was 6291, 351 and 151 for liver, pancreas and intestine, respectively. These figures clearly illustrates that many patients will never benefit from transplantation and die on the waiting list^[1]. The problem is even greater than these figures suggest as not all those who might benefit from transplantation are listed. In the United Kingdom, patients with chronic liver disease are listed only when estimated likelihood of death without transplant is greater than likelihood of death following transplantation. This estimation of survival is calculated based on validated models using objective laboratory data incorporated in scores such as developed by the Model for End-Stage Liver Disease (MELD) or United Kingdom End-stage Liver Disease (UKELD). The UKELD score includes, besides the MELD parameters, serum creatinine, bilirubin and INR, also serum sodium. The latter score seems to have an improved predictive accuracy. A UKELD score of over 49 predicts a > 9% 1-year mortality without liver transplant; this is the minimum criteria for entering the waiting list in the United Kingdom^[2]. In the United States, survival benefit starts when MELD score exceeds 17, unless the patient has other co-morbid factors, such as liver cancer, affecting prognosis or unacceptable quality of life because of liver disease. In the United Kingdom, candidates for LT must also have a greater than 50% probability of surviving for 5 years post transplant with a quality of life acceptable to the patient^[3].

Although not used as a direct criterion for selection or allocation, development of the concept of transplant survival benefit, i.e. the extra years of life attributable to transplant, might facilitate more effective use of scarce organs and restrict access to those whose lives will be

Table 1 Deaths in United Kingdom¹ and United Kingdom Transplant Activity²

	Overall deaths	New registrations	Waiting list at 31/3/10	Re-movals	Transplants	3 yr post transplant survival
Liver	7503	962	371	123	679	85%
Pancreas	5223 ³	300 ⁴	335	22	200	74%
Small bowel	4 ⁵	NA	NA	NA	21	NA

¹United Kingdom deaths from United Kingdom Mortality Statistics, London National Statistical Office, 2010; ²United Kingdom Transplant Activity from National Health Service Blood and Transplant Activity Report 2010, Bristol; ³Deaths for diabetes; ⁴Including 243 kidney and pancreas transplantations; ⁵Deaths in 2005. Note: Overall deaths include all causes and all ages so many of those who died would not be suitable for transplantation. NA: Not available.

extended minimally or not at all. However, it has proved very difficult to develop robust modelling on which to base such a benefits based approach to liver allocation.

In case of diabetes mellitus, numbers on waiting lists also underestimate the need for transplantation as this therapeutic modality is primarily applied to patients in need for a combined pancreas-kidney transplantation. Transplantation for diabetes alone is restricted, in many centers, to some patients, with hypoglycemia unawareness and brittle diabetes. The adverse consequences of immunosuppression, such as the increased risk of some infections and malignancies need to be balanced against the potential benefits of improved glycemic control so it is not clear which patients with diabetes mellitus might benefit from transplantation.

In part because of cultural and logistic issues, deceased donor rates vary considerably between countries, ranging from 2 per million population in Greece to 35 per million population in Spain^[4]. The success of public health initiatives, leading to better awareness for vascular diseases such as hypertension and reduction in fatal road accidents, resulted in a fall in the number of “traditional ideal” (young post-traumatic) organ donors and an increase in the use of “high risk” donors, such as older and obese donors and donors after circulatory arrest^[5]. The increasing donor risk profile partly negates the benefits made by better surgery and peri-transplant care.

To increase the number of available organs, several technical advances and policies have been adopted. Using extended criteria donors (ECD) and donation after circulatory death (DCD) [also referred to as non-heart beating donors (NHBD)] and implementing split LT (SLT), domino or living donor LT can contribute to enlarging the donor pool. All these techniques and policies are however not free from additional risk and therefore their use raises medical and ethical concerns as welfare of both the living donor and recipient may be compromised. Indeed all these allografts from deceased donors carry an increased risk of primary non-function, early or delayed dysfunction and possibly a greater risk trans-

mission of infection and cancers^[6]. It has been clearly shown that ECD organs, defined as organs originating from donors dying from cerebrovascular catastrophe, DCD, longer ischemia time, older age and steatosis compromise outcome. Severe allograft steatosis, defined as > 60% fatty infiltration, is associated with a greater risk of primary graft dysfunction and lower patient and graft survival^[7]. Grafts with more than 30% steatosis have been reported to be safe in low-risk recipients but associated with more risk in recipients with MELD scores greater than 30^[8]. Clinical estimation of the degree of fatty infiltration correlates poorly with histologic assessment.

Guarrera *et al*^[9] have undertaken a pilot study designed to evaluate the safety and feasibility of liver preservation with hypothermic machine perfusion (HMP), a technique widely used in kidney transplantation^[10] although its potential has been shown in animal models of LT. Compared to standard cold preservation in human LT, HMP appears safe, may improve graft function and is reported to be associated with a reduction in preservation injury (PI). This strategy is likely to be most beneficial in older, steatotic and DCD grafts, which are most susceptible to PI.

Models investigating the interaction between donor and recipient risk profiles have been developed to predict the likelihood of graft and patient survival after LT. Feng *et al*^[11] recently identified, in a large donor cohort study, nine factors (age, height, DCD donors, split liver grafts, black race, cause of death from cerebrovascular accident, regional sharing and cold ischemia time) which were associated with graft failure. As a corollary, a donor risk index predicting the effect of these variables on graft survival was developed^[11]. It is clear that it will become more and more important to match donor risk score with recipient risk score in order to assure an acceptable outcome for the recipient.

SLT has been developed as a strategy to increase the number of liver grafts by creating 2 grafts from 1 donated liver. The bipartition of a liver is especially important in the small group of pediatric patients for whom size-matched whole liver allografts are scarce. Indeed the use of split grafts has been associated with a reduction in the risk of death on the pediatric waiting list; although some centers have reported an increased risk of graft failure, the split procedure for adult-pediatric pair is now accepted as a valuable technical variant in pediatric LT^[11,12]. Donor selection for splitting, technical and logistic expertise to decrease total ischemia time are all important factors for a successful outcome of the procedure. This technique is much less successful in the adult-adult split constellation.

In order to expand further the donor pool, organs from DCD donors are increasingly used in liver and pancreas transplantation, especially in the United Kingdom and the Netherlands. Liver and pancreas grafts are usually restricted to those originating from controlled

donors - those donors in Maastricht category III (awaiting cardiac arrest)^[13]. However in Europe, legal constraints do not allow use of NHBD in all countries^[14]. Although, DCD LT can have good outcome, their use is associated with a significantly higher risk of graft failure^[13-16], severe biliary complications and higher costs^[17-19]. However, increasing understanding of the pathophysiology of the events surrounding DCD and better selection and timing may improve outcome in the future.

Several reports described successful islet isolation and transplantation from DCD donors^[20-24]. These donors could provide an important resource for islet transplantation if used under strict criteria and in multiple transplantation, particularly in countries where heart-beating donors are not readily available.

The use of DCD organs has not been deemed suitable to intestinal recipients because of concerns about organ quality.

With respect to transplantation of organs of the gastrointestinal system, living donation is essentially confined to LT. Better understanding of the anatomy and increasing surgical skills has allowed living donor liver transplants (LDLT) to become a routine procedure in some, especially Asian, centres. LDLT has been widely adopted in Asia because of the very low rates of deceased organ donation and because of the very high incidence of liver cancer. LDLT accounts for over 95% liver transplants in Asia. In 2008, a Chinese series of 234 right-liver living donor liver transplants showed 1-, 3-, and 5-year overall survival rates of 93.2%, 85.7%, and 82.4%, respectively, comparable with deceased donor liver transplant outcomes^[25]. Good outcomes have been shown even when using grafts with a graft-to-recipient weight ratio (GRWR) < 0.8%, with a rate of small-for-size syndrome similar to those receiving graft with a GRWR > 0.8%, provided the recipient is receiving the graft from a young donor^[26].

In the Western world LDLT is practised much less frequently because of the greater availability of deceased donors but also because of major concerns with donor mortality, especially when transplanting the right lobe which is associated with an estimated risk of donor death of 0.08% and a morbidity around 20%. The reported morbidity and mortality data underestimate the real risk. There have been anecdotal reports of donors requiring a transplant for hepatic failure. The outcome of LDLT is good with a 1-, 5- and 10-years graft survival of 81%, 70% and 68%, respectively^[27-30]. The survival rates after LDLT are better than full size deceased donor LT in children but somewhat lower in adults^[29].

However, not all liver transplant candidates have suitable donors. Altruistic (non-directed) liver donation is done very rarely although the number of altruistic kidney donations, while still small, is increasing slowly.

Living donor bowel transplantation has been reported as an additional resource for patients with intestinal failure with total parenteral nutrition-related life-

threatening complications^[31]. However the very limited data from the Intestinal Transplant Registry do not demonstrate a clear advantage of living donor intestine donation over deceased donor intestine transplant^[32-34]. The early outcomes of combined intestinal and LT using living donors are promising and the elimination of the high mortality on the cadaver waiting list (30%) for this category of patients represents a substantial advantage^[35].

Limitations of solid organ transplantation

Transplantation is associated with a significant improvement in both quality and quantity of life for most organ recipients but does not reach normal values^[36]. Patient and graft survival is limited by many factors including recurrent disease, immune mediated graft damage, technical problems and long-term infectious, malignant and cardiovascular consequences of immunosuppression. It is noteworthy that recurrent hepatitis C virus (HCV) allograft infection is almost universal and associated with a worse outcome compared with most other indications, yet HCV related cirrhosis is one of the leading indication for LT in the Western world^[37].

Although operational tolerance is found in a small proportion of highly selected liver allograft recipients, most patients will require life-long immunosuppression. Attempts to induce tolerance strategies which are successful in the laboratory, have yet to be reliably achieved in man^[38].

Transmission of donor-related disease, especially some infections and cancers, can be mitigated but not abolished^[39].

XENOTRANSPLANTATION

Research in xenotransplantation has grown in the last decades^[40,41]. The use of knock-out pigs with multiple gene modifications reduced the frequency of hyperacute rejection which was a major problem in earlier models^[42,43]. Many physiological restraints, as evidenced by a systemic inflammatory response involving the innate immune system, by platelet, leucocyte and complement activation, by coagulation dysfunction associated with coagulation-anticoagulation incompatibilities of primates and pigs, remain. The transplantation of porcine organs has been carried out in non-human primates with better outcomes with pig hearts or kidneys compared with pig livers, the main problems being in the latter a coagulation dysfunction with thrombocytopenia leading to spontaneous bleeding^[44]. However, pig livers may provide sufficient function to maintain short term support and might so be used in patients with acute liver failure, either until the native liver recovers or as a bridge to liver allograft. Only one clinical pig-to-human LT has been reported so far by a surgical team in Los Angeles headed by Leonard Makowka, in a patient with fulminant hepatitis. The patient underwent preoperative plasmapheresis to remove

circulating xenoantibodies and the porcine liver graft was placed in a heterotopic position. The pig liver was rejected in few hours and the patient died before a human liver became available^[45].

Significant roadblocks, such as the immunologic hurdle of cross species transplantation and transmission of infections, particularly endogenous retroviruses, need to be overcome before pig organ xenotransplantation can become a clinical reality. Furthermore, the physiological impact of xenotransplantation remains unclear as shown by the immunogenicity and uncertain physiological functioning of pig proteins in the maintenance of homeostasis.

Progress is being made in this difficult field of transplantation as shown by the report of encouraging outcome of pig hepatocyte xenotransplantation with the benefit of the lack of an acute humoral xenograft rejection, the immediate restoration of the liver function and the resistance to specific human viruses^[46]. Are equally encouraging the results of xenotransplantation of encapsulated pig islet cells with the aim to prevent antibody or T-cell contact with islets, allowing though insulin to reach the systemic circulation^[47]. However, this xenotransplantation remains illegal at this time.

CELLULAR TRANSPLANTS

Cellular transplants are being used in a very limited number of indications, such as use of pancreatic islets for diabetes mellitus^[48] and hepatocytes for some metabolic liver diseases and parenchymal liver diseases^[49]. The Collaborative Islet Transplant Registry reports that the majority of islet transplant procedures are performed in the islet transplantation alone setting, with Islet-after-Kidney-Transplantation representing only a small fraction of all islet transplants performed^[50].

Islet transplantation is limited to a highly selected group of patients with type 1 diabetes (T1DM) and aims to achieve adequate glycemic control and removal of need for exogenous insulin^[48] (Table 2). Islet transplantation is associated with short-term benefits of insulin independence, normal or near normal HbA1C levels, sustained marked decrease in severe hypoglycemic episodes and a return of hypoglycemia awareness. However the long-term efficacy is disappointing. In 2006, an international, multicenter trial reported on 36 subjects with T1DM; insulin independence with adequate glycemic control at 1 year after the final transplantation was achieved in only 44% of patients and 31% remained insulin-independent at 2 years^[51]. Follow-up of a larger cohort of 65 patients showed that insulin independence was achieved in 69% at 1 year, 37% at 2 years and only 7.5% at 5 years^[52].

A successful islet transplantation can effectively reverse and stabilize the risk of secondary diabetic complications. The Vancouver group compared islet recipients to best medical therapy and found that islet transplanta-

Table 2 Conditions where human cell transplants have been used clinically

	Indications	Ref.
Islet transplantation	Patients with type 1 diabetes with severe glycaemic lability, recurrent hypoglycemia, and a reduced hypoglycaemia awareness	Shapiro <i>et al</i> ^[84]
		Shapiro <i>et al</i> ^[51]
		Ryan <i>et al</i> ^[52]
		Matsumoto <i>et al</i> ^[54]
		Strom <i>et al</i> ^[57]
Hepatocyte transplantation	Inherited metabolic disorders: Familial hypercholesterolemia Urea cycle deficit α 1 antitrypsin-deficiency Glycogen storage dz 1a Infantile refsum's dz Factor VII deficiency Crigler-Najar type 1 syndrome	Grossman <i>et al</i> ^[86]
		Strom <i>et al</i> ^[87]
		Horlsen <i>et al</i> ^[88]
		Mitry <i>et al</i> ^[89]
		Fox <i>et al</i> ^[90]
		Muraca <i>et al</i> ^[91]
	Progressive familial intrahepatic cholestasis Chronic liver failure (Child A-C) Acute liver failure (as bridge to transplant)	Sokal <i>et al</i> ^[92]
		Dhawn <i>et al</i> ^[93]
		Hughes <i>et al</i> ^[62]
		Ambrosino <i>et al</i> ^[94]
		Sterling <i>et al</i> ^[61]
		Mito <i>et al</i> ^[60]
Strom <i>et al</i> ^[95]		
Strom <i>et al</i> ^[57]		
Sterling <i>et al</i> ^[61]		
Habibullah <i>et al</i> ^[96]		
Bilir <i>et al</i> ^[97]		

tion had a better impact upon risk of secondary complication^[53].

In contrast to the time consuming and expensive islet isolation from donor pancreas, islet transplantation is minimally invasive and carries a low morbidity and mortality compared to whole pancreas transplantation because the islets can be infused percutaneously into the hepatic portal vein. However, its use is limited because of the shortage of high quality donor pancreases, the high cost of the isolation procedure, the maintenance of a specialised human islet isolation laboratory, the need for life-long immunosuppression and the need of multiple organs to obtain enough islets. The ability to achieve single-donor islet transplantation would provide many more islet grafts and also increase the number of recipients with T1DM. Although Matsumoto *et al*^[54] recently published a protocol describing successful single-donor islet transplantation, further studies are needed to confirm the benefit of this protocol. Recipients of IT are also exposed to a wide range of human leukocyte antigens from multiple donors due to repeated islet infusions, which are still, in many units, matched for ABO blood group only. The development of antibodies may be an important issue in end stage diabetic patients also requiring a kidney transplant, as their appearance possibly limits the chance to find a compatible kidney^[55]. It may therefore be difficult to resolve the competing requirements of islet and renal transplantations.

The research in hepatocyte transplantation (HT) has shown encouraging results in particular in the treatment of some inherited metabolic disorders, and have raised expectations for a new therapeutic approach as a pos-

sible alternative to LT (Table 2)^[56]. About 30 patients had HT for liver-based inborn errors of metabolism; the main indication was urea cycle defects. About 5% of newly formed, exogenous-derived, cells natively expressing the gene involved in the disease, suffice to significantly alleviate the consequences of many congenital liver metabolic diseases^[57]. The procedure seems to be safe and results are encouraging; however, as seen in the field of islet transplantation, cell function often declines within 1 year ending finally up in LT.

A new approach of gene targeting technology has been recently proposed with the potential of combining human induced pluripotent stem cells with genetic correction to generate clinically relevant cells for autologous cell-based therapies for the treatment of some inherited metabolic disorders^[58].

HT in patients with chronic liver failure has been associated with dismal results^[59-63]. This might be related, at least in part, to the presence of fibrosis, which may impair cell engraftment in the liver^[62]. HT has also been applied in patients with acute and acute-on-chronic liver failure, in the former with the aim to support the liver function as a bridge to LT. More than 40 patients with acute liver failure have been treated worldwide; a part from an improvement in bilirubin levels and hepatic encephalopathy, there was no significant impact on outcome^[59]. Two large clinical trials assessed the effectiveness of bio-artificial liver devices including both a biological component and an artificial scaffold, and both failed to have a beneficial effect on survival^[63,64].

HT is also limited by the availability of livers for cell isolation. The recipient's cirrhotic liver as a source for donor hepatocytes, would be an easily available option, but it is uncertain whether these cells behave *in vivo* as they do in culture.

Establishing banks of cryopreserved hepatocytes is challenging, because isolated liver cells are very sensitive to damage. Furthermore, cryopreserved hepatocytes engraft less efficiently than freshly-isolated cells. Another major issue is their time-limited efficacy, which may limit the use of HT as a bridge to LT. In contrast, the use of stem/precursor cells is associated with relatively easy procurement and as they proliferate well *in vitro*, such cells may provide sufficient cell mass available for transplantation. A further issue in this field is related to the mechanism of parenchymal integration and repopulation of exogenous hepatocytes^[65]. "Making space" is a prerequisite in order to provide an initial regenerative stimuli; partial hepatectomy, partial embolization and irradiation of the liver all represent effective regenerative stimuli in animal models, but their efficacy in humans requires further investigation. Moreover, the exogenous cells should have a proliferative advantage with respect to autologous liver parenchymal cells; in order to proliferate some authors suggest that this problem may be overcome with the use of foetal hepatic progenitors cells that exhibit an intrinsic biological advantage^[66].

REGENERATIVE MEDICINE AND TISSUE ENGINEERING

The manufacturing of bioengineered organs in the laboratory starting off from autologous differentiated cells and/or stem cells is undoubtedly ground-breaking and exciting for the transplant community. As there will always be more potential recipients than donors, many researchers are working in the field of artificial tissue engineering (TE) and regenerative medicine (RM).

In 2006, Atala *et al.*^[67] of the Wake Forest Institute for Regenerative Medicine in Wiston-Salem, North Carolina, implanted bladders engineered *ex vivo* from the seeding of autologous cells onto artificial supporting scaffolds; in 2008 the same group implanted a trachea manufactured from human components^[68]. However, solid organs with lots of blood vessels, such as liver, pancreas and bowel are harder to grow.

Progresses in the development of clinically feasible liver TE approaches, has been hampered mainly by insufficient cell-to-cell contact of the engrafted hepatocytes. Ohashi *et al.* developed a method of cell sheet technology to engineer a uniformly continuous sheet of hepatic tissue using isolated primary hepatocytes cultured on temperature-responsive surfaces. Sheets of hepatic tissue transplanted into the subcutaneous space resulted in efficient engraftment to the surrounding cells, with the formation of two-dimensional hepatic tissues that stably persisted for longer than 200 d and showed several characteristics of liver-specific functionality.

Decellularization-recellularization technology has done steps forward in order to manufacture liver organoids. Uygun *et al.*^[69] decellularized rat livers and repopulated them with rat primary hepatocytes, showing hepatic function. Atala's group implemented the technique by bioengineering livers with human cells^[70,71].

Extracellular matrix (ECM) scaffolds might provide the desired natural environment to enhance current cell-based approaches aimed at producing large quantities of functional pancreatic endocrine cells^[72,73]. However, compared to islet transplantation, whole organ transplantation using ECM scaffolds is clearly more invasive, requiring revascularization and possibly even exocrine drainage.

Primary hurdles to intestinal bioengineering are the functional regeneration of diverse motility patterns and the complexity of intestinal anatomy.

The implantation of organoid units of intestine has been successful in rats and pigs; however, this technology is time consuming and expensive, as several centimetres of bowel are needed to obtain a sufficient number of organoid units able to repopulate just a few centimetres of engineered intestine. Moreover, organoid units cannot be cultured and grown easily *in vitro*^[74,75]. Decellularization-recellularization technology has been used to engineer less complex gut structures such as the esophagus. A 10-cm segment of porcine jejunum was decellularized

and repopulated with autologous cells. After maturation, the construct was implanted in the arm of a patient suffering from a major esophago-tracheal defect and retrieved after 7 d; the construct showed patent vessels and viable cells, showing that sustained the implantation^[76].

The attraction of a failing organ being replaced by a bioengineered organ generated from a decellularized scaffold and seeded with autologous stem cells is obvious but not without limitations. It will take time to isolate and grow such organs or sufficient cells to provide adequate function so this approach would not be an option for those with acute liver failure where liver replacement is required within some days and liver assist devices have yet to demonstrate an effective role. Similar time constraints may also preclude the use of such organs in patients with primary liver cancer. The use of autologous stem cells, able to differentiate into the kind of required cells and with the potential to expand without limitation, may resolve the issue of rejection and long-term immunosuppression requirement, even though very little is understood regarding the host immune response to bio-engineered constructs.

However, organs derived from autologous stem cells may be subject to the same risk of damage from virus or immune mechanisms that result in the failure of the native organ. Recurrent HCV allograft infection remains a major cause of graft failure despite major advances in antiviral treatments and recurrent autoimmune diseases such as diabetes or autoimmune hepatitis, contribute to graft loss despite associated immunosuppression. Without genetic modification, autologous stem cell derived organs or cells will not correct the disease resulting from metabolic conditions that result in cirrhosis and so leading to organ failure, such as is seen in Wilson's disease or tyrosinosis. Organs or cells derived from allogeneic stem cells would provide an alternative to deceased donor organs and so mitigate against the organ shortage and allow greater access to life-saving care. The application of bone marrow-derived stem cells has shown good results in small groups of patients however in the absence of control groups^[71,72,77,78].

In addition to supplementing or replacing the traditional abdominal organs from living or deceased donors, RM could be of help in other patient groups such as those with inflammatory bowel disease and selected small bowel disease refractory to treatment. Non-responding patients frequently require surgery to control symptoms, although such interventions will not necessarily resolve their disease. An approach based on new findings of RM could drive major changes in management and treatment of such diseases. RM therapy can become effective either in repairing damaged intestinal tissue and correcting immunological underlying disorders. Therefore, RM should not only be considered as a potential therapy for patients with inflammatory disease refractory to standard medical and biological therapy but, hopefully, as a curative treatment that allows achievement of long-lasting

remission.

RM could also have a great impact in the area of gastrointestinal motility disorders, particularly those associated with the aganglionic gut or Hirschsprung's disease and other congenital or acquired enteric nervous system disorders, and may obviate the need for surgical therapies that, although life-saving, are associated with an unsatisfactory long-term prognosis for many.

RM and TE have the enormous potential to help not only those patients who would otherwise be candidates for liver, pancreas or bowel transplantation but also those who would not, under current restrictions, be eligible for listing. Despite promising pre-clinical results^[69,73-75,79-83], many critical aspects of cell therapy and TE need to be further addressed, including long-term safety, tolerability and efficacy in the clinical setting, and last but not least the development of an European Medicines Agency/Food And Drug Administration approved product, before they become protagonists of a new scientific era. Doubts whether such aspirations can be fulfilled in the near future remain, despite the significant advances already made, uncertain. Lessons from other technologies, such as gene therapy, suggest that expectations must be managed carefully.

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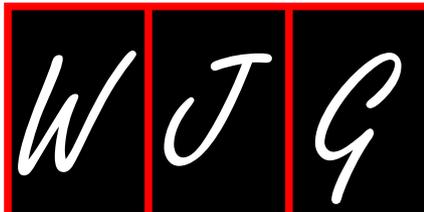
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Tissue engineering for neuromuscular disorders of the gastrointestinal tract

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Abstract

The digestive tract is designed for the optimal processing of food that nourishes all organ systems. The esophagus, stomach, small bowel, and colon are sophisticated neuromuscular tubes with specialized sphincters that transport ingested food-stuffs from one region to another. Peristaltic contractions move ingested solids and liquids from the esophagus into the stomach; the stomach mixes the ingested nutrients into chyme and empties chyme from the stomach into the duodenum. The to-and-fro movement of the small bowel maximizes absorption of fat, protein, and carbohydrates. Peristaltic contractions are necessary for colon function and defecation.

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NEUROMUSCULAR FUNCTION OF THE GUT: NEUROMUSCULAR TUBES AND SPHINCTERS

The digestive tract is designed for the optimal processing of food that nourishes all organ systems. The esophagus, stomach, small bowel, and colon are sophisticated neuromuscular tubes with specialized sphincters that transport ingested food-stuffs from one region to another. Peristaltic contractions move ingested solids and liquids from the esophagus into the stomach^[1]; the stomach mixes the ingested nutrients into chyme and empties chyme from the stomach into the duodenum^[2]. The to-and-fro movement of the small bowel maximizes absorption of fat, protein, and carbohydrates^[3]. Peristaltic contractions are necessary for colon function and defecation^[4].

Sphincters of the digestive tract, on the other hand, have intrinsic muscle tone that produces sustained (tonic) pressure zones. The sphincteric pressures regulate movement of luminal content produced by the peristaltic contractions. Relaxation of the lower esophageal sphincter (LES), for example, allows the entry of esophageal content into the stomach^[5]; contraction and relaxation of the pyloric sphincter regulates movement of gastric content

into the duodenum^[6]. The ileocecal valve regulates flow of content from the ileum into the cecum^[7]. The internal anal sphincter (IAS) regulates the elimination of rectal-sigmoid content^[8]. Sphincters not only provide resistance to flow in the areas distal to the sphincter but also limit the retrograde movement of intraluminal content into the areas proximal to the sphincter. Thus, normal neuromuscular function of the esophagus, stomach, small bowel, and colon requires the coordination of the tubular neuromuscular structures and the relevant sphincters. Symptoms such as dysphagia, nausea and vomiting, diarrhea, constipation, or incontinence may occur if neuromuscular function of the esophagus, stomach, intestine, and colon is disturbed, resulting in neuromuscular disorders such as achalasia, gastroesophageal reflux, gastroparesis, intestinal pseudo-obstruction, colonic inertia, and fecal incontinence, respectfully^[1-4,9,10].

The wall of gastrointestinal tract organs contains circular and longitudinal smooth muscle layers, the enteric nervous system including the myenteric plexus, and the interstitial cells of Cajal (ICCs) which control the rhythmicity of contractions^[11,12], all of which are targets for tissue bioengineering^[13]. The gastrointestinal tract wall is innervated by vagal afferent and efferent fibers and inputs from the sympathetic nervous system. Thus, neuromuscular disorders of the gastrointestinal tract may involve damage to the smooth muscle, enteric nerves, ICCs, extrinsic neurons, or all cell types. Neuromuscular diseases such as achalasia, gastroparesis, intestinal pseudo-obstruction, and colonic inertia represent the most severe forms of neuromuscular diseases of the gastrointestinal (GI) tract^[14-17]. These disorders are very difficult to treat, and very few drugs are designed to improve GI neuromuscular function. Table 1 shows a summary of the human neuromuscular disorders from selected areas of the GI tract, the key neuromuscular abnormalities, the clinical neuromuscular diagnosis, and the medical and surgical treatments currently available for these diseases.

In the most severe neuromuscular diseases, surgery is often required to improve symptoms. For example, a myotomy of the LES may be necessary to treat severe dysphagia due to achalasia. Partial resection of the colon and pull-through operations may also be necessary to treat drug-refractory, severe constipation or marked colonic dilation. Colectomy may be needed to correct aganglionic segments of colon as seen in Hirschsprung's disease. While a surgical approach may relieve one set of symptoms, the consequence is often secondary neuromuscular disorders. After myotomy is performed for achalasia, for example, gastroesophageal reflux may occur and require drug treatment or fundoplication. Problems with fecal incontinence may also occur after colonic resection.

In this review, the normal neuromuscular function and disorders of neuromuscular function of the digestive organs in humans are discussed. We will focus on advances in regenerative medicine as an innovative and potential cure for GI neuromuscular diseases. The goal of this novel technology is restoration of the neuromus-

cular function of the bowel wall. A regenerative medicine approach aims to bioengineer "functional" circular and longitudinal smooth muscle, enteric neurons, ICCs, and mucosa in the correct anatomical configurations in order to produce normal physiologic gut functions. GI neuromuscular disorders are particularly suited to regenerative medicine approaches because drug and surgical therapies are extremely limited.

ESOPHAGUS: NEUROMUSCULAR FUNCTION AND DYSFUNCTION

The esophagus is a neuromuscular tube with an upper esophageal sphincter formed by the cricopharyngeus muscle, the esophageal body, and the lower esophageal sphincter at the esophagogastric junction. Peristaltic waves in the esophageal body move ingested solid and liquid foods from the proximal esophagus to the distal esophagus and into the stomach during relaxation of the lower esophageal sphincter (The upper esophageal sphincter is striated muscle and disorders will not be addressed). Neuromuscular disorders of the esophagus involve the esophageal body, the lower esophageal sphincter, or both and are listed on Table 1. For example, peristaltic esophageal contractions with abnormally high amplitudes are associated with chest pain and dysphagia and are termed the "nutcracker esophagus"^[18]. On the other hand, low amplitude and simultaneous contractions of the esophageal body are associated with achalasia and scleroderma^[19]. High amplitude contractions of the nutcracker esophagus may be treated with nitrates, but there are no medical or surgical treatments for low-amplitude simultaneous contractions of the esophageal body.

Neuromuscular disorders also occur in the LES. Increased LES pressure and decreased LES relaxation after swallows are the key neuromuscular features of achalasia^[19]. The hypertensive lower esophageal sphincter may be treated with nitrates, Botox injections, balloon dilation, or surgical myotomy. In contrast, a low amplitude LES pressure is associated with gastroesophageal reflux. There are no specific drug treatments available to increase LES pressure, but radiofrequency ablation^[20] and augmentation of the LES are device-related treatments that increase LES pressure^[21].

Regenerative medicine methods to produce patches or segmental portions of the esophageal wall have been studied in dogs. In a study by Nakase *et al.*^[22], keratinocytes and fibroblasts (KF+ group) were cultured on human amniotic membrane opposed to smooth muscle tissue on a polyglycolic acid scaffold which was rolled around a polypropylene tube. Three weeks after implantation of this construct in the omentum, the KF (+) constructs had developed into tubes with stratified squamous epithelium and smooth muscle-like tissue. The KF (-) constructs lacked keratinocytes and fibroblast layers and developed luminal obstructions 2-3 wk after implantation into the esophagus. On the other hand, the 3 cm

Table 1 Neuromuscular disorders of the gastrointestinal tract, current treatments and future regenerative medicine approaches

	Disorder	Neuromuscular diagnosis	Medical treatment	Surgical treatment
Esophagus				
Body	↑ Contraction	Nutcracker	Nitrates	Myotomy
	↓ Contraction	Scleroderma	None	None
Lower esophageal sphincter	↑ Contraction	Achalasia	Botox	Myotomy
	↓ Relaxation	GERD	Nitrates	Fundoplication, RFA
	↓ Contraction		None	
Stomach				
Fundus	Failure of relaxation	Fundic dysfunction	None	None
	Excess relaxation	Fundic dysfunction	None	None
Body/antrum	↓ Contraction	Gastroparesis	Prokinetics	None
	↑ Contraction	Dumping syndrome	Anticholinergic drugs	Reverse small bowel segment
Pylorus	↑ Contraction	Pylorospasm	Botox	Pyloroplasty
	↓ Contraction	Dumping syndrome	Anticholinergic drugs	Reverse small bowel segment
Small intestine				
Jejunum/ileum	↑ Contraction	Obstruction	None	Surgical resection
	↓ Contraction	CIPO	Prokinetics/antibiotics/STN	None
Ileocecal valve (sphincter)	↑ Contraction	Obstruction	None	Surgical resection
	↓ Contraction	Backwash ileitis	antibiotics	None
Colon				
Ascending/transverse	↑ Contraction	IBS	Fiber, anticholinergic drugs	None
Sigmoid/rectum	↓ Contraction	Colonic inertia	Laxatives, lubiprostone	Colectomy
Anal sphincter (internal)	↑ Contraction	Anal outlet obstruction	Laxatives, botox	Myotomy
	↓ Contraction	Fecal incontinence	Fiber, injection	Sphincteroplasty Sacral nerve stimulation

↑: Increase; ↓: Decrease; CIPO: Chronic intestinal pseudo-obstruction; GERD: Gastroesophageal reflux disease; IBS: Irritable bowel syndrome; RFA: Radiofrequency ablation; STN: Somatostatin.

length KF (+) constructs showed distensibility, but no peristaltic contractions, when implanted in the esophagus. Shrinkage of the keratinocytes and smooth muscle also resulted in a shorter segment of restored esophagus that was durable up to 420 d in some dogs. Two of the six KF (+) dogs eventually developed esophageal strictures. Scar formation and shrinkage of tissue engineered oral mucosa epithelial sheets were also major problems in bioengineered esophageal constructs^[22].

Takimoto *et al.*^[23] used 5 cm silicone tubes to shape a collagen sponge matrix into an artificial esophagus. The construct was implanted in 43 dogs. Strictures occurred in 27 dogs if the Teflon support tube was removed 2-3 wk after implantation; but, the regenerated esophageal tissue replaced the esophageal defect if the stent was removed 4 wk after implantation. These regenerated tissues showed stratified epithelium, glands, and striated muscle, suggesting that cervical esophageal muscle had grown into the implant area, not smooth muscle of the esophageal body.

Regenerative medicine approaches to bioengineering the LES have been reported. In canine studies, the baseline LES pressure increased after skeletal muscle derived stem cells were injected into the LES area. The cells integrated within the native GI smooth muscle, but no differentiation of the cells into smooth muscle genotypes was noted^[24]. Regenerated lower esophageal sphincters need adequate myogenic tone to prevent gastroesophageal reflux, but also need to relax in response to swallows and the arrival of food boluses in the distal esophageal body. Furthermore, the LES must relax in response to

distension of the fundus to allow physiological venting of stomach air. At this time, functioning neuromuscular constructs to restore animal or human esophageal body or sphincter functions are not available.

STOMACH: NEUROMUSCULAR FUNCTION AND DYSFUNCTION

Fundus and corpus-antrum

The key functional neuromuscular regions of the stomach are the fundus, body/antrum, and pylorus. In healthy subjects, the fundus relaxes through vagal mediated nitric oxide pathways in response to swallowing and in response to solids and liquids entering the gastric fundus from the esophagus^[2]. Ingested solids are triturated by the corpus/antrum until appropriate particle size is achieved. In humans, peristaltic activities are controlled by 3 cycle per minute gastric myoelectric activity or gastric slow waves^[2]. The triturated food is termed “chyme” and is emptied through the pylorus into the duodenum by body/antral peristaltic contractions.

Patients with paralysis of the stomach (or gastroparesis) may have neuromuscular dysfunction of the fundus, body/antrum, pylorus, or all of these areas. Symptoms of gastroparesis include early satiety, prolonged fullness, nausea, and the vomiting of undigested food^[25]. Table 1 summarizes major gastric neuromuscular defects and the associated clinical diagnoses and treatments in gastroparesis. In patients with gastroparesis, the fundus fails to relax appropriately in response to ingestion of meals;

other patients have excessive relaxation of the fundus. Abnormalities of fundic relaxation affect the ability of the stomach to accommodate ingested food. There are no established medical or surgical treatments for fundic dysfunction.

Low amplitude contractions of the corpus and antrum and abnormalities of gastric slow waves (e.g., presence of tachygastrias) results in disturbed peristalsis and gastroparesis^[2,26]. There are few medications available to treat gastroparesis^[27]. Gastric electrical stimulation is a device used to treatment for the nausea and vomiting symptoms of severe gastroparesis^[28].

Regenerative medicine approaches for the development of gastric tissues are evolving. Araki *et al.*^[29] implanted a 5 cm collagen scaffold with silicone layers on the mucosa side and fabric on the serosa side. The scaffold was soaked in blood or bone marrow aspirate before implantation. Seven dogs were implanted. During the 16-wk study, the construct developed ulcers that healed, showed 59%-77% shrinkage in length, and developed alpha-smooth muscle actin positive cells, but no calponin staining cells. Maemura *et al.*^[30] used rat stomach epithelium organoid cells, a preparation of minced rat stomach wall that was centrifuged, washed, and placed onto a tubular mesh. The organoid cells were wrapped in omentum *in vivo*, harvested after 3 wk, and used to replace the resected stomach in the same rat. Barium studies showed emptying from the neo-stomach, but weight in these rats was no better than rats with total gastrectomy. Histology showed neo-mucosa and smooth muscle orientation similar to the normal stomach. In the study by Hori *et al.*^[31], mucosal and smooth muscle layers of the canine stomach were bioengineered, but the construct exhibited no contractions. Ultimately, bioengineered constructs need to restore peristaltic contractions in the corpus/antrum for restoring gastric mixing and emptying of food.

Pylorus

On the other hand, dumping syndrome results when ingested foods are emptied exceedingly rapidly from the stomach into the duodenum^[32]. Increased antral contractions or decreased pyloric resistance to emptying are putative underlying mechanisms of dumping syndrome. Dumping syndrome may develop after resection of the antrum-pylorus for treatment of ulcers or cancer. Thus, pyloric function affects gastric emptying rates. Hypotensive pylorus is another mechanism that may underlie the dumping syndrome. There are no medical approaches to increasing pyloric pressure at this time. On the other hand, a hypertensive pyloric sphincter which does not relax (example: pylorospasm) is associated with gastroparesis^[33]. Medical approaches to the treatment of pylorospasm include injection of Botox or balloon dilation of the pylorus^[34]. In some patients, a myotomy may be performed to reduce pyloric pressure and increase gastric emptying.

Micci *et al.*^[35] described transplantation of neural stem cells to the pylorus and results showed improved

relaxation of pylorus muscle strips and improved gastric emptying in neural nitric oxide synthetase (nNOS) deficient mice. Results suggested the stem cells restored nNOS function regarding relaxation of the pylorus. The pylorus is a target for stem cell and regenerative medicine treatments. At this time, the bioengineered stomach and pylorus have not reached development for clinical deployment.

SMALL INTESTINE: NEUROMUSCULAR FUNCTION AND DYSFUNCTION

The small intestine is a hollow neuromuscular tube that comprises the duodenum, jejunum, and ileum^[36]. In the small intestine, chyme is broken down into smaller nutritional fragments: amino acids, carbohydrates, and fatty acids. Normal small intestinal motility and digestive enzymes from the pancreas and bile from the gallbladder are needed for normal digestion and absorption of nutrients. The ileum has special features for absorption of bile salts^[37]. The ileocecal valve is positioned between the distal ileum and the cecum, controls emptying of ileal effluent into the cecum, and prevents the reflux of cecal contents into the ileum. The regulation of this process is poorly understood because the ileocecal valve area is difficult to study in humans. Normal small intestinal motility includes phase 3, the interdigestive migrating motor complex, which consists of strong peristaltic contractions that move distally from the duodenum to the ileum in a cycle every 90 min during fasting^[38]. These contractions transport indigestible fibers from the small bowel into the colon.

Neuromuscular disorders of the small intestine are uncommon in adults. Scleroderma is associated with low amplitude, poorly organized small intestinal contractions and idiopathic chronic intestinal pseudo-obstruction involves disorders of the enteric nervous system and smooth muscle of the small bowel. In disorders with poor small intestinal peristaltic contractions, as seen in chronic intestinal pseudo-obstruction, bacterial overgrowth of the small bowel occurs and leads to malabsorption and diarrhea. There are no treatments for hypocontractility of the jejunum and ileum; however, small bowel bacterial overgrowth is treated with antibiotics and somatostatin which may increase the incidence of phase 3 migrating motor complexity. Jejunal diverticula can be resected surgically if they are thought to be the cause of symptomatic bacterial overgrowth. Short bowel syndromes occur in adults when the jejunum is resected due to ischemia and in neonates with severe enterocolitis that required resection^[39].

In regards to the ileocecal valve, a narrow nipple-like structure is positioned between the distal ileum and the cecum^[7]. Specific neuromuscular disorders have not been described. Mechanical obstructions due to tumors such as adenocarcinoma of the ileum or cecum may cause ileal obstruction and are treated with appropriate operations. Dysfunction or loss of the ileocecal valve may lead to

“backwash ileitis”.

Regenerative medicine approaches to small bowel neuromuscular diseases include studies to increase the length of the small bowel to treat short bowel syndromes. Kim *et al*^[40] used polyglycolic tubes seeded with intestinal fragments (epithelial organoid units) which were implanted in the omentum of rats. These constructs developed into neointestinal cysts that were well vascularized, had a neomucosa with crypts, and some areas had smooth muscle^[40]. Anastomosis of the constructs with native small intestine was successful and no stenoses or obstructions developed^[41].

The small intestinal submucosa (SIS) was used to form a scaffold for bioengineered small intestine in dogs^[42]. SIS sheets were wrapped around glass tubes to form segments which were used to patch defects, approximately 7 cm × 3 cm in size, or for tubular implants in the small intestine. Most dogs with tubular implants had obstructions, anastomotic leaks, or appeared ill. Dogs with patch implants survived, but the implants shrunk approximately 35% in size, the mucosal layers were not well organized, and the amount of smooth muscle was variable or absent. Hori *et al*^[43] used autologous mesenchymal stem cells (MSC) from bone marrow seeded onto a collagen scaffold to induce smooth muscles to regenerate small intestine. A 5 cm area was resected and replaced with scaffold and a silicone tube stent to prevent contraction and stenosis. All six dogs survived. Although mucosa developed, no smooth muscle layers regenerated. Grikscheit *et al*^[44] used intestinal organoid units loaded into polymer tubes to form intestine-like segments. The units were implanted into the omentum. Four weeks later, small bowel resections were performed and the regenerated cysts and the native small bowel were connected with anastomoses. The regenerated cysts contained villi, crypts, ganglion cells, and some muscularis. Transit time was 1825 min in animals with regenerated cysts *vs* 982 min in those without implants, but weight gain was better in the former group. Nakase *et al*^[45] seeded a collagen scaffold with autologous smooth muscle cells isolated from the canine stomach. After implantation in the ileum, mucosal villi and circular smooth muscle cells developed in orderly alignment at 12 wk in animals with ileal reanastomosis at 8 wk after implantation. Scaffolds coated with basic fibroblast growth factor enhanced smooth muscle growth and angiogenesis, but distinct smooth muscle layers and contractions were not seen in these constructs^[46]. Some smooth muscle cells differentiated into fibroblast-like cells. More recently, Koga *et al*^[47] showed that the porcine small bowel was successfully lengthened with a hydraulic-lengthening device. The elongated jejunum retained near normal motility and absorption when implanted in the native jejunum.

No studies using human tissue have been reported for small bowel regeneration, although there is an extensive clinical need for small intestine segments as therapies for short gut syndromes. In adults with short bowel syndrome, increased length of small bowel would help

ongoing problems with malabsorption and dehydration.

COLON: NEUROMUSCULAR FUNCTION AND DYSFUNCTION

The anatomical regions of the colon include the cecum, ascending colon, transverse colon, descending colon, sigmoid colon, and anorectum. The cecum and ascending colon receives up to 1.5 L of ileal effluent each day and absorbs 90% of the effluent. Subsequently, approximately 200 g of semi-solid stool are normally eliminated by defecation each day. Evacuation of the rectum requires coordination between peristaltic waves in the rectal-sigmoid area of the colon and relaxation of the anal sphincter and the pelvic floor muscles^[4,8].

Neuromuscular diseases related to the colon include irritable bowel syndrome in which increased and decreased contractility of the colon produces alternating constipation and diarrhea. Treatments for irritable bowel syndrome are confined to fiber supplements, cholinergic agents, and tricyclic antidepressants. There are no surgical options for irritable bowel syndrome. Colonic inertia is a disorder with weak contractions throughout the colon resulting in severe constipation. For patients with colonic inertia, there are very few medications other than laxatives and lubiprostone. In severe cases of colonic inertia, a colectomy may be performed. The colectomy (with ileo-sigmoid or ileo-rectal anastomosis) often results in loose stools four to six times per day. In some patients, the recto-sigmoid segment has significant neuromuscular dysfunction and constipation persists despite the colectomy^[48,49]. Similarly, functional constipation with associated fecal incontinence, often due to colonic dilation and megarectum, generally responds poorly to medical management. Surgical approaches such as cecostomy or appendicostomy (ACE) have been widely used in the treatment of refractory constipation. However, some patients are unable to have a bowel movement on their own despite the use of the cecostomy/ACE, while others demonstrate recurrent symptoms after discontinuation of antegrade enemas^[50]. When this occurs, further surgical intervention including placement of a diverting colostomy or colonic resection becomes an option^[51].

Regenerative medicine approaches to the colon have been explored in rats. A bioengineered colon segment, colonic mucosa, and smooth muscle was produced by seeding colon organoid units from autologous sigmoid colon onto polymer scaffolds. After implantation for 12 wk, the regenerated cysts showed correct colon micro-architecture^[52]. Hecker *et al*^[53] bioengineered colon tissues with normal three dimensional smooth muscle orientation around a central lumen; the tissue exhibited peristalsis. These experiments suggest that multiple smooth muscle rings may be an alternative method compared to regenerating long segments of the colon organ itself. There is much work remaining to be done to prepare the way for human studies related to the regeneration of the colon.

IAS: NEUROMUSCULAR FUNCTION AND DYSFUNCTION

The IAS is a smooth muscle that is under anonomic nervous system control, whereas the external sphincter is striated muscle under voluntary control^[54]. A hypertensive IAS with poor relaxation results in rectal outlet obstruction associated with severe constipation^[4,8]. Medical treatments include physical therapy, laxatives, Botox injections, and myotomy of the sphincter^[55]. On the other hand, a hypotensive or weak anal sphincter results in fecal incontinence. Fecal incontinence results in significant psychosocial issues and poor quality of life. Treatments of fecal incontinence include stool bulking agents, sphincteroplasty, injection of the sphincter with bulking agents, and sacral nerve stimulation^[56].

Neuronal disorders of the gut such as Intestinal Neuronal Dysplasia and Hirschsprung's disease involve a developmental or degenerative loss of enteric neurons^[57]. Aganglionosis results in serious motility problems due to lack of adequate peristalsis. Symptoms include painful abdominal distention, stool retention, and fecal incontinence. Currently, surgical excision of the aganglionic segment is the most effective treatment strategy. Despite technical advances in surgery, the complications in Hirschsprung's patients after corrective surgery include fecal incontinence (10%-16%) and enterocolitis (10%)^[58,59].

Considerable progress has been made using regenerative methods to bioengineer an internal anal sphincter that has properly aligned circular smooth muscle, and importantly, neural innervation. Hecker *et al.*^[53] showed that IAS smooth muscle cells in fibrin-based gels developed into three dimensional smooth muscle rings which showed relaxation and contraction in response to 8-bromo-c AMP and acetylcholine, respectively. Somara *et al.*^[60] used human IAS cells to form bioengineered three dimensional smooth muscle rings which exhibited basal myogenic tone that was dependent on prokekin kinase C (PKC) pathways not rho kinase. Bioengineered mouse IAS constructions were grown for 28 d after implantation in the backs of mice. The harvested rings developed basal tone, responded normally to vascularization, had no ICCs, but smooth muscle cells (smooth muscle actin and h-caldesmon positive cells) were present and responded normally to stimulating and relaxing drugs^[61]. Thus, the implanted IAS constructions had traits of the native IAS. Raghavan *et al.*^[62] reported the successful implantation of the bioengineered IAS comprised of human smooth muscle cells and immortalized fetal enteric neurons. The harvested construct, implanted into RAG1-1-mice for 25-28 d developed neovascularization, myogenic tone, and normal contraction and relaxation characteristics in response to testing. These advances in bioengineering the IAS reflect increasing sophistication in combining the enteric nerve and GI smooth muscle components that will be critical in providing treatment for clinical neuromuscular diseases such as fecal incontinence.

CONCLUSION

Neuromuscular disorders of the GI tract are excellent but complex targets for regenerative medicine approaches designed to restore function. In many of these human diseases, the symptoms are severe, but treatments are extremely limited. Many challenges for regenerative medicine approaches remain: identifying sources for cells, construction of scaffolds that result in the proper three-dimensional growth of the selected cells into the desired organ, physiological orientation of the component layers of the wall of the GI organs, and the functional integration of the key cells (smooth muscle, enteric nerves, ICCs). Implantation, integration, and growth of the regenerated patches, grafts, and organs require growth factors, vascularization of the bioengineered tissues, and restoration of neuromuscular function. Many challenges must be overcome to bioengineer functional regions of the GI tract, but the needs and the opportunities are great.

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Liver bioengineering: Current status and future perspectives

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Abstract

The present review aims to illustrate the strategies that are being implemented to regenerate or bioengineer livers for clinical purposes. There are two general pathways to liver bioengineering and regeneration. The first consists of creating a supporting scaffold, either synthetically or by decellularization of human or animal organs, and seeding cells on the scaffold, where they will mature either in bioreactors or *in vivo*. This strategy seems to offer the quickest route to clinical translation, as demonstrated by the development of liver

organoids from rodent livers which were repopulated with organ specific cells of animal and/or human origin. Liver bioengineering has potential for transplantation and for toxicity testing during preclinical drug development. The second possibility is to induce liver regeneration of dead or resected tissue by manipulating cell pathways. In fact, it is well known that the liver has peculiar regenerative potential which allows hepatocyte hyperplasia after amputation of liver volume. Infusion of autologous bone marrow cells, which aids in liver regeneration, into patients was shown to be safe and to improve their clinical condition, but the specific cells responsible for liver regeneration have not yet been determined and the underlying mechanisms remain largely unknown. A complete understanding of the cell pathways and dynamics and of the functioning of liver stem cell niche is necessary for the clinical translation of regenerative medicine strategies. As well, it will be crucial to elucidate the mechanisms through which cells interact with the extracellular matrix, and how this latter supports and drives cell fate.

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Key words: Liver; Regenerative medicine; Tissue engineering; Extracellular matrix; Scaffold; Stem cells

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INTRODUCTION

With the many recent advances in the general area of liver regenerative medicine, there have been a multitude of significant improvements regarding the technology of liver bioengineering and regeneration. The purpose of the present review is to illustrate the two main strategies that are currently being implemented to manufacture liver organoids for clinical purposes.

DECELLULARIZATION-RECELLULARIZATION TECHNOLOGY

Several studies have provided evidence that this technology offers a valuable platform for liver bioengineering through the repopulation of an acellular liver with appropriate fresh cells.

The first report addressed the methodology for the decellularization of rodent livers^[1]. Livers were cannulated through the inferior vena cava, with the portal vein severed and the superior vena cava clamped. The decellularization process began with rinsing of the liver with 100 mL of phosphate buffered saline (PBS) to clear the blood followed by perfusion of three 300 mL isotonic solutions of 1%, 2%, and 3% Triton X-100 at a rate of 5 mL/min. This was followed by perfusion of a 300 mL PBS solution containing 0.1% sodium monododecyl sulfate (SDS) and a 300 mL PBS wash. The disruption of the lipid membranes cleared most of the cellular components of the organ except for intact nuclear cages containing DNA, which was further removed by a solution of SDS. Hematoxylin and eosin staining of the intact decellularized liver showed a fine web of matrix remaining in the acellularized liver, which was further analyzed by immunohistochemical staining of collagen IV and laminin. The stains showed the presence of collagen within the matrix and that laminin was present within the basement membrane of the vessels. After the decellularization process, the scaffold remained intact and strong enough to maintain further cannulation for the perfusion of cells. 10^6 cells of the rat liver progenitor cell line WB344 in Roswell Park Memorial Institute medium were infused into the decellularized liver through the cannulated inferior vena cava. Further histological analysis of the center of the intact recellularized scaffold indicated that the intrahepatic vasculature was able to traffic cells from the inferior vena cava. This report demonstrated the necessary process of using SDS in the decellularization process to truly remove any cellular components, specifically DNA.

Another similar subsequent report that used a similar decellularization method showed vascular patency through portal vein dye^[2]. The decellularization process was performed by sequential perfusion of different concentrations of detergents through the portal vein at a flow rate of 1 mL/min. The livers were perfused for 72 h with SDS in distilled H₂O: 0.01% SDS for 24 h, 0.1% SDS for 24 h, and 1% SDS for 24 h. The livers were then perfused with distilled H₂O for 15 min and with 1%

Triton X-100 for 30 min to cleanse the livers of any remaining SDS. After rinsing the decellularized livers with PBS for 1 h, only the median lobe was sterilized in 0.1% peracetic acid in PBS for 3 h and kept for recellularization after further extensive PBS washing. The decellularized scaffolds were histologically analyzed to demonstrate that the scaffolds were acellular and functionally similar to an intact normal liver, in order for recellularization to be possible. Histological analysis showed that there were no nuclei or cytoplasmic staining in the decellularized liver compared to a normal rat liver. Immunohistochemical analysis of four extracellular matrix (ECM) proteins (collagen type I, collagen type IV, fibronectin and laminin- β 1) showed that the structural and basement membrane components of ECM remained intact similarly to the normal liver. DNA analysis of the decellularized scaffold showed that less than 3% of residual DNA remained after the decellularization process. They also reported intact functional vascular beds and microvasculature through the perfusion of the Allura Red dye. The dye flowed through the vasculature just as expected in a functioning liver. The acellular translucent scaffold was then infused with rat-derived hepatocytes through perfusion of the portal vein at 15 mL/min. The perfusion system consisted of a peristaltic pump, bubble trap, and oxygenator from a donors-after-cardiac-death organ resuscitation perfusion system. They introduced approximately 12.5 million cells during each of the four steps in ten-minute intervals, which showed superior engraftment efficiency when compared to a single-step infusion. The recellularized grafts were maintained in a perfusion chamber for up to 2 wk *in vitro*, with histological staining of the recellularized sections at 4 h, 1 d, 2 d, and 5 d of perfusion. At 4 h, the majority of the cells remained in and around the vessels; however, at 1 d and 2 d, the cells leave the vessels and become distributed throughout the matrix.

It should be emphasized that this is the first report that contains data showing the level of function exhibited by the hepatocytes grown on the decellularized matrix. They report that hepatocyte viability was maintained during culture and that cell death was kept to a minimum. They were also able to determine that the cells migrated beyond the matrix barrier to reach decellularized sinusoidal spaces through scanning electron microscopy (SEM) and histological analysis. They also determined that albumin synthesis was not increased in the recellularized matrix compared to an intact liver; however, urea synthesis was significantly higher in the recellularized liver than the hepatocyte sandwich during culture. The analysis of the expression of drug metabolism enzymes showed that the levels of Cyp2c11, Gstm2, Ugt1a1, and Cyp1a1 that were expressed in the recellularized grafts were similar to those of the sandwich hepatocyte cultures. The recellularized liver grafts were then transplanted into recipient rats that underwent unilateral nephrectomy for auxiliary liver graft transplantation. The recellularized liver grafts were perfused quickly with blood and the appropriate efflux

occurred only after 5 min. The graft was maintained *in vivo* for 8 h, and then harvested for further Tdt-mediated dUTP Nick-End Labeling staining analysis. This staining demonstrated that the cells were minimally damaged and further histological staining showed that the hepatocytes reserved normal morphology and parenchymal positions.

While it is extremely important to have these previous promising reports on liver decellularization and recellularization, the ultimate necessary technology that needs to be expanded upon is the decellularization and recellularization of whole organs—specifically human organs, and subsequently human derived cell lines—in order to create a transplant graft for possible human functioning. The report by Baptista *et al.*³¹ demonstrated the potential for the colonization of human hepatocyte progenitors on a decellularized liver matrix. This is one of the first reports to show the decellularization and recellularization process with a whole liver instead of thin slices or lobes of the liver, as well as the first report to recellularize successfully with human liver cells. They attempted to decellularize whole livers from multiple species as well, including mice, rats, ferrets, rabbits, and adult pigs.

All of the dissected livers were cannulated with different gauged cannulas, depending on the species, through the inferior vena cava and the portal vein, which were then hooked up to a Masterflex peristaltic pump in preparation for decellularization. There was approximately 40 times the volume of the liver perfused with distilled water at a flow rate of 5 mL./min. The decellularization process was performed by perfusion of approximately 50 times the volume of the liver with 1% Triton-X 100/0.1% Ammonium Hydroxide. The approximate perfusion times for the decellularization process were 1 h for mice, 2 h for ferret, 3 h for rat, and 24 h for pig livers. It was visibly clear after the perfusion period that the parenchyma became transparent and the vascular tree was visible under low magnification microscopy (Figure 1).

Spectrophotometric and agarose gel DNA analysis showed the removal of approximately 97% of the DNA from the tissue, indicating efficiency of the decellularization process. SEM was performed to determine that that ultrastructure was preserved. The SEM analysis showed that reticular collagen fibers that support the hepatic tissue were present and the “portal triad” structures remained intact, as well as the lack of any cells. Histological analysis of acellular ECM was performed to further characterize the scaffold composition. The staining showed that there was no cellular nuclear material or any other cellular material present. The staining also showed that collagen layers with vascular channels were present, along with collagen fibers, elastin fibers, and glycosaminoglycans (Figure 2).

Quantification of the ECM components showed higher levels of collagen and glycosaminoglycans in the decellularized scaffold compared to native tissue, which can be explained by the absence of cellular components, while there was no difference in elastin presence. The localization of the specific extracellular matrix proteins

collagen I, collagen III, collagen IV, laminin, and fibronectin were all observed around the vascular structures, specifically denser around the larger vessels, and the parenchymal areas of the acellular liver, as well as the fresh tissue. Vascular preservation and patency was demonstrated by the ability of the network of vascular remnants to retain labeled dextran that had a similar molecular weight to that of blood proteins.

The recellularization methods used in this report show that perfusion through the vena cava or the portal vein (preferred) both allow the green fluorescent protein-labeled MS1 endothelial cells to line the vascular network, including the larger vessels to the capillary sized vessels. Portal vein-seeded endothelial cells were primarily deposited in the periportal regions of the liver lobule while the vena cava-seeded endothelial cells were primarily concentrated in the regions of the central veins and in smaller branches and vessels. Through fluorescent microscopy and transmission electron microscopy they were able to determine that the lumens of the acellular vascular remnants could be colonized by endothelial cells that were able to actively spread and cover the vessel basement membrane while forming appropriate cell-cell junctions. They also determined that the surface of the vascular lumen was non-thrombogenic, which was confirmed by the lower quantification of platelets in the bioscaffold compared to the fresh tissue. The reseeding experiments performed in this report utilized the coseeding of human umbilical vein endothelial cells and freshly isolated human fetal liver cell's, while using similar recellularization protocols previously mentioned. Immunohistochemical analysis was used to assess the proliferation and analyze the presence of hepatocytic lineage markers. Staining of Ki67 to assess proliferation showed a high number of positive cells throughout the bioscaffold, which was 3 times higher than the number of apoptotic cells present. The staining also showed that the hepatocytic markers α -fetoprotein, CYP2A, and CYP3A were expressed in the parenchyma. Cytokeratin 19 was strongly seen throughout the bioscaffold in biliary tubular structures while clusters of albumin-expressing hepatocytes were distributed in the parenchyma. The small amount of co-expression of these specific markers implies that there are specific niches within the bioscaffold for bile duct and hepatocytes. Immunohistochemical staining also detected CK19+/CK18-/ALB-tubular structures and clusters of ALB+/CK18+ cells in the parenchyma, which suggests that the bioscaffold is able to support the differentiation of the fetal hepatoblasts into biliary or hepatocytic lineages. The ability of cells with immunophenotypes consistent with hepatocytes, cholangiocytes, and endothelial cells to form discrete pockets in the bioscaffold suggests that some of the micro-architectural “blueprint” was retained within the scaffold. This suggests that not only does the bioscaffold provide a three-dimensional vascularized scaffold (previously described) but it also retains the necessary environmental cues, further explained by the retention of the glycosaminoglycans that serve as

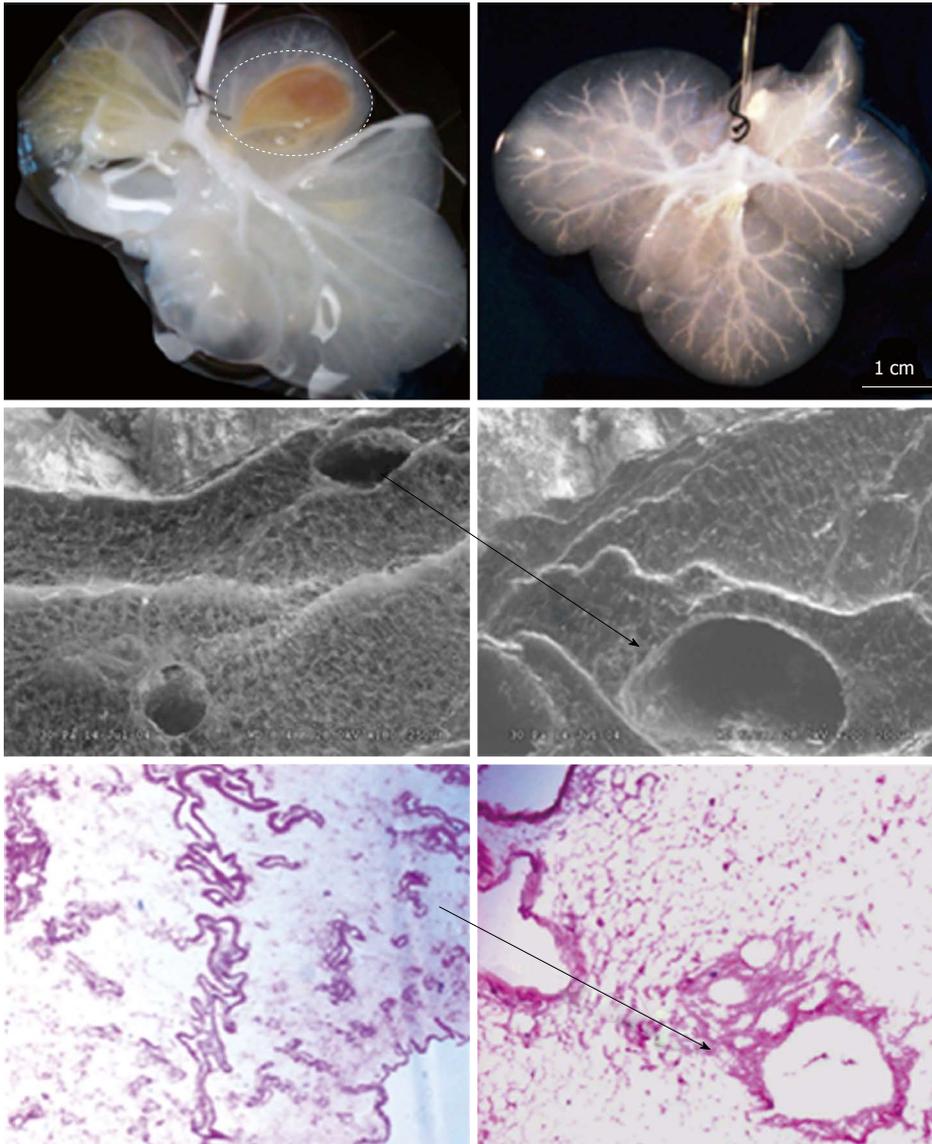


Figure 1 Gross and microscopic anatomy of acellular ferret livers. Upper row: The liver on the left is almost entirely decellularized, however it remains a segment still cellular (interrupted line); on the right, instead, the liver is fully acellular as expression of successful decellularization; Middle row: Scanning electronic microscopic ruling out the presence of any cell remnant and showing the triad completely acellular (arrow); Lower row: Hematoxylin and eosin confirms the lack of cellular element within the remaining liver extracellular matrix (arrow).

active binding sites for growth factors that regulate cell phenotype, for progenitor hepatic and endothelial cells to grow, differentiate, and maintain functionality.

A related study reports on a refined decellularization procedure. This study demonstrated the ability of liver progenitor cells to differentiate to both the hepatocyte and cholangiocyte lineages while seeded on the decellularized scaffold^[4]. The strategy for recellularizing the bioscaffold was aimed at creating a more rapid and efficient differentiation of the stem cells using tissue-specific extracts enriched in extracellular matrix and a hormonally specific defined medium using associated growth factors and cytokines. They reseeded the scaffold with human hepatic stem cells in a hormonally defined medium specific for adult liver cells. The stem cell markers were expressed in the cells after the reseeded process and the

cells differentiated into mature functional parenchymal cells in approximately one week. These cells remained viable and presented stable mature phenotypes for more than 8 wk.

Similar results have been obtained by other groups^[5,6], however in all the above reported investigations liver ECM was produced from rodent livers. Instead, Barakat *et al*^[7] recently developed a method to decellularize porcine livers, which were eventually repopulated with human cells^[8,9]. The goal was to produce a clinically relevant model of liver bioengineering. Livers from Yorkshire pigs were decellularized with SDS. The ECM of the posterior segment of the right liver lobe was used as scaffold for cell seeding. Fetal hepatocytes co-cultured with fetal stellate cells were expanded, collected, resuspended in appropriate medium supplemented with hepatocyte growth factor and seeded

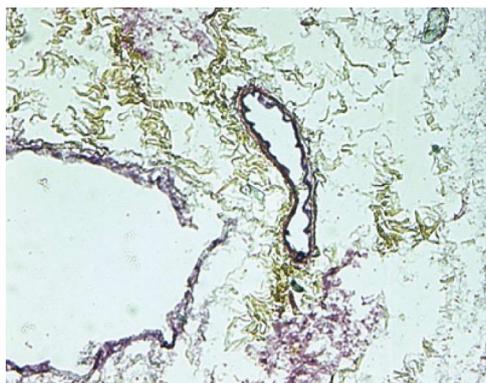


Figure 2 Movat-Pentachrome staining of acellular liver sections shows yellow staining for collagen and dark staining for elastin surrounding the vascular structures.

within the ECM. The so-obtained constructs were perfused for 3 d, 7 d and 13 d. During perfusion, pH, PO₂, PCO₂, lactate, glucose, urea nitrogen and albumin were measured to assess metabolic and synthetic functions. Of note, some constructs were implanted *in vivo* and perfused for 2 h to determine the behavior of the matrix *in vivo* and its ability to withstand the shear stress produce by the blood flow in physiologic conditions. Results were encouraging. Liver organoids showed active metabolism and preserved capability to synthesize albumin, and were able to sustain blood pressure without harm. Notably, immunohistochemical analysis revealed cell differentiation into mature hepatocytes. This latter finding provides evidence that ECM is essential in that it supports cells and may drive the differentiation of progenitor cells into an organ-specific phenotype^[10]. Badyal's group confirmed this information in an elegant model of liver hepatectomy in rats^[11], in which he demonstrated that liver ECM implanted in intact and amputated livers enhances hepatocyte proliferation and ultimately liver regeneration.

While the primary goal for the majority of the research pertaining to decellularizing and recellularizing an organ is the functional transplantation of a bioengineered organ into a recipient host, there are the possibilities of using this technology in *in vitro* studies for advanced preclinical drug development^[12]. This report provided a 60-min rapid natural decellularization method for a 3-dimensional scaffold prepared from a rat liver that maintained the microvascular system and was able to withstand fluid flowing through all three hepatic circular systems. The method utilized two thirty-minute perfusion periods; a 1% Triton-X 100 solution followed by a 1% SDS solution. The development of a novel *in vitro* 3-dimensional model that closely represents the *in vivo* liver could present the potential for toxicity testing of key compounds in preclinical drug developments since the liver is the main metabolizing organ that is usually the target of toxicity.

CELLS FOR LIVER REGENERATION

The liver is able to regenerate itself with the ability to

maintain adequate volume and function after undergoing up to 70% resection. However, the way the liver regenerates after a more or less extended amputation is not a true recapitulation of liver ontogenesis. In fact, resumption of the original volume is accomplished by cellular hyperplasia of the remaining liver rather than true regeneration of the amputated portion whose original anatomy will not be resumed^[13]. Therefore, from an evolutionary perspective, liver hyperplasia is a mechanism of repair that has developed to restore normal function, not normal anatomy. Unfortunately, the actual system that regulates the hepatic regeneration after injury remains mostly obscure. When the liver regenerates after amputation, cellular hyperplasia occurs spontaneously through a complex cascade of events and pathways. This cascade of regulation involves the inflammatory signaling, the recruitment of inflammatory cells, the stimulation of hepatobiliary cell proliferation, and the ultimate aim of cell migration and neo-angiogenesis. The restoration of the tissue mass is thus carried out by the division of mature hepatocytes. If the mature hepatocytes are unable to maintain sufficient proliferative potential to restore the organ, or if there is complete inhibition of this process, intervention occurs from the liver progenitor cells, known as oval cells^[14-18].

There are many techniques that address regenerating the liver, without actually fully regenerating and replacing the organ, by attempting to enhance the natural regeneration of the injured liver. The basic idea behind this technique is to enhance the liver's natural ability to regenerate itself through the transplantation and mobilization of liver progenitor cells that are isolated from bone marrow. The studies that address this technique base the idea off the fact that it has been found that the cells resident to the bone marrow are able to aid in liver regeneration by differentiating into fully functional hepatocytes^[19-22]. While these studies have yet to fully characterize these cells, it has been clearly established that there are bone marrow populations that could have the ability to increase the quality of the clinical conditions regarding patients that have chronic liver disease or injury. In these clinical trials the initial goal was to determine whether or not the infusion of autologous bone marrow cells, through perfusion of the peripheral vein or the hepatic artery, into patients who have liver cirrhosis, was safe. Some of these studies were able to achieve more than just safety results, and showed that there was statistically significant clinical improvement in the patients^[23-25].

More recent studies have attempted to determine the clinical safety of administering patients with the hematopoietic stem cell mobilizing cytokine, granulocyte colony stimulating factor (G-CSF), which has been shown to improve the functioning of the liver in patients with liver disease. It is thought that the function of the G-CSF is to primarily activate cells that are within the bone marrow that have hepatocyte lineage differentiation potential. G-CSF not only interacts with the bone marrow cells, but it has also been shown to increase the ability of resident progenitor cells that have the receptor for the cytokine to respond to injuries. In these studies it has been deter-

mined that the G-CSF is able to maintain the ability to mobilize cells from the bone marrow and the peripheral circulation, while there is an increase in the circulating hepatocyte growth factor that plays a major role in liver regeneration^[26-29]. The bone marrow and peripheral blood are great sources for this because they are easily accessible while having a large source of stem cells and progenitor cells that are able to proliferate *in vitro*. Since these studies primarily aimed to focus on the safety potential for administering the cytokine, there have been two large studies that have been conducted in order to actually determine the therapeutic treatment potential of this technique. These trials were performed by administering G-CSF to the patient with liver disease, which was then followed by the isolation of stem cells from both the peripheral circulation and the bone marrow. These isolated cells were then infused back into the patient through the already established perfusion methods. The trials clearly showed significant improvement in the serum bilirubin and the liver enzyme levels, while there was no improvement noticed in the untreated control group^[30,31].

As previously mentioned, despite the clearly seen therapeutic potential for bone marrow cells to help the regenerative process of a diseased liver, the findings from these trials have yet to be able to determine the specific cell in the bone marrow that is actually aiding in the regeneration. There have been a few *in vivo* animal models that have demonstrated the ability for bone marrow derived mesenchymal stem cells and hematopoietic stem cells (CD34⁺/Lin) to have ability to differentiate into hepatocytes^[32-36]. Fetal liver progenitor cells have also been shown to improve the condition of cirrhotic patients^[37,38]. Therefore, the use of these cells with the previously described isolation and infusion techniques presents multiple advantages for creating a potential therapy. This presents the possibility of having an easily obtainable source of cells that are from the isolated G-CSF mobilized bone marrow cells. The concern of the patient having a rejection to the treatment would be absent because all of the cells used in the therapy are autologous. A portion of these cells used could also possibly carry a progenitor phenotype following infusion, which could help participate in the liver repopulation over time when the damage to the native hepatocyte population is chronic. The corrective gene could therefore be slowly increased in the native cells with as little as a repopulation of 10% of the cells expressing the factor^[39].

Other cell sources are also available, namely fetal hepatoblasts and stem cells from adult or fetal tissue. As reported above, Baptista *et al.*^[3] used fetal liver hepatoblasts to recellularize liver ECM scaffolds. Once in this three-dimensional environment, these liver progenitors were able to expand and differentiate into biliary and hepatocytic lineages. In the fetal liver, these cells are the main parenchymal cell type and are identified by their expression of α -fetoprotein (AFP). These cells are rare in the normal adult liver, except in livers with severe injury or disease^[40,41]. Because these cells are able to originate the

two hepatic cell lineages, hepatocytes and cholangiocytes, they are named bipotential progenitors.

AFP-negative hepatic stem cells are the precursors to hepatoblasts that can mature into AFP-positive hepatoblasts^[42-44]. Human fetal hepatoblasts are then the putative transient amplifying progenitors in the liver lineage and can be cultured long-term and clonally, contributing to liver parenchyma when transplanted into SCID mice^[45]. Hepatoblasts express biliary and hepatocyte markers such as CK19, CK14, α -GT, glucose-6-phosphatase, glycogen, albumin, AFP, E-cadherin^[46], α -1 microglobulin, Hep-Par1, glutamate dehydrogenase, and DPP-IV^[42,47]. These progenitors do not express mesenchymal or hematopoietic markers like CD90, vimentin, and CD34^[46]. The therapeutic potential and safety of these cells has already been successfully tested in human patients with end-stage chronic liver disease^[48]. In these patients, there was significant clinical improvement in terms of biochemical and overall clinical parameters. Moreover, mean MELD score decreased ($P < 0.01$) over the following 6 mo after stem cell therapy. Thus, fetal derived stem/progenitor cells have the potential to provide supportive therapy to organ transplantation in the management of end-stage liver diseases^[18,48-54].

This notwithstanding, it is the authors' conviction that cell transplantation alone may not be appropriate. In fact, clinical transplantation provides incontrovertible evidence that the outcome of cell transplantation is very poor when compared to whole organ transplantation. Therefore, it cannot be proposed as an alternative to whole organ transplantation, rather it should be considered still an experimental treatment, as it has been proposed by Cravedi's^[55] in the case of islet transplantation and by from a regenerative medicine perspective, the poor outcome may be attributed to the fact that cells welfare is dramatically impaired when cells are extrapolated by their natural niche—namely the ECM—despite encapsulation. Therefore, research should direct efforts to bioengineer a suitable supporting scaffold, which would recapitulate the same characteristics of the natural environment.

Interestingly, some authors have proposed a different bioengineering method, which does not require any supporting scaffolds. However, cells are not manipulated alone but are grown in order to produce cell sheets. Hara-guchi's group^[56] employs temperature-responsive culture surfaces onto which poly (N-isopropylacrylamide) is covalently immobilized to control cell adhesion/detachment with simple temperature change. Cells adhere, spread, and proliferate on temperature-responsive surfaces at 37 °C, which is the normal temperature for mammalian cell culture. By reducing temperature below 32 °C, cells spontaneously detach from the surfaces without requiring proteolytic enzyme such as trypsin, since the grafted polymer becomes hydrophilic. When temperature is reduced after cells reach confluency, all the cells are harvested as a single contiguous cell sheet. The advantage of this method is that, as trypsin is not used, all cell membrane proteins including growth factor receptors, ion

channels, and cell-to-cell junction proteins are intact after the harvest. Furthermore, the ECM deposited during cell culture is retained under cell sheets, and therefore, cell sheets easily integrate to transplanted sites. In a murine model, sheets of hepatic tissue transplanted into the subcutaneous space resulted in efficient engraftment to the surrounding cells, with the formation of two-dimensional hepatic tissues that stably persisted for more than 6 mo, while showing several liver-specific functions^[57].

FUTURE PERSPECTIVES

The need for improved treatment modalities for patients with diseased or absent tissues or organs is evident. Regenerative medicine holds the promise of regenerating tissues and organs by either stimulating previously irreparable tissues to heal themselves, or manufacturing them *ex vivo*^[58-64]. In the first scenario, cells with regenerative potential are targeted to the diseased bodily district. Given the multitude of available sources of these cells, it is still a mystery as to which are the most appropriate and best cell sources. Although this may vary depending on the tissue or organ of interest, it is important to fully understand the biological mechanisms controlling differentiation along a specific lineage of all cell types. Ideally, it is desirable to have the ability to harvest autologous cells and employ them with minimal *ex vivo* manipulation. Ultimately, the goal is to identify cells that can be easily harvested and differentiated consistently along the lineage of interest. At the same time, research should aim to in-depth understanding of all environmental stimuli that are required by liver SC niches to be activated and allow hepatocyte and/or biliary cell regeneration aiming to compensate tissue loss.

In the second scenario, differentiated, adult liver cells or SC are seeded on supporting scaffolds and allowed to mature in custom-made bioreactors. Human or animal-derived whole tissue ECM scaffolds are preferred, compared to artificial homogeneous materials, because they preserve an intact vascular network that will allow regeneration of the vascular system for optimal delivery of nutrients and oxygen. The utilization of autologous cells holds the theoretical potential to rule out immunological breakdowns and concerns, and limits the response of the immune system to a non-harmful inflammatory reaction.

In both cases, there are clearly a lot of gray areas that need to be colored in^[58,59,63,65-69]. There has been a greater understanding of the cell types and numbers of cells used for repopulation, but it is still lacking the perfected elements to produce optimal results. Even when this is fully understood and developed, there also needs to be an established standard or test on the bioengineered organ that would reveal the successful incorporation of all the necessary items that the organ requires in order to be fully functional *in vivo*. The actual functionality of the cells within the decellularized matrix and of the organoid as a whole, as well as the biocompatibility of the so-obtained construct, absolutely must be confirmed before

transplantation can ever be a feasible option. Importantly, it will be crucial to understand the mechanisms through which cells interact with the environment and in particular how the liver ECM drives and regulates cell fate and which additional molecules (namely growth factors) are essential to achieve this goal.

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Impaired suppressive activities of human MUTYH variant proteins against oxidative mutagenesis

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Abstract

AIM: To investigate the suppressive activity of MUTYH variant proteins against mutations caused by oxidative lesion, 8-hydroxyguanine (8OHG), in human cells.

METHODS: p.R154H, p.M255V, p.L360P, and p.P377L MUTYH variants, which were previously found in patients with colorectal polyposis and cancer, were selected for use in this study. Human H1299 cancer cell lines inducibly expressing wild-type (WT) MUTYH (type 2) or one of the 4 above-mentioned MUTYH variants were established using the piggyBac transposon vector system, enabling the genomic integration of the trans-

poson sequence for MUTYH expression. MUTYH expression was examined after cumate induction using Western blotting analysis and immunofluorescence analysis. The intracellular localization of MUTYH variants tagged with FLAG was also immunofluorescently examined. Next, the mutation frequency in the *supF* of the shuttle plasmid pMY189 containing a single 8OHG residue at position 159 of the *supF* was compared between empty vector cells and cells expressing WT MUTYH or one of the 4 MUTYH variants using a *supF* forward mutation assay.

RESULTS: The successful establishment of human cell lines inducibly expressing WT MUTYH or one of the 4 MUTYH variants was concluded based on the detection of MUTYH expression in these cell lines after treatment with cumate. All of the MUTYH variants and WT MUTYH were localized in the nucleus, and nuclear localization was also observed for FLAG-tagged MUTYH. The mutation frequency of *supF* was 2.2×10^{-2} in the 8OHG-containing pMY189 plasmid and 2.5×10^{-4} in WT pMY189 in empty vector cells, which was an 86-fold increase with the introduction of 8OHG. The mutation frequency (4.7×10^{-3}) of *supF* in the 8OHG-containing pMY189 plasmid in cells overexpressing WT MUTYH was significantly lower than in the empty vector cells ($P < 0.01$). However, the mutation frequencies of the *supF* in the 8OHG-containing pMY189 plasmid in cells overexpressing the p.R154H, p.M255V, p.L360P, or p.P377L MUTYH variant were 1.84×10^{-2} , 1.55×10^{-2} , 1.91×10^{-2} , and 1.96×10^{-2} , respectively, meaning that no significant difference was observed in the mutation frequency between the empty vector cells and cells overexpressing MUTYH mutants.

CONCLUSION: The suppressive activities of p.R154H, p.M255V, p.L360P, and p.P377L MUTYH variants against mutations caused by 8OHG are thought to be severely impaired in human cells.

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Key words: 8-hydroxyguanine; Mutation; MUTYH; MUTYH-associated polyposis; Oxidative mutagenesis; *supF* forward mutation assay; piggyBac transposon; Colorectal polyposis

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INTRODUCTION

8-hydroxyguanine (8OHG) is an oxidatively damaged form of guanine^[1], and because 8OHG can pair with adenine as well as cytosine, the formation of 8OHG in DNA causes a G:C to T:A transversion mutation^[2]. To prevent such mutations, excision repair proteins, such as MUTYH (OMIM 604933), that act on 8OHG are present in human cells. The MUTYH protein is a DNA glycosylase that catalyzes the removal of adenine that is mispaired with 8OHG in double-stranded DNA^[3-7]. Two major MUTYH proteins, type 1 and type 2, are expressed in human cells as a result of multiple transcription initiation sites and the alternative splicing of mRNA transcripts^[4,7]. Because the type 1 protein contains a mitochondrial targeting signal (MTS) in its N-terminal, it is localized in the mitochondria. In contrast, the type 2 protein lacks the N-terminal 14 amino acids of type 1, and this absence leads to the destruction of the MTS; consequently, the type 2 protein is localized in the nucleus^[4,7].

Biallelic germline mutations in the *MUTYH* gene are responsible for MUTYH-associated polyposis (MAP) (OMIM 608456), which is a hereditary disease characterized by multiple colorectal adenomas and carcinomas^[8-12]. Most biallelic *MUTYH* carriers have between 10 and a few hundred colorectal polyps, meaning that MAP shows a phenotypic overlap with two other hereditary colorectal polyposis syndromes: familial adenomatous polyposis (FAP: OMIM 175100) and attenuated FAP (AFAP: OMIM 175100), both of which are caused by inactivation of the *APC* gene (OMIM 611731)^[13,14]. Therefore, screening for germline mutations in *MUTYH* and *APC* is important in candidate patients with multiple colorectal polyps. However, even when *MUTYH* gene variations are detected in the mutation screening, if information regarding the level of the repair activities of the MUTYH variants is lacking, a correct diagnosis of MAP is impossible to make. Thus far, 300 unique DNA variants of the

MUTYH gene have been reported in the Leiden Open Variation Database (http://www.lovd.nl/2.0/index_list.php)^[15], and the proportion of missense *MUTYH* variations in the database is larger than nonsense mutations or truncating mutations. For most of the genes, a functional analysis is needed to determine whether the activity of a protein encoded by a missense variant is severely reduced. Thus, the effect of *MUTYH* variations on repair activity should be examined; however, so far, only a small number of *MUTYH* variations has been investigated^[16-27]. In most of these studies, the DNA glycosylase activities of the variant recombinant proteins were analyzed using a DNA cleavage assay to test the abilities of the variants to cleave double-stranded oligonucleotides containing an A:8OHG mispair *in vitro*^[18,19,21,23-27]. However, because examining the repair activity of MUTYH variant proteins from multiple aspects would lead to a more definitive judgment of the pathogenicity of MUTYH variants and MUTYH has the ability to regulate the mutation frequency in human cells *in vivo*^[28-30], evaluating the mutation frequency in human cells is also valuable. However, at present, the activities of MUTYH variants in the regulation of mutation suppression in human cells *in vivo* have not been previously reported. Therefore, in this paper, we evaluated the suppressive activities of MUTYH variant proteins against oxidative mutagenesis in human cells. We recently determined the DNA glycosylase activities of 14 type 2 (nuclear form) MUTYH variants using a DNA cleavage assay^[27], and based on those results, p.R154H, p.M255V, p.L360P, and p.P377L type 2 proteins were chosen from the tested variants, and their abilities to suppress mutations caused by 8OHG in human cells were analyzed in this study. As far as we know, this is the first report to analyze the suppressive activities of MUTYH variants against oxidative mutagenesis in human cells.

The Human Genome Variation Society (<http://www.hgvs.org/>) recommends using the transcript variant $\alpha 5$ (NM_001128425.1), which encodes the longest isoform (549 amino acids), as a reference sequence. Therefore, the type 2 proteins p.R154H, p.M255V, p.L360P, and p.P377L used in this study correspond to the reference proteins p.R182H, p.M283V, p.L388P, and p.P405L, respectively.

MATERIALS AND METHODS

Cell line

The human cancer cell line H1299 was obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained at 37 °C in RPMI1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin under a 5% CO₂ atmosphere. The study design was approved by an institutional review board.

Construction of expression plasmid

Human wild-type (WT) and variant (p.R154H, p.M255V, p.L360P, and p.P377L) MUTYH type 2 cDNAs were polymerase chain reaction-amplified with *PfuUltra* Hot-

start DNA polymerase (Stratagene, La Jolla, CA) and the MUTYH-type 2/pET25b(+) expression vector^[27] as a template; the amplified sequence was then inserted into a piggyBac cumate switch inducible vector (System Biosciences, Mountain View, CA) at the *NheI* and *NotI* restriction enzyme sites. A WT MUTYH type 2 expression vector with a C-terminal FLAG tag was previously constructed using the pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA)^[31]; in this study, the variants were generated using site-directed mutagenesis with a QuikChange Site-directed Mutagenesis kit (Stratagene). All of the vectors were confirmed using DNA sequencing with a BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Tokyo, Japan) and an ABI 3100 Genetic Analyzer (Applied Biosystems).

Transfection

A plasmid vector was transfected into H1299 cells using Lipofectamine 2000 reagent (Invitrogen) according to the supplier's recommendations.

Establishment of stable inducible cell lines

H1299 cells were transfected with the cumate switch inducible vector for MUTYH expression together with the piggyBac transposase vector (System Biosciences). To establish stable inducible cell lines, positively transposed cells were selected using puromycin (1 µg/mL). Because the inducible piggyBac vector features a tight cumate switch combined with the EF1-CymR repressor-T2A-Puro cassette to establish stable cell lines, the addition of cumate solution (System Biosciences) to the puromycin-selected cells led to the induction of MUTYH expression.

Western blotting analysis

Cells were harvested in lysis buffer containing 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 0.1% sodium dodecyl sulfate (SDS), 1.0% Triton X-100, 0.5% sodium deoxycholate, 100 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), and the whole-cell extracts were mixed with an equal volume of 2 × SDS sample buffer and boiled. The extract was then subjected to SDS-polyacrylamide gel electrophoresis, and the proteins obtained were electrophoretically transferred to a polyvinylidene fluoride membrane (GE Healthcare Bio-Science, Piscataway, NJ). The membrane was blocked with nonfat milk at room temperature (RT) for 1 h and incubated with an anti-MUTYH monoclonal antibody (clone 4D10; Abnova, Taipei, Taiwan) or an anti-β-tubulin monoclonal antibody (clone 2-28-33; Sigma-Aldrich) at RT for 1 h. After washing with PBS containing 0.05% Tween-20 (PBS-T), the membrane was incubated with an anti-mouse HRP-conjugated secondary antibody (GE Healthcare Bio-Science) at RT for 1 h. The membrane was then washed with PBS-T, and immunoreactivity was visualized using an ECL chemiluminescence system (GE Healthcare Bio-Science).

Indirect immunofluorescence analysis

Cells were fixed with 10% formalin at RT or 4% paraformaldehyde at 4 °C. The cells were permeabilized with 1% Nonidet P-40 in PBS for 5 min and incubated with 10% normal goat serum blocking solution (DAKO, Kyoto, Japan) for 30 min. The cells were then probed with mouse anti-MUTYH monoclonal antibody (4D10) or mouse anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) at RT for 1 h. Indirect immunofluorescence labeling was performed by incubation with an Alexa Fluor 594-conjugated secondary antibody (Molecular Probes, Eugene, OR) at RT for 1 h, and the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). The immunostained cells were examined under a fluorescence microscope (Olympus BX-51-FL; Olympus, Tokyo, Japan) equipped with epifluorescence filters and a photometric CCD camera (Sensicam; PCO Company, Kelheim, Germany). The captured images were digitized and stored using an image analysis program (MetaMorph; Molecular Devices, Palo Alto, CA).

Shuttle vector plasmid and an indicator bacterial strain

The plasmid pMY189 and the indicator *Escherichia coli* (*E. coli*) strain KS40/pKY241 were used for the *supF* forward mutation assay, as reported previously^[30,32]. pMY189 is a shuttle vector containing the bacterial suppressor tRNA (*supF*) gene. KS40 is a nalidixic acid-resistant (*gyrA*) derivative of MBM7070 with genotype *lacZ* (*am*), *CA7070*, *lacY1*, *hsdR*, *hsdM*, Δ (*araABC-leu*)7679, *galU*, *galK*, *rpsL*, *thi*. The pKY241 plasmid contains a chloramphenicol resistance marker and the *gyrA* (amber) gene. *E. coli* KS40/pKY241 cells carrying the active *supF* gene are sensitive to nalidixic acid and form blue colonies on LB plates containing ampicillin, chloramphenicol, isopropyl-β-D-thiogalactopyranoside (IPTG), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), whereas cells carrying the mutated *supF* gene form white colonies on plates containing nalidixic acid, ampicillin, chloramphenicol, IPTG, and X-gal.

Construction of a shuttle vector plasmid containing an 8OHG residue

pMY189-8OHG, which is the shuttle plasmid pMY189 containing a single 8OHG:cytosine pair at nucleotide position 159 of the *supF* gene, was prepared according to a previously described method^[30]. Briefly, *E. coli* XL1-Blue MRF⁺ (Stratagene) and R408 Helper Phage (Stratagene) were used to prepare single-stranded pMY189 DNA, and 30 µg of the single-stranded pMY189 plasmid and a 5-fold molar excess of a 5'-phosphorylated 24-mer oligonucleotide with a single 8OHG at nucleotide position 159 of the *supF* gene [5'-CGA CTT CGA A (8OHG) G TTC GAA TCC TTC-3'] were annealed in a reaction mixture. Forty units of T4 DNA polymerase (New England Biolabs, Beverly, MA), 600 µmol/L of deoxynucleotide triphosphate (GE Healthcare Bio-Science), 36 Weiss units of T4 DNA ligase (New England Biolabs) and 1 mmol/L of ATP (Nacalai Tesque, Kyoto, Japan) were added to the

reaction mixture, and the mixture was incubated at 37 °C for 4 h. Closed circular pMY189-8OHG was isolated using cesium chloride-ethidium bromide density gradient centrifugation.

SupF forward mutation assay

Cells were cultured in the presence of cumate for 3 d for the induction of MUTYH expression, and they were then transfected with the shuttle plasmid pMY189 or pMY189-8OHG. After 48 h, the propagated plasmids were extracted from the cells using a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) and digested with *DpnI* restriction enzyme (New England Biolabs) to eliminate unreplicated plasmids with the bacterial methylation pattern. After purification with Amicon Ultra Centrifugal Filter Units (Millipore, Bedford, MA), the plasmids were introduced into the KS40/pKY241 indicator *E. coli* strain using electroporation. The transformants were plated onto LB agar plates containing 50 µg/mL of nalidixic acid, 150 µg/mL of ampicillin, and 30 µg/mL of chloramphenicol together with IPTG and X-gal. White colonies on were counted as *supF* mutants. The mutation frequencies were calculated as the number of *supF* mutants per the total number of transformants, which were counted on LB plates containing ampicillin, chloramphenicol, IPTG and X-gal.

Statistical analysis

The statistical analysis was performed using an unpaired *t*-test and JMP software, version 9 (SAS Institute, Cary, NC). *P*-values less than 0.05 were considered statistically significant.

RESULTS

Establishment of human cells inducibly expressing MUTYH variants

To investigate the ability of MUTYH variants to suppress mutations caused by 8OHG in human cells, we used the piggyBac transposon vector system^[33] to establish human cells capable of inducibly expressing MUTYH variants and performed a *supF* forward mutation assay using the shuttle plasmid pMY189, which contains a single 8OHG in the *supF* gene. First, H1299 human cancer cells were transfected with a piggyBac cumate switch inducible vector for the expression of WT, p.R154H, p.M255V, p.L360P, or p.P377L MUTYH together with the piggyBac transposase vector; positively transposed cells were then selected with puromycin. We also transfected cells with an empty (parental) piggyBac cumate switch inducible vector and transposase vector. The expression of MUTYH protein after cumate induction was examined using Western blotting analysis using an anti-MUTYH monoclonal antibody (Figure 1A). MUTYH protein was abundantly expressed in cells in which a WT, p.R154H, p.M255V, p.L360P, or p.P377L MUTYH expression vector but not an empty vector was transposed. Immunofluorescence analysis also showed abundant MUTYH pro-

tein expression in cells in which a WT, p.R154H, p.M255V, p.L360P, or p.P377L MUTYH expression vector but not an empty vector was transposed (Figure 1B). In accordance with the previous finding that the MUTYH type 2 protein is the nuclear form^[4,7], WT MUTYH protein was localized in the nucleus. All of the MUTYH variants were also localized in the nucleus, suggesting that the amino acid changes in p.R154H, p.M255V, p.L360P, and p.P377L did not alter the subcellular localization of these proteins in human cells. With regard to endogenous MUTYH expression, low levels were detected in the immunofluorescence analysis, as shown in the panel of empty vector-transposed cells (Figure 1B). When the intensity of the MUTYH protein signal was enhanced with image-editing software, the signal was observed in both the nucleus and cytoplasm (Figure 1C), which is compatible with the existence of both the type 1 mitochondrial form and the type 2 nuclear form^[4,7]. Next, to confirm the nuclear localization of MUTYH type 2 variant forms, we constructed a vector to express MUTYH tagged with a FLAG peptide at the C-terminus and examined the localization of the MUTYH variants using immunofluorescence analysis with an anti-FLAG antibody (Figure 2). All of the variants showed nuclear localization, further suggesting that the amino acid changes in p.R154H, p.M255V, p.L360P, and p.P377L did not alter their subcellular localization in human cells. Together, the above findings indicate that human cells inducibly expressing the MUTYH variants (p.R154H, p.M255V, p.L360P, or p.P377L) and their control cells were properly prepared and were appropriate for evaluating the suppressive activities of MUTYH variants against oxidative mutagenesis in human cells.

Impaired activities of MUTYH variants in the suppression of oxidative mutagenesis in human cells in vivo

Next, mutation frequencies were compared for the empty vector-transposed human cells and the cumate-inducible stable cells expressing WT or variant MUTYH using a *supF* forward mutation assay with the shuttle plasmid pMY189. In this assay, we introduced a single 8OHG residue at position 159 of the *supF* gene in pMY189. The mutation frequency of *supF* was 2.2×10^{-2} in the 8OHG-containing pMY189 plasmid and 2.5×10^{-4} in WT pMY189 in empty vector-transposed cells (Figure 3), which was an 86-fold increase in mutation frequency with the introduction of 8OHG. The mutation frequency (4.7×10^{-3}) of *supF* in the 8OHG-containing pMY189 plasmid in cells overexpressing WT MUTYH was significantly lower than in the empty vector-transposed cells. However, the mutation frequencies of *supF* in the 8OHG-containing pMY189 plasmid in cells overexpressing the p.R154H, p.M255V, p.L360P, or p.P377L MUTYH variant were 1.84×10^{-2} , 1.55×10^{-2} , 1.91×10^{-2} , and 1.96×10^{-2} , respectively, meaning that no significant difference was observed in the mutation frequency between the empty vector-transposed cells and the cells overexpressing MUTYH variants. These results suggested that the suppressive activities of p.R154H, p.M255V, p.L360P,

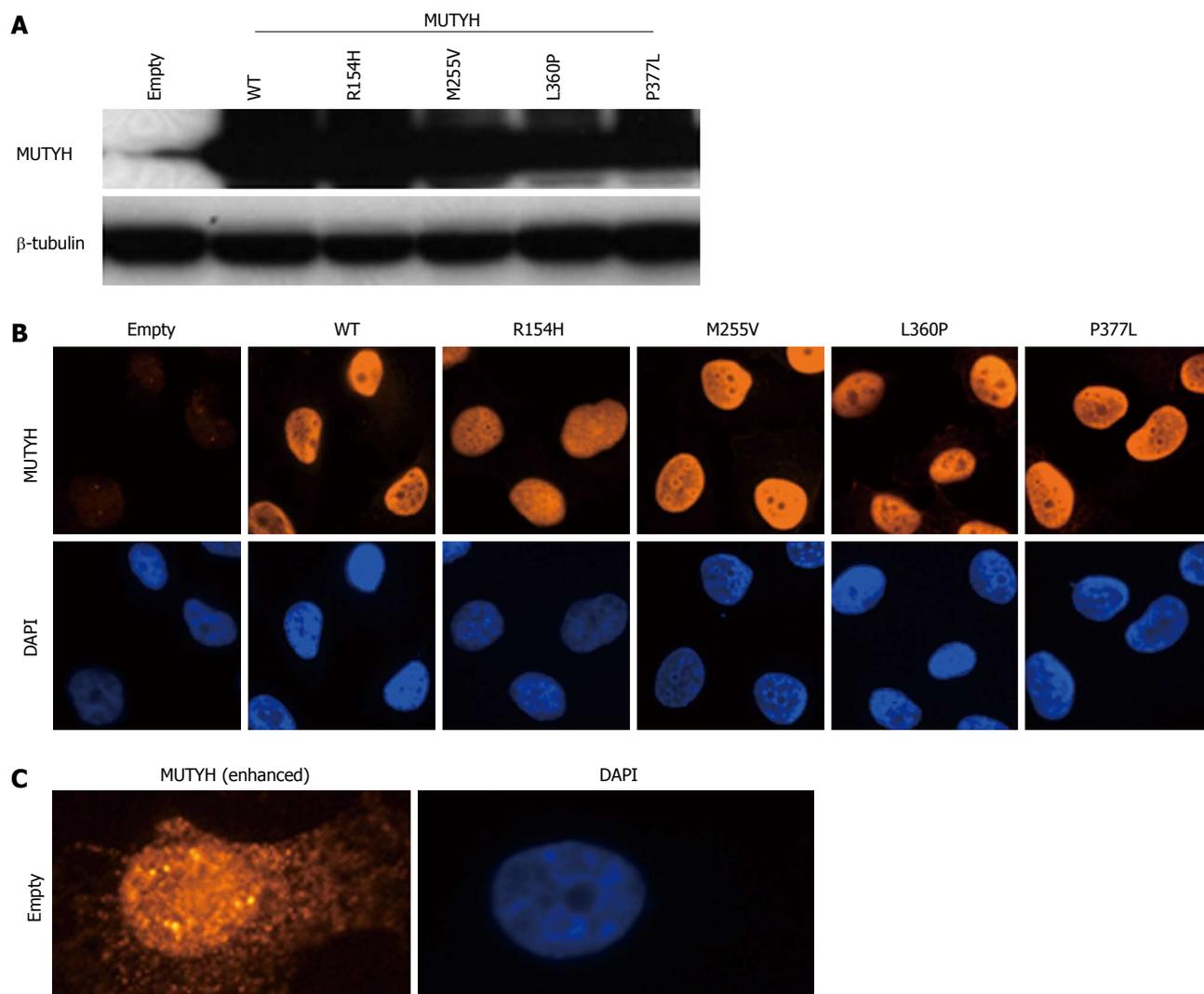


Figure 1 Establishment of H1299 human cell lines inducibly expressing MUTYH variant proteins. A: Detection of MUTYH proteins in cumate-inducible stable cell lines expressing MUTYH in the presence of cumate using Western blotting analysis with an anti-MUTYH antibody. Lysates from empty vector-transposed cells and cells inducibly expressing wild-type (WT) MUTYH or p.R154H, p.M255V, p.L360P, or p.P377L MUTYH variants were analyzed. β -tubulin protein was also analyzed as an internal control; B: Immunofluorescence detection of MUTYH proteins expressed in the cell lines used in (A) in the presence of cumate. The MUTYH protein (red) was stained with anti-MUTYH as the primary antibody and Alexa Fluor 594-conjugated goat anti-mouse IgG as the secondary antibody. The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue); C: Immunofluorescence detection of endogenous MUTYH proteins in the empty vector-transposed cells as described in (B). The intensity of the signals of MUTYH protein (red) was enhanced with image-editing software to determine the subcellular localization of endogenous MUTYH protein. The nuclei were counterstained with DAPI (blue).

and p.P377L MUTYH variants against mutations caused by 8OHG were severely impaired in human cells.

DISCUSSION

In this study, human cell lines inducibly expressing MUTYH variants (p.R154H, p.M255V, p.L360P, or p.P377L) were established, and the abilities of these cells to suppress mutations caused by 8OHG were compared using a *supF* forward mutation assay with a shuttle vector containing an 8OHG residue in the *supF* gene. The assay showed that the suppressive activities of p.R154H, p.M255V, p.L360P, and p.P377L MUTYH variants against mutations caused by 8OHG were severely impaired in human cells. To the best of our knowledge, this is the first analysis of the suppressive activities of MUTYH variants against oxi-

dative mutagenesis in human cells *in vivo*.

The type 2 protein is the nuclear form of MUTYH^[4,7], and somatic *APC* and *KRAS* (OMIM 190070) mutations occur in the nuclear DNA of MAP tumors^[8,9,12]; therefore, we believed that it would be more appropriate to investigate type 2 rather than type 1 and we established cell lines expressing the type 2 form in this study. In the *supF* forward mutation assay using a shuttle vector containing 8OHG, no significant difference was observed in the mutation frequencies between empty vector-transposed cells and cells expressing 1 of the 4 MUTYH variants, indicating the severe impairment of the suppressive activities of the MUTYH variants against mutations caused by 8OHG in human cells *in vivo*. We previously showed that the adenine DNA glycosylase activity of the p.M255V protein was 10.7% of the level of the WT protein and that the DNA

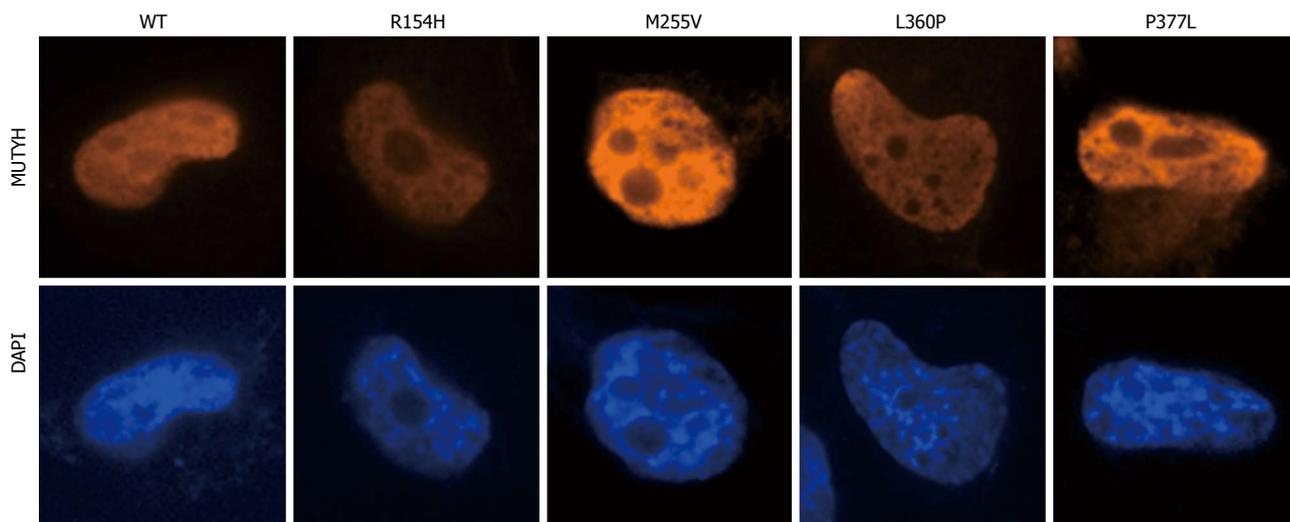


Figure 2 Nuclear localization of MUTYH variant proteins (p.R154H, p.M255V, p.L360P, and p.P377L). H1299 cells were transiently transfected with a vector expressing various types of MUTYH proteins tagged with FLAG, and MUTYH-FLAG protein (red) was stained with anti-FLAG M2 as the primary antibody and Alexa Fluor 594-conjugated goat anti-mouse IgG as the secondary antibody. The nuclei were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) (blue). WT: Wild-type.

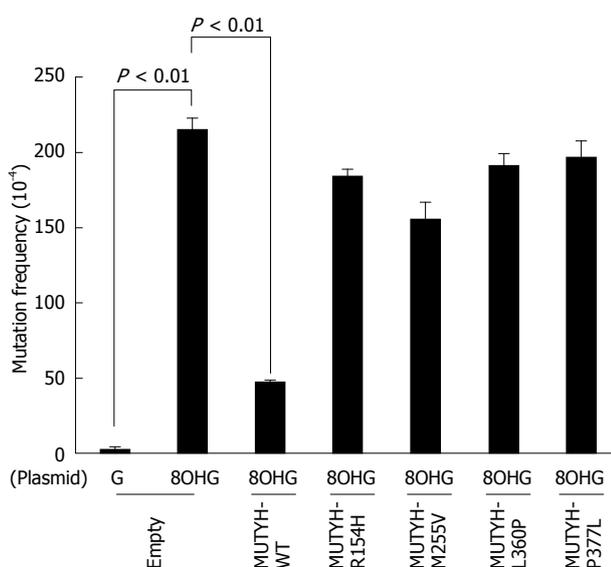


Figure 3 Measurement of the mutation frequency of the *supF* gene in the pMY189 plasmid using a *supF* forward mutation assay in H1299 human cell lines inducibly expressing MUTYH variant proteins. Empty vector-transfected cells and cells inducibly expressing wild-type (WT) MUTYH or p.R154H, p.M255V, p.L360P, or p.P377L MUTYH variants in the presence of cumate were transfected with a pMY189 shuttle plasmid, and the mutation frequency of *supF* in these human cell lines was measured. "8-hydroxyguanine (8OHG)" indicates a pMY189 plasmid containing an 8OHG residue at position 159 of *supF*, while "G" indicates a pMY189 plasmid containing the WT *supF* gene. The data are shown as the means \pm SE.

glycosylase activities of the p.R154H, p.L360P, and p.P377L proteins were severely impaired^[27]. Thus, the results regarding the regulation of the mutation frequency in the present study are in agreement with DNA glycosylase activity in the previous study. A combination of the results of two distinct analyses, i.e., *in vitro* and *in vivo* analyses, would provide more definitive proof of the pathogenicity of the p.R154H, p.M255V, p.L360P, and p.P377L MUTYH variants. Because the diagnosis of MAP depends on whether

(1) the clinical phenotypic characteristics of MAP are present in a candidate patient; and (2) the repair activities of the MUTYH variants encoded in the two *MUTYH* alleles of the patient are decreased, even when gene variations are found in the patient by *MUTYH* mutation screening, information on the levels of the repair activities of MUTYH variants is indispensable for properly diagnosing MAP. Regarding this point, our results are clinically useful.

Previous studies have provided contradictory results regarding the subcellular localization of MUTYH protein in MAP patients; one paper insisted that MUTYH protein was predominantly localized in the cytoplasm of colorectal tumor cells in MAP patients but not in non-MAP patients, while the other papers denied this localization^[34,35]. In the present study, the nuclear localization of the p.R154H, p.M255V, p.L360P, and p.P377L MUTYH type 2 variants was shown using two distinct experiments. Therefore, it seems that the amino acid changes of p.R154H, p.M255V, p.L360P, and p.P377L did not alter the subcellular localization of the MUTYH protein. Similarly, Molatore *et al.*^[26] recently reported that 3 missense MUTYH variants other than our variants were all localized in the nucleus.

In this paper, we successfully established cumate-inducible stable human cell lines by utilizing the piggyBac transposon vector system. Transposon technology is an attractive non-viral gene delivery model that allows for efficient genomic integration in a variety of cell types^[36]. Among the several transposon systems available, the piggyBac transposon, which was isolated from the genome of the cabbage looper moth (*Trichoplusia ni*), has been optimized for gene transfer into mammalian cells^[36,37]. In practice, the MUTYH expression status in our cell lines after puromycin selection in the presence of cumate clearly demonstrated abundant MUTYH expression in almost all of the cells. Because we performed transient transfection with a shuttle plasmid in the *supF* forward

mutation assay in this study, the genomic integration of the sequence for MUTYH expression using the piggyBac transposon system was well suited to our experiment.

In our experiments, the level of expression of exogenously introduced MUTYH was much higher than the level of expression of endogenous MUTYH. This scenario allowed us to effectively evaluate the activities of MUTYH variants to regulate the mutation frequency, and we believe that such an evaluation was successfully performed. However, we cannot completely exclude the possibility that the functional difference observed under experimental conditions of high MUTYH expression levels does not reflect a true functional difference.

The impaired activity of MUTYH variants was shown using H1299 human lung cancer cells in this study. We used this cell line because we believed that the ability of MUTYH variants to suppress mutations in H1299 cells is not different from their ability in human cells derived from the colorectum. If there are no organ type-specific systems to modulate MUTYH activity, then MUTYH activity is dependent on the MUTYH expression level and MUTYH variation. Moreover, we studied overexpressed and exogenous MUTYH variant proteins in this paper. Therefore, we believe our results can most likely be applied for colorectal cells. However, because it might be possible that the difference in organ type has an effect on the results of functional evaluation, we would like to evaluate this activity in human colorectal cells in the future.

Genetic screening for *MUTYH* mutations in the diagnosis of colorectal polyposis continues to be performed worldwide, and technological progress in genome sequencing analysis has contributed to efficient and rapid screening protocols. Therefore, increasing *MUTYH* nucleotide variants are likely to be detected in the future. For appropriate patient management, the levels of the repair activities of MUTYH variant proteins should be evaluated, and our system for determining the abilities of these variants to suppress oxidative mutagenesis in human cells *in vivo* may be of great use for such evaluations.

COMMENTS

Background

The *MUTYH* gene is responsible for MUTYH-associated polyposis (MAP), a relatively recently identified hereditary disease. Although 300 *MUTYH* variants have been found, only a small number of variants has been functionally characterized. Therefore, evaluations of the activities of MUTYH variant proteins are needed for the correct diagnosis of MAP.

Research frontiers

An *in vitro* DNA cleavage assay was performed to evaluate the repair activities of MUTYH variants. Despite the clinical importance of the multiplicity of functional analytical methods for evaluating the activities of MUTYH variant proteins, until now, the ability of MUTYH variants to suppress oxidative mutagenesis in human cells *in vivo* has not been previously analyzed.

Innovations and breakthroughs

Human cumate-inducible stable cell lines expressing various MUTYH variants were established using the piggyBac transposon vector system. This is the first report to utilize human cells expressing MUTYH variants encoded by an ectopically transposed gene. Moreover, this is the first report to analyze the suppressive activities of MUTYH variants against oxidative mutagenesis in human cells.

Applications

The results of the present study suggest that the suppressive activities of p.R154H, p.M255V, p.L360P, and p.P377L MUTYH variant proteins against mutations caused by 8-hydroxyguanine (8OHG) are severely impaired in human cells. These *in vivo* results combined with results from our previous *in vitro* analysis provide definitive proof of the pathogenicity of p.R154H, p.M255V, p.L360P, and p.P377L MUTYH variants. This conclusion is valuable for the appropriate diagnosis of MAP.

Terminology

The base excision repair protein MUTYH is involved in the repair of the oxidative base lesion 8OHG in DNA. Patients with biallelic inactivating germline mutations in the *MUTYH* gene are predisposed to MAP, which is characterized by the development of multiple colorectal adenomas and carcinomas.

Peer review

This is a good study in which authors analyze the suppressive activity of MUTYH variant proteins against mutations caused by 8OHG in human cells. Towards understanding the impact of having so many missense mutations among MAP patients, the authors have steadily developed an infrastructure for serving the patients in the future. Through expression of MUTYH WT and 4 variants, subcellular localization, and mutation frequency counting, they suggested that anti-mutation activity of the four MUTYH variants were severely impaired in human cells.

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Long-term treatment outcomes of clevudine in antiviral-naive patients with chronic hepatitis B

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Abstract

AIM: To evaluate the treatment outcomes of clevudine compared with entecavir in antiviral-naive patients with chronic hepatitis B (CHB).

METHODS: We retrospectively analyzed the clinical

data of CHB patients treated with clevudine 30 mg/d and compared their clinical outcomes with patients treated with entecavir 0.5 mg/d. The biochemical response, as assessed by serum alanine aminotransferase (ALT) activity, virologic response, as assessed by serum hepatitis B virus DNA (HBV DNA) titer, serologic response, as assessed by hepatitis B e antigen (HBeAg) status, and virologic breakthrough with genotypic mutations were assessed.

RESULTS: Two-hundred and fifty-four patients [clevudine ($n = 118$) vs entecavir ($n = 136$)] were enrolled. In clevudine-treated patients, the cumulative rates of serum ALT normalization were 83.9% at week 48 and 91.5% at week 96 (80.9% and 91.2% in the entecavir group, respectively), the mean titer changes in serum HBV DNA were -6.03 and -6.55 \log_{10} copies/mL (-6.35 and -6.86 \log_{10} copies/mL, respectively, in the entecavir group), and the cumulative non-detection rates of serum HBV DNA were 72.6% and 83.1% (74.4% and 83.8%, respectively, in the entecavir group). These results were similar to those of entecavir-treated patients. The cumulative rates of HBeAg seroconversion were 21.8% at week 48 and 25.0% at week 96 in patients treated with clevudine, which was similar to patients treated with entecavir (22.8% and 27.7%, respectively). The virologic breakthrough in the clevudine group occurred in 9 (7.6%) patients at weeks 48 and 15 (12.7%) patients at week 96, which primarily corresponded to genotypic mutations of rtM204I and/or rtL180M. There was no virologic breakthrough in the entecavir group.

CONCLUSION: In antiviral-naive CHB patients, long-term treatment outcomes of clevudine were not inferior to those of entecavir, except for virologic breakthrough.

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Key words: Chronic hepatitis B; Hepatitis B virus; Clevudine; Entecavir; Treatment outcomes

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a serious health problem worldwide and a major risk factor for the development of liver cirrhosis and hepatocellular carcinoma (HCC), two grave complications that have a high morbidity and mortality^[1,2]. Although the seroprevalence rate of HBV in the South Korean population has gradually decreased since the development of the HBV vaccine in the 1980s, the rate is still high compared to Western countries^[3,4].

Since the first oral antiviral agent against HBV, lamivudine, was introduced in 1997^[5-7], there has been a significant evolution in antiviral fields for patients with chronic hepatitis B (CHB).

Among HBV-targeted oral agents available in Korea, such as lamivudine, adefovir, clevudine, entecavir, and telbivudine, clevudine (CLV) and entecavir (ETV) are now prescribed to a large extent, and they were approved by the South Korean Food and Drug Administration in 2006 and subsequently marketed in Korea as a 1st-line treatment for patients with chronic HBV infection. CLV, which was developed in Korea, is a pyrimidine nucleoside analog with potent antiviral activity against HBV^[8]. CLV therapy for 24 wk showed durable off-treatment virologic suppression and excellent on-treatment virologic response in both hepatitis B e antigen (HBeAg)-positive CHB patients and HBeAg-negative CHB patients^[9,10]. However, CLV-related myopathy was found in a previous global clinical trial, which was controlled due to unfavorable safety profiles despite a tolerable, manageable, and self-limited level of adverse effects.

In contrast, after the clinical establishment of ETV, an analog of the deoxyguanosine nucleoside, as an antiviral agent against HBV, ETV has become the most frequently prescribed drug in clinical fields for the treatment of CHB patients both globally and in Korea, with advantages of potent HBV suppression and low geno-

typic resistance^[11-13].

This study was performed to evaluate the long-term treatment efficacy and safety of CLV in antiviral-naive patients with chronic HBV infection on the basis of those of ETV as a comparative standard of the present study.

MATERIALS AND METHODS

Study subjects

The study subjects were CHB patients who were treated with CLV or ETV as a 1st-line treatment at six University Hospitals in Daejeon and Chungcheong provinces in Korea between January 2007 and March 2010. CHB was diagnosed either histologically or clinically. The clinical diagnosis of CHB was determined through the presence of hepatitis B surface antigen over at least a 6-mo period, a high titer of serum HBV DNA, and sustained or fluctuating elevations of serum alanine aminotransferase (ALT) activity at the corresponding times. In this study, we enrolled CHB patients with a serum ALT value ≥ 80 IU/L and serum HBV DNA titer $> 100\ 000$ copies/mL who had been taking CLV 30 mg or ETV 0.5 mg daily. We followed the global guideline for ALT value, which is 2 times over the normal value. Patients with the following criteria were excluded: age less than 18 years; interferon therapy within 6 mo; HCC diagnosis prior to entrance to this study; alcohol consumption more than 80 g per week; elevated serum ALT value due to other causes, such as drugs, toxins, autoimmune-related or metabolic-related liver diseases, co-infection with hepatitis C virus or human immunodeficiency virus; creatinine clearance less than 50 mL/min; or severe systemic illnesses. During the treatment with antiviral agents, drug compliance was recorded, and patients who did not take the antiviral drug for more than 12 wk were also excluded from the study. The selection of antiviral drug was determined by the patients after they were provided with information on the drugs and their relative merits. The study was conducted in accordance and compliance with ethical guidelines of the 1975 Declaration of Helsinki.

Among the 352 total CHB patients treated with CLV or ETV, we analyzed the clinical data of 254 patients in this study after excluding 67 patients with serum ALT value < 80 IU/L and 31 patients with inadequate follow-up, poor compliance, or insufficient data (Figure 1).

Clinical variables for study evaluation

Clinical baseline characteristics, such as age, gender, history of alcohol consumption, presence of cirrhosis, history of prior antiviral therapy, and other medical history, were retrospectively reviewed and analyzed. Cirrhosis was diagnosed based on histologic evidence through liver biopsy or clinical manifestations of portal hypertension, such as gastroesophageal varices or other collaterals, ascites, encephalopathy, splenomegaly, thrombocytopenia, and radiologic features suggestive of cirrhosis on liver

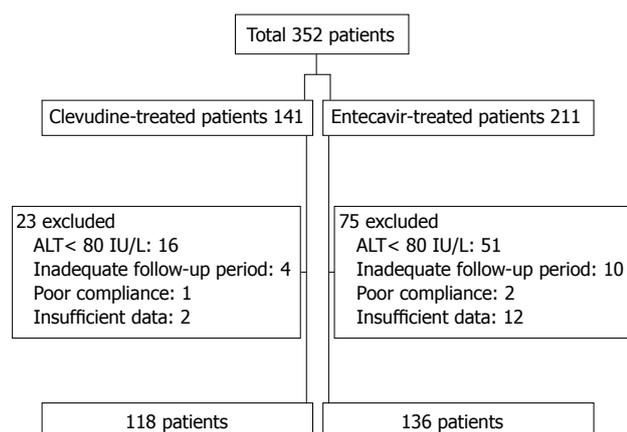


Figure 1 Flow chart of patient enrollment. ALT: Alanine aminotransferase.

ultrasound or computed tomography.

Serum ALT, creatinine, HBeAg/anti-HBe, and HBV DNA titer were recorded approximately 3-mo interval following CLV or ETV administration, and if the examination times were missed, the latest results within a month were used for analysis.

Serum HBV DNA titers were determined by real-time polymerase chain reaction (PCR) with two types of detection indicators: one model with a minimum detection level of 316 copies/mL (Cobas Amplicor PCR, Roche Molecular Systems, United States) and the other model with a minimum detection level of 70 copies/mL (Cobas Taqman, Roche Diagnostics, United States). Mutant analysis of HBV DNA for detecting genotypic resistance was performed by polymerase chain reaction-restriction fragment length polymorphism or direct sequencing in patients showing virologic breakthrough.

Evaluation of treatment efficacy

We evaluated the biochemical responses, including the median change and cumulative normalization rate of the serum ALT value, the virologic responses, including the decrement degree and cumulative non-detection rate of the serum HBV DNA titer, the serologic responses, including the cumulative rates of HBeAg clearance and HBeAg seroconversion, and the antiviral resistance, including the cumulative rate of virologic breakthrough with genotypic mutation.

Non-detection of HBV DNA was defined as a serum HBV DNA titer less than 316 copies/mL. ALT normalization was defined as a serum ALT value less than 40 IU/L. HBeAg clearance was defined as a loss of HBeAg in a patient who was previously positive for HBeAg, and HBeAg seroconversion was defined as a loss of HBeAg and an acquisition of anti-HBe in a patient who was previously positive for HBeAg and negative for anti-HBe. Virologic breakthrough was defined as an increase in serum HBV DNA titer by $> 1 \log_{10}$ (10-fold) above nadir after achieving a virologic response during continued treatment. Genotypic resistance was defined as the detection of mutations that have been shown *in vitro*

to confer resistance to an antiviral agent. In this study, rtM204V/I and rtL180M were considered CLV-related mutations due to common profiles of genotypic mutation for CLV and lamivudine^[14,15]. We also defined no virologic response as a decrease $< 2 \log_{10}$ copies/mL in serum HBV DNA titer after 24 wk of drug administration.

Evaluation of adverse effects

When the patients experienced constitutional symptoms, such as weakness, fatigue, or muscle pain, during the administration of antiviral agents, the serum creatinine phosphokinase (CK) activity was measured for the detection of clinical myopathy. Clinical myopathy was defined as a presence of the above-mentioned symptoms with a simultaneous elevation of serum CK activity.

Statistical analysis

SPSS software (Windows version 15.0, Chicago, IL, United States) was used for statistical analysis for comparison. Student's *t*-test was applied for consecutive variables, and the χ^2 test or Fisher's verification was applied for categorical variables. Statistical significance was determined when the *P*-value was < 0.05 .

RESULTS

Baseline characteristics of the patients

Of the 254 patients with CHB enrolled in this study, 118 patients (70 men and 48 women) were treated with CLV, and 136 patients (86 men and 50 women) were treated with ETV (Figure 1). The mean age of the patients was 42 ± 11 years. None of the patients were previously treated with any antiviral drugs including interferon. No significant difference was noted in the number of patients with cirrhosis (12.7% in the CLV group *vs* 22.1% in the ETV group, $P = 0.052$), HBeAg positivity (74.6% in the CLV group *vs* 69.1% in the ETV group, $P = 0.322$), median serum ALT value [158 (107-263) IU/L in the CLV group *vs* 139.5 (101.5-288.5) IU/L in the ETV group, $P = 0.206$], and serum HBV DNA titer ($7.3 \pm 1.1 \log_{10}$ copies/mL in the CLV group *vs* $7.4 \pm 1.1 \log_{10}$ copies/mL in the ETV group, $P = 0.556$) between the CLV and ETV groups. The median follow-up period was similar in the two groups (74 ± 24 wk in the CLV group and 75 ± 21 wk in the ETV group, $P = 0.922$) (Table 1).

Biochemical responses assessed by serum ALT

The median values of serum ALT at weeks 24, 48, 72, 96, and 120 after treatment were 27, 27, 27, 26, and 16 IU/L in the CLV group and 29, 24, 23, 21, and 16 IU/L in the ETV group, respectively (Figure 2A). The cumulative rates of serum ALT normalization at the same points of time were 81.9% (95/116), 83.9% (99/118), 83.9% (99/118), 91.5% (108/118), and 94.1% (111/118) in the CLV group and 73.9% (99/134), 80.9% (110/136), 89.7% (122/136), 91.2% (124/136), and

Characteristics	Clevudine (<i>n</i> = 118)	Entecavir (<i>n</i> = 136)	<i>P</i> -value
Age ¹ , yr	42 ± 11	41 ± 11	0.358
Male gender	70 (59.3)	86 (63.2)	0.528
Cirrhosis	15 (12.7)	30 (22.1)	0.052
ALT ² , IU/L	158 (107-263)	139.5 (101.5-288.5)	0.206
HBeAg-positive	88 (74.6)	94 (69.1)	0.322
HBV DNA level ³ , log ₁₀ copies/mL	7.3 ± 1.1	7.4 ± 1.1	0.556
Follow-up period ³ , wk	74 ± 24	75 ± 21	0.922

Data are presented by ¹mean ± SD; ²median (interquartile rang); ³median ± SD. ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen.

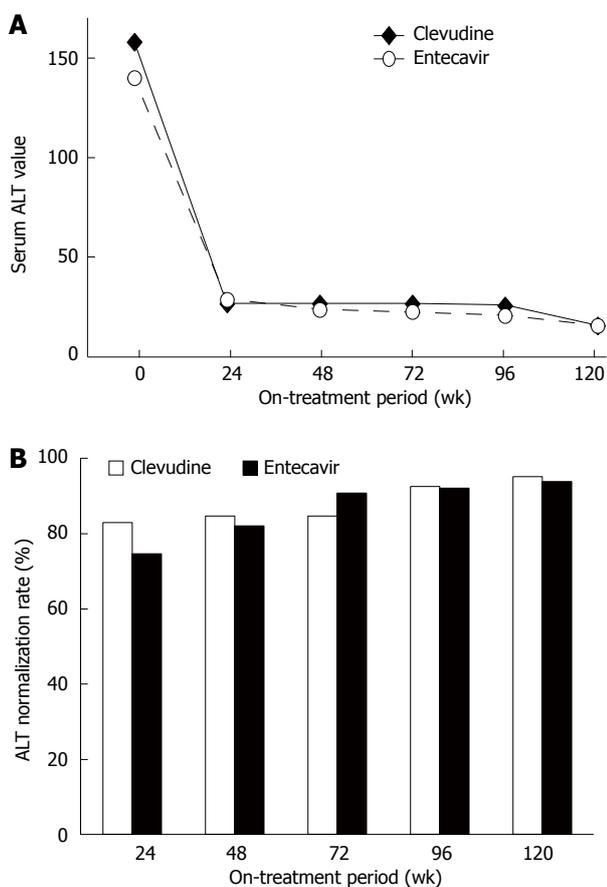


Figure 2 Median changes in serum alanine aminotransferase values and cumulative rates of alanine aminotransferase normalization during the on-treatment period. A: Alanine aminotransferase (ALT) values; B: Cumulative rates of ALT normalization.

92.6% (126/136) in the ETV group, respectively (Figure 2B). No significant difference was noted in either the median value or cumulative normalization rate of serum ALT between the two groups.

Virologic responses assessed by serum HBV DNA

The titer decrements of serum HBV DNA were -5.83, -6.03, -5.66, -6.55, and -7.28 log₁₀ copies/mL in the

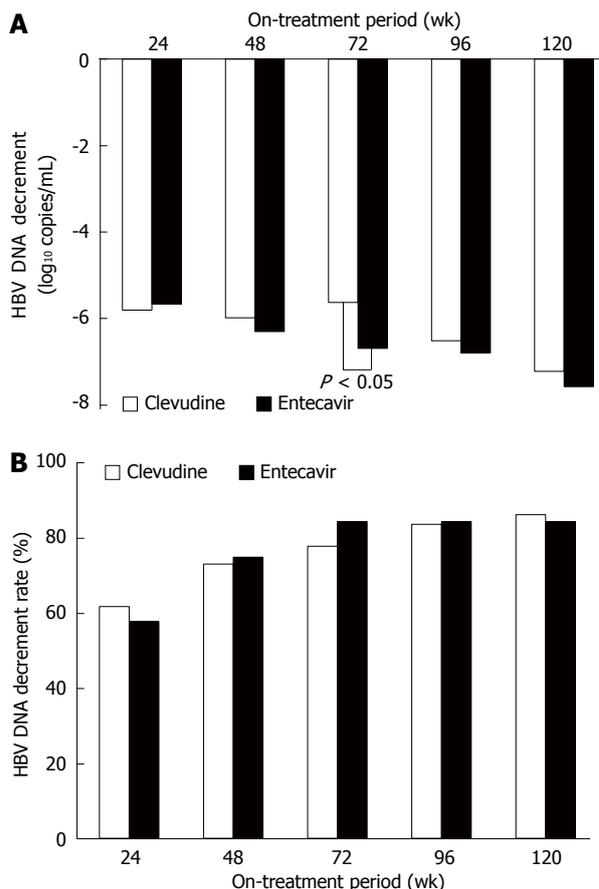


Figure 3 Decrement changes in serum hepatitis B virus DNA titer and cumulative rates of hepatitis B virus DNA non-detection during the on-treatment period. A: Hepatitis B virus (HBV) DNA decrement changes; B: HBV DNA non-detection rate.

CLV group and -5.70, -6.35, -6.74, -6.86, and -7.63 log₁₀ copies/mL in the ETV group at weeks 24, 48, 72, 96, and 120 after treatment, respectively. There was no significant difference between the groups, except for serum HBV DNA titer at week 72 (CLV group *vs* ETV group, *P* < 0.05)(Figure 3A). At the same time points, the cumulative non-detection rates of serum HBV DNA were 61.3% (68/111), 72.6% (85/117), 77.1% (91/118), 83.1% (98/118), and 85.6% (101/118) in the CLV group and 57.3% (71/124), 74.4% (99/133), 83.8% (114/136), 83.8% (114/136), and 83.8% (114/136) in the ETV group, respectively, showing no significant difference (Figure 3B).

Serologic responses assessed by HBeAg

In patients who were positive for HBeAg, the cumulative rate of HBeAg clearance was 19.5% (16/82), 33.3% (29/87), 37.5% (33/88), 40.9% (36/88), and 40.9% (36/88) in the CLV group and 20.2% (17/84), 28.3% (26/92), 34.0% (32/94), 40.4% (38/94), and 40.4% (38/94) in the ETV group at weeks 24, 48, 72, 96, and 120 after drug administration, respectively (Figure 4A). The cumulative rate of HBeAg seroconversion was

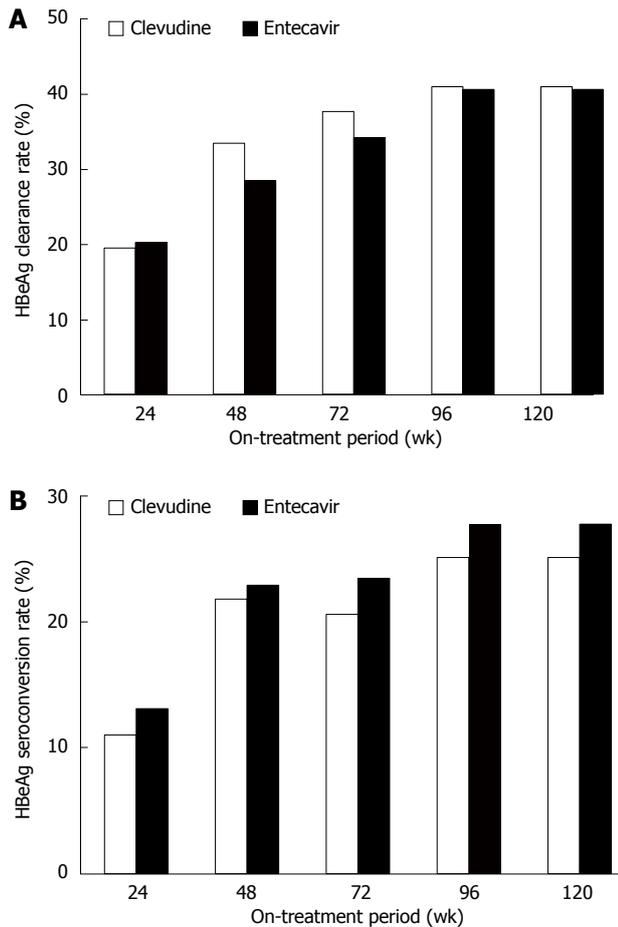


Figure 4 Cumulative rates of hepatitis B e antigen clearance and hepatitis B e antigen seroconversion during the on-treatment period. A: Hepatitis B e antigen (HBeAg) clearance rate; B: HBeAg seroconversion rate.

11.0% (9/82), 21.8% (19/87), 20.5% (18/88), 25.0% (22/88), and 25.0% (22/88) in the CLV group and 13.1% (11/84), 22.8% (21/92), 23.4% (22/94), 27.7% (26/94), and 27.7% (26/94) in the ETV group, respectively, at the same time points (Figure 4B). No significant difference was observed in the cumulative rates of HBeAg clearance or HBeAg seroconversion between the two groups.

Virologic breakthrough and adverse effects

Virologic breakthrough in the CLV group occurred in 7.6% (9/118) of the patients at 1-year after administration and 12.7% (15/118) of the patients at 2-years after administration, while no virologic breakthrough occurred in the ETV group throughout the treatment period (CLV group *vs* ETV group, $P < 0.05$). Most of the patients with virologic breakthrough showed a genotypic mutation at sites rt204 and rt180, either singly or combined, except for two patients, who showed mutation at site rt80. Only two patients (1.7%) in the CLV group experienced clinical myopathy, and they showed self-limited recovery with conservative management without dose reduction or discontinuation of CLV.

DISCUSSION

1-(2-deoxy-2-fluoro- β -L-arabinofuranosyl)thymine (CLV) is a nucleoside analogue with powerful antiviral activity against HBV; it inhibits DNA polymerase through the binding of clevudine-triphosphate to the polymerase^[16]. CLV is one of the currently available antiviral agents used as a 1st-line treatment for CHB patients in Korea, an HBV-endemic area. Although CLV was reported to be beneficial in virologic suppression on-treatment and off-treatment in CHB patients, these results were reported in a few clinical studies with small study populations and/or short follow-up periods^[9,10,17,18], and a large-scale, long-term comparative study should have been required for verifying the efficacy of CLV. With these considerations, the present study was conducted to assess relatively long-term (median follow-up period of 74 wk) clinical outcomes of CLV in antiviral-naïve CHB patients compared with ETV-treated patients during the same period, and this study was conducted in multiple regional institutes.

ETV is strongly recommended as a 1st-line treatment agent in the AASLD and EASL guidelines^[19,20], because it effectively suppresses HBV DNA and has a low genotypic resistance. Because of the globally established effects of ETV on HBV infection, the present study used clinical outcomes of ETV as a comparative standard for estimating the treatment efficacy of CLV in patients with CHB.

In this study, the cumulative rate of serum ALT normalization in CLV-treated patients increased from 83.9% at week 48 to 91.5% at week 96, which was similar to ETV-treated patients. Previous studies have reported that CLV showed a wide-range of ALT normalization (68.2% to 98.1% at week 24 and 77.3% to 87.5% at week 48)^[9,10,17,21-23]. The cumulative non-detection rate of serum HBV DNA in the CLV group also increased from 61.3% at week 24 to 72.6% at week 48 and 83.1% at week 96, which was similar to the ETV group. These rates seem to be slightly lower than previously reported HBV DNA non-detection rates^[17,21,24,25], most likely resulting from the use of a stricter detection assay of HBV DNA in our study. HBV DNA detection is mainly affected by the detection limit of the quantitation tool of HBV DNA. The real-time polymerase chain reaction method used in our study institutes is the most sensitive method for the quantitation of serum HBV DNA, and the lower limit of detection was 70 copies/mL.

CLV-treated patients showed excellent biochemical and virological responses during treatment and these response were not inferior to the responses shown in ETV-treated patients. Specifically, the titer decrement of serum HBV DNA was rapid and potent, as it was nearly at the 90% level of decrement at 96 wk after treatment for 24 wk. The rapid and potent suppression of HBV DNA is generally a significant indicator for maintaining an on-treatment virological response and sustained off-

treatment virological response^[26].

This study revealed that the HBeAg clearance rate in the CLV group increased from 33.3% at week 48 to 40.9% at week 96, and this pattern was similar to the ETV group and the results of previous CLV-related studies (11.1%-25.7% at 6 mo and 23.5%-48.0% at 12 mo)^[9,10,21,24]. The HBeAg seroconversion rate of CLV-treated patients, which was 21.8% at week 48 and 25.0% at week 96, was in agreement with ETV-treated patients and comparable to results from previous studies (7.6%-21.4% at 6 mo and 11.8%-16.9% at 12 mo)^[9,10,18,25]. Our study did not show a significant difference either HBeAg clearance or HBeAg seroconversion rates between the two groups treated with CLV or ETV.

In CLV-treated patients, the cumulative rate of virologic breakthrough increased from 7.6% at week 48 to 12.7% at week 96, while no virologic breakthrough occurred in ETV-treated patients during treatment. Most of the patients with virologic breakthrough had genotypic mutations of rtM204I or rtL180M, as previously reported in other CLV-associated studies. Rt204 of HBV DNA polymerase/reverse transcriptase is the main site relevant to CLV-associated genotypic mutation, with cross-resistance to other antiviral agents, such as lamivudine and telbivudine^[14]. The add-on or mono-switch to nucleotide analogs, such as tenofovir or adefovir, is a possible rescue therapy for CLV-resistant CHB patients; however, the choice of drug should be validated through further clinical studies. In addition to rt204, rt80, rt180, rt181, rt184, and rt270 have also been infrequently reported to be mutation sites conferring resistance to CLV. The cumulative rate of virologic breakthrough induced by CLV-associated genotypic mutation has been reported to be 0.7%-14.5% up to 12 mo^[18,21,27,28] and 24.4% up to 24 mo^[29], which is similar to the results of the present study. Biochemical breakthrough after virologic breakthrough may occur, but several months are required for ALT to increase^[30]. We did not compare the interval between biochemical breakthrough and virologic breakthrough because it only occurred in the CLV group. Most of the patients who showed virologic breakthrough were changed to or supplemented with another drug before ALT increased. The current treatment for virologic breakthrough for CLV is similar to that of lamivudine^[31].

The biologic mechanism of myopathy related to CLV has not been clearly identified, but mitochondrial dysfunction induced by mtDNA polymerase γ suppression of the phosphorylated CLV metabolite has been hypothesized to be involved^[32-34]. Although the incidence rate of myopathy has been reported to be variable (1%-14.6%)^[15,17,21,22,25,35], only 1.7% of CLV-treated patients in this study showed clinical myopathy to the tolerable extent of compliance without any modification of drug administration.

The present study has several restrictions. First, this is a retrospective study, which could not entirely escape

a non-intentional withdrawal of a number of study candidates prior to study analysis; therefore selection bias was not completely eliminated. This matter could be overcome through a comparative prospective study; Second, the detection limit of the serum HBV DNA titer was different in each institute that participated in this study, which could affect the cumulative non-detection rate of serum HBV DNA, despite the adoption of real-time PCR with a relatively sensitive quantitation indicator; Third, the analysis of clinical myopathy was insufficient because of irregular check-up for obtaining clinical information and data review only through medical records of the patients, which could not exclude the presence of myopathy of a subclinical nature; Fourth, the lack of off-treatment clinical responses suggests that an advanced clinical study sufficient to assess long-term outcomes, such as response maintenance, relapse, and disease progression after treatment, is needed.

In conclusion, we evaluated the clinical outcomes of CLV as a first-line treatment agent in antiviral-naive CHB patients. The on-treatment biochemical, virologic, and serologic responses of CLV, except for genotypic resistance with virologic breakthrough, were as good as and not inferior to the responses of ETV up to a 96-wk period. In the future, clinical data for the rescue therapy of CLV-related antiviral resistance should be mandatory and should be acquired through a large-scale prospective study. A study to determine the conditions under which virologic resistance could be minimized should be performed.

COMMENTS

Background

Chronic hepatitis B virus (HBV) infection is a serious health problem worldwide and a major risk factor for the development of liver cirrhosis and hepatocellular carcinoma, two grave complications that have a high morbidity and mortality. Although the seroprevalence rate of HBV in the South Korean population has gradually decreased since the development of the HBV vaccine in the 1980s, the rate is still high compared to Western countries.

Research frontiers

Clevudine (CLV) is one of the currently available antiviral agents used as a 1st-line treatment for chronic hepatitis B (CHB) patients in Korea, an HBV-endemic area. Although CLV was reported to be beneficial in virologic suppression on-treatment and off-treatment in CHB patients, these results were reported in a few clinical studies with small study populations and/or short follow-up periods, and a large-scale, long-term comparative study should have been required for verifying the efficacy of CLV.

Innovations and breakthroughs

Authors evaluated the clinical outcomes of CLV as a first-line treatment agent in antiviral-naive CHB patients. The on-treatment biochemical, virologic, and serologic responses of CLV, except for genotypic resistance with virologic breakthrough, were as good as and not inferior to the responses of entecavir up to a 96-wk period. In the future, clinical data for the rescue therapy of CLV-related antiviral resistance should be mandatory and should be acquired through a large-scale prospective study.

Applications

The study results suggest that long-term treatment outcomes of clevudine were not inferior to those of entecavir, except for virologic breakthrough in antiviral-naive CHB patients.

Peer review

Authors reported that long-term treatment outcomes of clevudine in antiviral-

naive patients with CHB. This report has the important information. The data is appropriately treated and text is clear although the retrospective study.

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Effects of hexahydrocurcumin in combination with 5-fluorouracil on dimethylhydrazine-induced colon cancer in rats

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Abstract

AIM: To investigate the effects of hexahydrocurcumin (HHC), and its combination with 5-fluorouracil (5-FU) on dimethylhydrazine (DMH)-induced colon cancer in rats.

METHODS: Male Wistar rats weighing 100-120 g were used as subject models. Aberrant crypt foci (ACF), early preneoplastic lesions of colon cancer, were induced

by subcutaneous injection of DMH (40 mg/kg) twice a week for two weeks. After the first DMH injection, rats were treated daily with vehicle ($n = 12$), curcumin (CUR) (50 mg/kg) ($n = 12$), HHC (50 mg/kg) orally ($n = 12$), and treated weekly with an intraperitoneal injection of 5-FU (50 mg/kg) ($n = 12$), or a combination of 5-FU plus CUR ($n = 12$) and HHC ($n = 12$) at the same dosage(s) for 16 wk. The total number of ACF and large ACF were assessed. Cyclooxygenase (COX)-1 and COX-2 expression were detected by immunohistochemistry in colon tissues. The quantitative data of both COX-1 and COX-2 expression were presented as the percentage of number of positive-stained cells to the total number of cells counted. Apoptotic cells in colon tissues were also visualized using the dUTP-biotin nick end labeling method. Apoptotic index (AI) was determined as the percentage of labeled nuclei with respect to the total number of nuclei counted.

RESULTS: The total number of ACF was highest in the DMH-vehicle group (1558.20 ± 17.37), however, the number of ACF was significantly reduced by all treatments, 5-FU (1231.20 ± 25.62 vs 1558.20 ± 17.37 , $P < 0.001$), CUR (1284.20 ± 25.47 vs 1558.20 ± 17.37 , $P < 0.001$), HHC (1086.80 ± 53.47 vs 1558.20 ± 17.37 , $P < 0.001$), DMH-5-FU + CUR (880.20 ± 13.67 vs 1558.20 ± 17.37 , $P < 0.001$) and DMH-5-FU + HHC (665.80 ± 16.64 vs 1558.20 ± 17.37 , $P < 0.001$). Interestingly, the total number of ACF in the combined treatment groups, the DMH-5-FU + CUR group (880.20 ± 13.67 vs 1231.20 ± 25.62 , $P < 0.001$; 880.20 ± 13.67 vs 1284.20 ± 25.47 , $P < 0.001$) and the DMH-5-FU + HHC group (665.80 ± 16.64 vs 1231.20 ± 25.62 , $P < 0.001$; 665.80 ± 16.64 vs 1086.80 ± 53.47 , $P < 0.001$) were significantly reduced as compared to 5-FU or each treatment alone. Large ACF were also significantly reduced in all treatment groups, 5-FU (111.00 ± 7.88 vs 262.20 ± 10.18 , $P < 0.001$), CUR (178.00 ± 7.33 vs 262.20 ± 10.18 , $P < 0.001$), HHC (186.60 ± 21.51 vs

262.20 ± 10.18, $P < 0.001$), DMH-5-FU + CUR (122.00 ± 5.94 *vs* 262.20 ± 10.18, $P < 0.001$) and DMH-5-FU + HHC (119.00 ± 17.92 *vs* 262.20 ± 10.18, $P < 0.001$) when compared to the vehicle group. Furthermore, in the DMH-5-FU + CUR and DMH-5-FU + HHC groups the formation of large ACF was significantly reduced when compared to CUR (122.00 ± 5.94 *vs* 178.00 ± 7.33, $P < 0.005$) or HHC treatment alone (119.00 ± 17.92 *vs* 186.60 ± 21.51, $P < 0.001$), however, this reduction was not statistically different to 5-FU monotherapy (122.00 ± 5.94 *vs* 111.00 ± 7.88, $P = 0.217$; 119.00 ± 17.92 *vs* 111.00 ± 7.88, $P = 0.619$, respectively). The levels of COX-1 protein after all treatments were not different from normal rats. A marked increase in the expression of COX-2 protein was observed in the DMH-vehicle group. Over-expression of COX-2 was not significantly decreased by 5-FU treatment alone (95.79 ± 1.60 *vs* 100 ± 0.00, $P = 0.198$). However, over-expression of COX-2 was significantly suppressed by CUR (77.52 ± 1.68 *vs* 100 ± 0.00, $P < 0.001$), HHC (71.33 ± 3.01 *vs* 100 ± 0.00, $P < 0.001$), 5-FU + CUR (76.25 ± 3.32 *vs* 100 ± 0.00, $P < 0.001$) and 5-FU + HHC (68.48 ± 2.24 *vs* 100 ± 0.00, $P < 0.001$) in the treated groups compared to the vehicle group. Moreover, CUR (77.52 ± 1.68 *vs* 95.79 ± 1.60, $P < 0.001$), HHC (71.33 ± 3.01 *vs* 95.79 ± 1.60, $P < 0.001$), 5-FU + CUR treatments (76.25 ± 3.32 *vs* 95.79 ± 1.60, $P < 0.001$) and 5-FU + HHC (68.48 ± 2.24 *vs* 95.79 ± 1.60, $P < 0.001$) markedly decreased COX-2 protein expression more than 5-FU alone. Furthermore, the AI in all treated groups, 5-FU (38.86 ± 4.73 *vs* 23.56 ± 2.12, $P = 0.038$), CUR (41.78 ± 6.92 *vs* 23.56 ± 2.12, $P < 0.001$), HHC (41.06 ± 4.81 *vs* 23.56 ± 2.12, $P < 0.001$), 5-FU + CUR (49.05 ± 6.75 *vs* 23.56 ± 2.12, $P < 0.001$) and 5-FU + HHC (53.69 ± 8.59 *vs* 23.56 ± 2.12, $P < 0.001$) significantly increased when compared to the DMH-vehicle group. However, the AI in the combination treatments, 5-FU + CUR (49.05 ± 6.75 *vs* 41.78 ± 6.92, $P = 0.192$; 49.05 ± 6.75 *vs* 38.86 ± 4.73, $P = 0.771$) and 5-FU + HHC (53.69 ± 8.59 *vs* 41.06 ± 4.81, $P = 0.379$; 53.69 ± 8.59 *vs* 38.86 ± 4.73, $P = 0.245$) did not reach significant levels as compared with each treatment alone and 5-FU monotherapy, respectively.

CONCLUSION: The combined effects of HHC with 5-FU exhibit a synergistic inhibition by decreasing ACF formation mediated by down-regulation of COX-2 expression.

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Key words: Hexahydrocurcumin; Curcumin analog; Colon cancer; Combination treatment; Cyclooxygenase-2; Apoptosis

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cancer in rats. *World J Gastroenterol* 2012; 18(47): 6951-6959 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i47/6951.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i47.6951>

INTRODUCTION

5-fluorouracil (5-FU) therapy is a conventional treatment for colorectal cancer and has been used for five decades. However, the toxicity of this agent to normal tissues is a major obstacle to successful cancer chemotherapy. To reduce its toxicity, combination treatment of 5-FU with minimally toxic substances derived from plants, such as genistein and geraniol, have been employed. While these are beneficial, they are also considered counterproductive in enhancing overall 5-FU efficacy^[1-3]. Curcumin (CUR), the major active substance in turmeric, which is derived from the rhizome of *Curcuma longa* L. may be an alternative^[4]. CUR is considered a suitable replacement for genistein and geraniol as it promotes higher 5-FU efficacy in the treatment of various cancer types, including colon cancer^[5,6]. The properties of CUR also highlight its usefulness, as it specifically inhibits the expression of cyclooxygenase (COX)-2, an enzyme highly expressed in a variety of human cancers, but does not suppress the expression of COX-1^[7,8]. CUR also has a low level of toxicity and is safe for the prevention/treatment of human colorectal cancer compared to other chemopreventive agents such as non-steroidal inflammatory drugs (NSAIDs)^[9]. Recently, Du *et al*^[3] showed that the synergistic effect of CUR combined with 5-FU inhibited the growth and reduced the level of COX-2 protein expression in HT-29 human colon cancer cells. However, while CUR is a very important agent in preventing and treating colorectal cancer, its characteristics-such as poor solubility, poor absorption in the gastrointestinal tract and rapid decomposition in human blood-make it unsuitable for pharmaceutical development^[10,11]. Therefore, CUR metabolites were synthesized in order to solve these problems^[12]. Hexahydrocurcumin (HHC) is one of the major metabolites of CUR. Previous studies found that HHC exhibited stronger antioxidant activity than CUR^[13]. Moreover, this compound inhibited the biosynthesis of prostaglandin (PGE₂) in lipopolysaccharide (LPS)-stimulated macrophages^[14]. PGE₂ is also a major product of COX-2 enzymes, which are involved in colorectal carcinogenesis. Interestingly, HHC decreased PGE₂ levels in phorbol ester-induced PGE₂ production in human colonic epithelial cells^[15]. Recently, our previous *in vitro* study showed that HHC markedly decreased the viability of HT-29 human colon cancer cells and down-regulated COX-2 mRNA production. Moreover, the combined effect of HHC with a low dose of 5-FU exerted a synergistic effect on the growth of HT-29 cells by markedly reducing cell viability and COX-2 mRNA to a greater degree than monotherapy^[16]. Therefore, the present study aimed to investigate the anti-colon carcinogenic effects of HHC in combination with 5-FU, with a focus on the expression of COX-2 in

dimethylhydrazine (DMH)-induced rat colon cancer.

MATERIALS AND METHODS

Animals

Male Wistar rats (National Animal Center, Salaya, Nakorn Pathom, Thailand) weighing 100-120 g were used. Animals were housed at 25 ± 2 °C under a 12-h light/dark cycle. All procedures were carried out in accordance with the Guidelines of Animal Care described by the Animal Center, Faculty of Medical Science, Naresuan University, Phitsanulok, Thailand.

Preparation of tested chemicals

CUR and HHC were prepared from *C. longa* L. Briefly, the curcuminoid mixture obtained from the rhizomes of *C. longa* L. was subjected to silica-gel column chromatography, using hexane-dichloromethane, dichloromethane and dichloromethane-methanol as eluents to afford CUR as the major constituent. Recrystallization was accomplished by dissolving the evaporated eluate with a small quantity of dichloromethane and ethanol was then added. CUR crystallized out as yellow needles with a melting point (m.p.) of 181-183 °C. HHC was synthesized from CUR by a catalytic hydrogenation reaction in ethanol for 5 h with palladium on charcoal as a catalyst. The product was isolated from tetrahydrocurcumin and octahydrocurcumin by silica-gel column chromatography followed by recrystallization with dichloromethane-*n*-hexane to provide a 45% yield of HHC as a white amorphous solid (m.p. 81-82 °C). The spectroscopic (IR, ¹H-NMR and mass spectra) data of the synthesized HHC were consistent with those in a previous report^[17]. CUR and HHC were then separately dissolved in propylene glycol and were ready for use.

Experimental protocols

The animals were randomly divided into seven groups. The animals in group 1, designated the negative control (normal group, $n = 10$), were fed daily with propylene glycol (PG). Group 2 to 7 served as the carcinogenic groups. In these groups, the animals were subcutaneously injected with DMH (Sigma-Aldrich, St. Louis, MO, United States) (40 mg/kg) twice a week for two wk in order to induce aberrant crypt foci (ACF). In group 2, (vehicle group, $n = 12$) rats were fed daily with PG. Group 3 (5-FU group, $n = 12$) received an intraperitoneal injection (*ip*) of 5-FU at a dose of 50 mg/kg body weight weekly. Group 4 (CUR group, $n = 12$) and 5 (HHC group, $n = 12$), were treated daily with intragastric administration of CUR and HHC at a dose of 50 mg/kg, respectively, whereas group 6 (5-FU + CUR, $n = 12$) and 7 (5-FU + HHC, $n = 12$) were the combined treatment groups, and were treated with 5-FU plus CUR and 5-FU plus HHC, respectively. All treatments were started after the first DMH injection and were given daily until week 16.

ACF analysis

Colons were dissected and washed with cold normal saline. They were then fixed in 10% buffer neutral formalin for 24 h. The colon tissues were stained with 2% methylene blue for 2 min and placed on a microscope slide with the mucosal surface up. ACF were examined under a light microscope at 40 × magnification and distinguished from surrounding normal crypts by the following characteristics: (1) increased size; (2) large luminal openings; (3) thickened epithelia; and (4) larger than adjacent normal crypts^[18,19]. In this study, the number of ACF and large ACF defined as containing 4 crypts or more (> 3 crypts/ACF)^[20], which represent advancing preneoplastic states, were counted.

Immunohistochemical analysis

COX-2: Paraffin sections from the distal portion of the colon were dewaxed in xylene and rehydrated through a graded series of alcohol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 15 min at ambient temperature. After washing in water, nonspecific binding sites were blocked with 5% bovine serum in phosphate-buffered saline (PBS) for 30 min at ambient temperature. The primary polyclonal antibody COX-2 (Cayman Chemical, Ann Arbor, MI, United States) was diluted in 1% BSA/Tris-Cl (at a 1:500 dilution) and incubated at 4 °C overnight. The slides were then gently rinsed with PBS and incubated with the secondary antibody (goat anti-rabbit immunoglobulins) conjugated with labeled polymer-horseradish (HRP) developed by the Envision system/HRP (DAKO cytometry, Glostrup, Denmark) as follows: the slides were incubated in peroxidase-labeled polymer for 30 min and substrate-chromogen for 10 min at ambient temperature. The nuclei were counterstained with Mayer's hematoxylin. COX-2 expression is presented as the percentage of the number of positive-stained cells to the total number of cells counted by image analysis (Image Pro[®]-plus).

COX-1: The paraffin sections were processed as in the COX-2 method up to the nonspecific binding sites step. The primary antibody COX-1 (Cayman Chemical, Ann Arbor, MI, United States) was diluted in 1% BSA/Tris-Cl (at a 1:1000 dilution) and incubated at 4 °C overnight. The slides were then rinsed with PBS and incubated with the secondary antibody conjugated with biotin (biotinylated anti-mouse immunoglobulins) for 1 h at ambient temperature. The slides were incubated with the ABC reagent (Vectastain, Burlingame, CA, United States) for 30 min at ambient temperature and developed by DAB peroxidase substrate (Vectastain, Burlingame, CA, United States). The nuclei were counterstained with Mayer's hematoxylin. The positive stained cells were then counted by image analysis software (Image Pro[®]-plus). COX-1 expression is presented as the percentage of the number of positive-stained cells to the total number of cells counted.

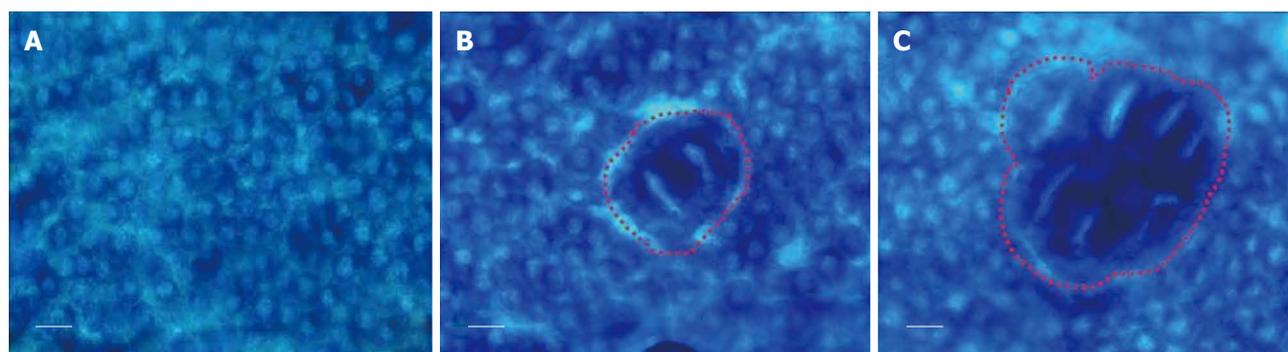


Figure 1 Topographical view of colon tissue. A: Normal colon tissue; B: Aberrant crypt foci (ACF) with 2 crypts; C: Large ACF (crypt multiplicity containing more than 3 crypts per ACF). Scale bars = 25 μ m.

Table 1 Effect of curcumin, hexahydrocurcumin, 5-fluorouracil and their combined treatment on aberrant crypt foci formation in rats exposed to dimethylhydrazine

Groups	Total ACF	Large ACF
Vehicle	1558.20 \pm 17.37	262.20 \pm 10.18
5-FU	1231.20 \pm 25.62 ^b	111.00 \pm 7.88 ^b
CUR	1284.20 \pm 25.47 ^b	178.00 \pm 7.33 ^b
HHC	1086.80 \pm 53.47 ^b	186.60 \pm 21.51 ^b
5-FU + CUR	880.20 \pm 13.67 ^{b,d,f}	122.00 \pm 5.94 ^b
5-FU + HHC	665.80 \pm 16.64 ^{b,d,h}	119.00 \pm 17.92 ^{b,h}

Each value is represented by mean \pm SE. ^b $P < 0.01$ vs vehicle group; ^d $P < 0.01$ vs 5-fluorouracil (5-FU) monotherapy; ^f $P < 0.01$ vs curcumin (CUR) monotherapy; ^b $P < 0.01$ vs hexahydrocurcumin (HHC) monotherapy. ACF: Aberrant crypt foci.

Apoptotic analysis

Apoptotic cells in the distal colon were visualized using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) method with the FragEL™ DNA fragmentation Detection kit (Calbiochem, Darmstadt, Germany). The deparaffinized tissue sections were permeabilized by Triton-X-100 and inactivated by endogenous peroxidases with 3% H₂O₂ in methanol at ambient temperature for 5 min. The tissue sections were incubated with TUNEL reaction mixture containing the TdT at 37 °C for 60 min. Slides were rinsed twice in PBS for 10 min and dried around the sample. The labeled DNA was detected by DAB solution for 10-15 min at ambient temperature. The nuclei were counterstained immediately with methyl green solution. Finally, the slides were washed and analyzed under a light microscope. Apoptotic index (AI) was determined as the percentage of the labeled nuclei with respect to the total number of nuclei counted.

Statistical analysis

The differences in mean values among the different groups were tested and the values are expressed as mean \pm SE. All data were tested by analysis of variance followed by the LSD test. All statistical calculations were carried out using SPSS (version 11.5) and a P -value < 0.05 was considered significant.

RESULTS

Effects of 5-FU, CUR, HHC and their combined treatments on ACF formation

ACF were distinguished from the surrounding normal crypts by their increased size, darker epithelial lining than normal crypts and large luminal opening (Figure 1). We assessed ACF formation in the colonic epithelia of experimental models by recording two parameters of ACF: (1) number of aberrant crypts and (2) large ACF containing 4 crypts or more (> 3 crypts/ACF), which are found in advancing preneoplastic states. The results showed that all rats developed ACF in the colon after DMH induction. The total number of ACF was highest in the DMH-vehicle group (1558.20 \pm 17.37) as shown in Table 1. HHC treatment alone markedly reduced the total number of ACF as compared to the vehicle group (1086.80 \pm 53.47 vs 1558.20 \pm 17.37, $P < 0.001$). In addition, the total number of ACF in the 5-FU (1231.20 \pm 25.62 vs 1558.20 \pm 17.37, $P < 0.001$) and CUR treatment alone groups (1284.20 \pm 25.47 vs 1558.20 \pm 17.37, $P < 0.001$) were significantly decreased as compared to the DMH-vehicle group. Interestingly, the total number of ACF in the combined treatment group, the DMH-5-FU + HHC group, was significantly reduced as compared to 5-FU or HHC treatment alone (665.80 \pm 16.64 vs 1231.20 \pm 25.62, $P < 0.001$; 665.80 \pm 16.64 vs 1086.80 \pm 53.47, $P < 0.001$). Similarly, the total number of ACF in the DMH-5-FU + CUR group was significantly reduced as compared to 5-FU or CUR treatment alone (880.20 \pm 13.67 vs 1231.20 \pm 25.62, $P < 0.001$; 880.20 \pm 13.67 vs 1284.20 \pm 25.47, $P < 0.001$). The large ACF were significantly reduced in all treatment groups, 5-FU (111.00 \pm 7.88 vs 262.20 \pm 10.18, $P < 0.001$), CUR (178.00 \pm 7.33 vs 262.20 \pm 10.18, $P < 0.001$), HHC (186.60 \pm 21.51 vs 262.20 \pm 10.18, $P < 0.001$), DMH-5-FU + CUR (122.00 \pm 5.94 vs 262.20 \pm 10.18, $P < 0.001$) and DMH-5-FU + HHC (119.00 \pm 17.92 vs 262.20 \pm 10.18, $P < 0.001$) when compared to the vehicle group. Furthermore, the DMH-5-FU + CUR and DMH-5-FU + HHC groups significantly reduced the formation of the large ACF when compared to CUR (122.00 \pm 5.94 vs 178.00 \pm 7.33,

$P < 0.005$) or HHC treatment alone (119.00 ± 17.92 vs 186.60 ± 21.51 , $P < 0.001$), however, this reduction was not statistically different to 5-FU monotherapy (122.00 ± 5.94 vs 111.00 ± 7.88 , $P = 0.217$; 119.00 ± 17.92 vs 111.00 ± 7.88 , $P = 0.619$, respectively).

Effects of 5-FU, CUR, and HHC alone and their combined treatments on COX-2 expression

Immunohistochemical assays demonstrated that COX-2 protein was mostly localized in the cytoplasm in a diffuse-granular pattern. Positive staining was seen as a brown stain at a low-power field (20X), indicated by the arrows in Figure 2A. The COX-2 protein was not observed in normal colon mucosal tissue. The over-expression of COX-2 was most marked in DMH-vehicle rats (Figure 2A). CUR and HHC alone and their combined treatments attenuated the over-expression of COX-2. The quantitative data from image analysis showed that marked COX-2 expression was found in the vehicle group ($100\% \pm 0.00\%$) (Figure 2B). Over-expression of COX-2 was not significantly decreased by 5-FU treatment alone ($95.79\% \pm 1.60\%$ vs $100\% \pm 0.00\%$, $P = 0.198$). However, over-expression of COX-2 was significantly suppressed by CUR ($77.52\% \pm 1.68\%$ vs $100\% \pm 0.00\%$, $P < 0.001$), HHC ($71.33\% \pm 3.01\%$ vs $100\% \pm 0.00\%$, $P < 0.001$), 5-FU + CUR ($76.25\% \pm 3.32\%$ vs $100\% \pm 0.00\%$, $P < 0.001$) and 5-FU + HHC ($68.48\% \pm 2.24\%$ vs $100\% \pm 0.00\%$, $P < 0.001$) compared to the vehicle group. Moreover, CUR monotherapy ($77.52\% \pm 1.68\%$ vs $95.79\% \pm 1.60\%$, $P < 0.001$), HHC monotherapy ($71.33\% \pm 3.01\%$ vs $95.79\% \pm 1.60\%$, $P < 0.001$), 5-FU + CUR ($76.25\% \pm 3.32\%$ vs $95.79\% \pm 1.60\%$, $P < 0.001$) and 5-FU + HHC ($68.48\% \pm 2.24\%$ vs $95.79\% \pm 1.60\%$, $P < 0.001$) markedly decreased COX-2 protein expression more than 5-FU treatment alone.

Effects of 5-FU, CUR, and HHC alone and their combined treatments on COX-1 expression

COX-1 expression was observed in the cytoplasm of most cells in colonic crypts in all animals. The COX-1 protein-labeled cells were analyzed by image analysis. The data revealed that the level of COX-1 protein in all treated rats was not different from the normal group (data not shown).

Effects of 5-FU, CUR, and HHC alone and their combined treatments on apoptosis

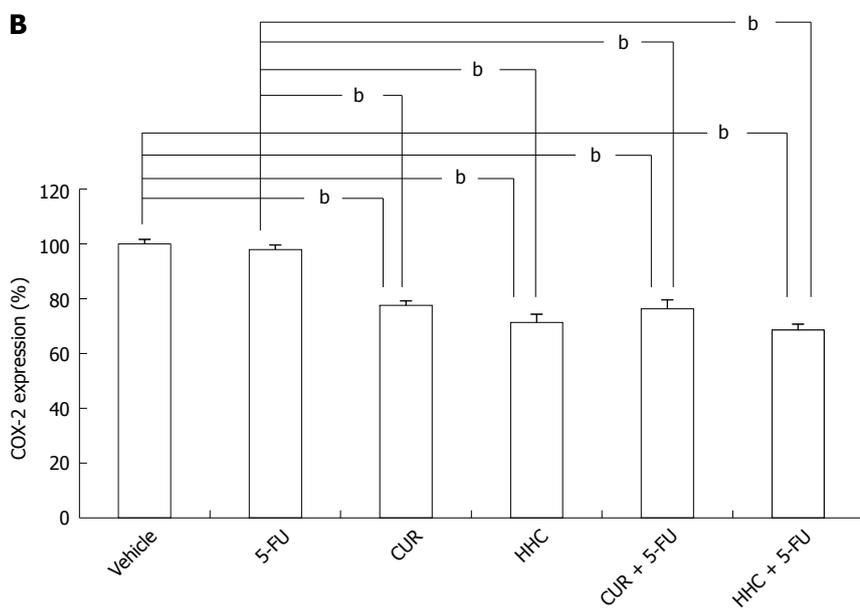
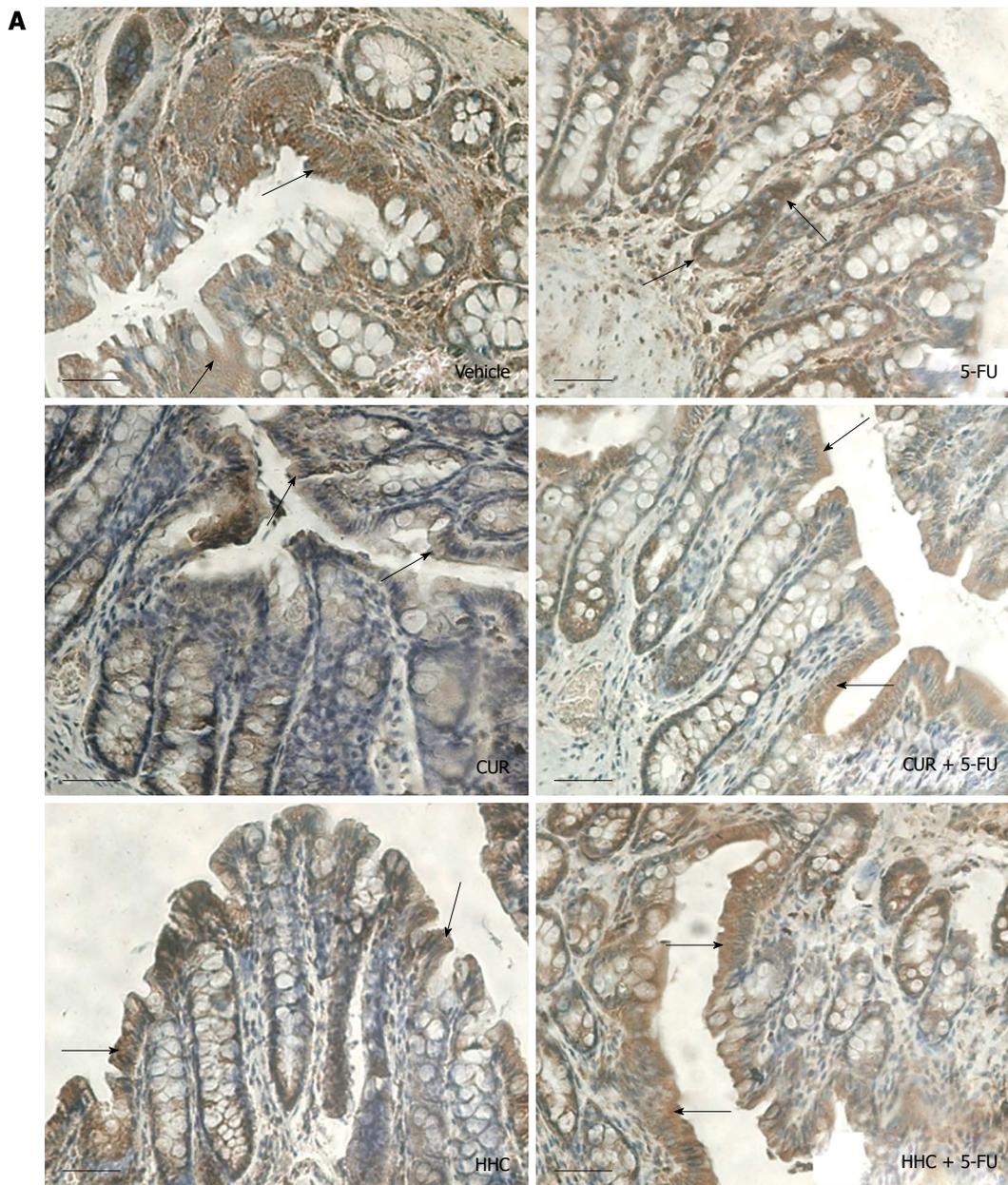
Apoptotic cells showed a strong dark or brown stain in normal colon mucosa (arrows in Figure 2C). In contrast, apoptotic cells were very low in DMH-vehicle group. 5-FU, CUR, and HHC alone and their combined treatments induced an increment of apoptotic cells in colon tissue. The quantitative data, as shown by the AI was highest in normal colon tissue ($75.71\% \pm 3.59\%$). In Figure 2D, the results show that AI was very low in the DMH-induced colon cancer vehicle group ($23.56\% \pm 2.12\%$). The AI in all treated groups were as follows: 5-FU ($38.86\% \pm 4.73\%$ vs $23.56\% \pm 2.12\%$, $P = 0.038$),

CUR ($41.78\% \pm 6.92\%$ vs $23.56\% \pm 2.12\%$, $P < 0.001$), HHC ($41.06\% \pm 4.81\%$ vs $23.56\% \pm 2.12\%$, $P < 0.001$), 5-FU + CUR ($49.05\% \pm 6.75\%$ vs $23.56\% \pm 2.12\%$, $P < 0.001$) and 5-FU + HHC ($53.69\% \pm 8.59\%$ vs $23.56\% \pm 2.12\%$, $P < 0.001$) and were significantly increased when compared to the DMH-vehicle group. However, the AI in the combined treatments which were: 5-FU + CUR ($49.05\% \pm 6.75\%$ vs $41.78\% \pm 6.92\%$, $P = 0.192$; $49.05\% \pm 6.75\%$ vs $38.86\% \pm 4.73\%$, $P = 0.771$) and 5-FU + HHC ($53.69\% \pm 8.59\%$ vs $41.06\% \pm 4.81\%$, $P = 0.379$; $53.69\% \pm 8.59\%$ vs $38.86\% \pm 4.73\%$, $P = 0.245$) did not reach significant levels as compared with each treatment alone and 5-FU monotherapy, respectively.

DISCUSSION

This study aimed to determine the anti-colon carcinogenic effects of HHC, a major metabolite of CUR, in combination with 5-FU therapy with a focus on the expression of COX-2 in DMH-induced colorectal cancer in rats. DMH induces DNA damage in the colon, ileum, and liver, and thus is a carcinogen in colorectal tissue^[21]. Several studies have reported that ACF are recognized as early preneoplastic lesions of colorectal origin. In addition, crypt multiplicity may indicate a step in colon carcinogenesis promotion^[22,23]. This study thus assessed the formation of ACF in DMH-induced colorectal cancer in a rat model. The results showed that both the total number of ACF and large ACF were highest in the DMH-vehicle group. Moreover, over-expression of COX-2 protein was most marked in this group. These findings agree with a previous study where an over-expression of COX-2 was detected in ACF, adenomas and carcinomas in azoxymethane-induced rat colon cancer^[24]. Several studies reported that COX-2 over-expression and up-regulation of the prostaglandins play a crucial role in carcinogenesis and angiogenesis^[25-27]. COX-2 is also known to have an anti-apoptotic effect on colon cancer cells *via* the activation of different signal transduction pathways^[28-30]. Therefore, ACF induction of COX-2 promotes colon carcinogenesis, and blockage of these processes is a strategy for colon cancer prevention and treatment.

In the present study, we demonstrated that HHC treatment alone significantly reduced the total number of ACF and large ACF compared with the vehicle. This result suggests that HHC has the ability to inhibit the initiation and promotion of steps in colorectal carcinogenesis which is similar to the property of CUR^[31]. Furthermore, HHC together with 5-FU exhibited a synergistic effect, with inhibition of total ACF numbers being higher than either HHC or 5-FU treatment alone. Therefore, it is reasonable to assume that HHC augments the growth inhibitory effect of 5-FU chemotherapy by inhibiting the initiation of colorectal carcinogenesis. This result also correlates with the synergistic inhibitory effect of CUR combined with 5-FU on the growth of HT-29 human colon cancer cells^[3].



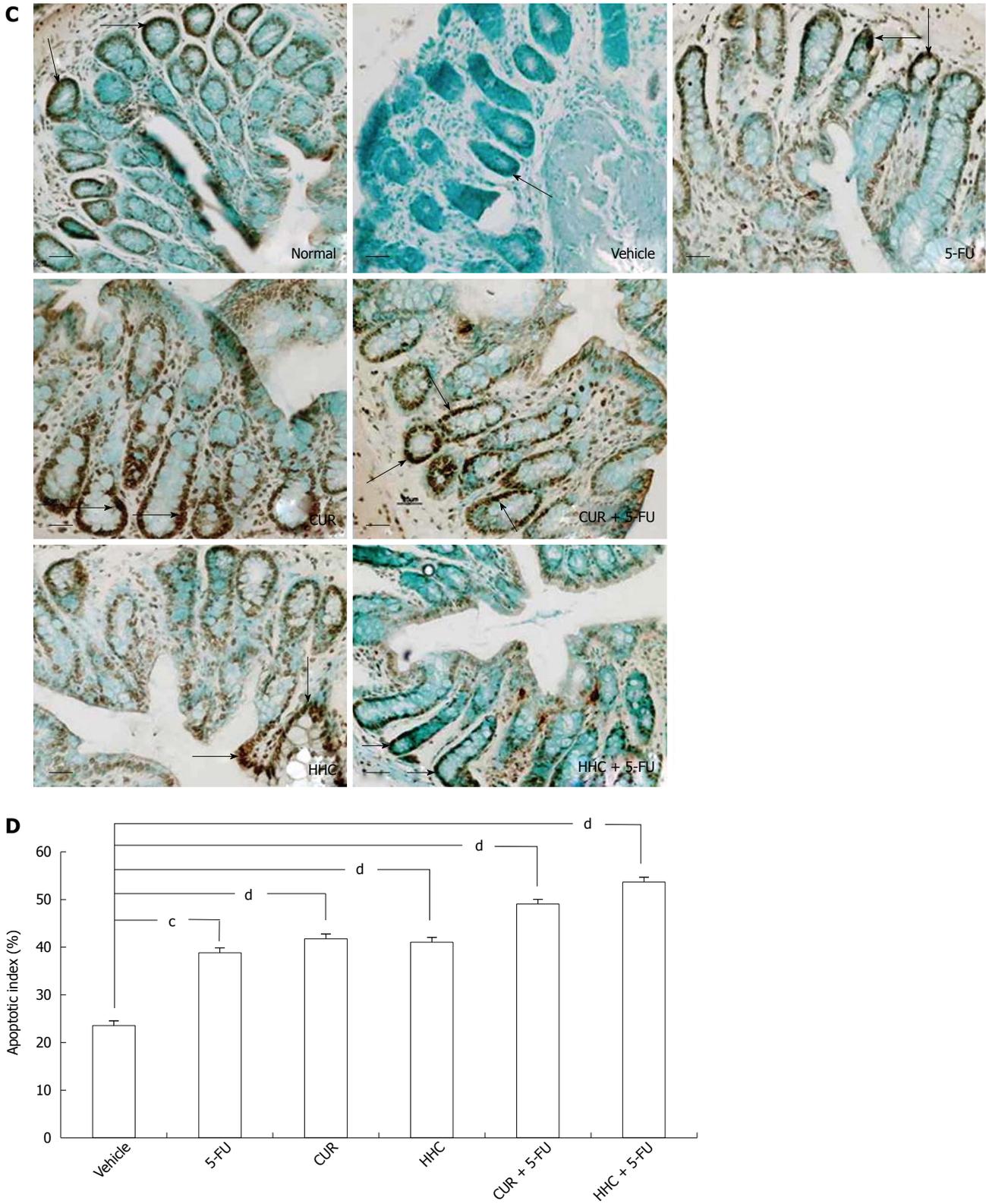


Figure 2 Effects of 5-fluorouracil, curcumin, and hexahydrocurcumin alone and their combined treatments on cyclooxygenase-2 protein expression and apoptosis. A: Immunohistochemical staining of cyclooxygenase-2 (COX-2) protein expression. Scale bars = 50 μ m; B: Quantitative data of COX-2 protein expression (%). Each value is represented by mean \pm SE. ^b $P < 0.01$ vs vehicle or 5-FU monotherapy; C: Apoptosis labeled using the dUTP-biotin nick end labeling method. Scale bars = 50 μ m; D: Apoptotic index (%). Each value is represented by mean \pm SE. ^c $P < 0.05$, ^d $P < 0.01$ vs vehicle group. 5-FU: 5-fluorouracil; HHC: Hexahydrocurcumin; CUR: Curcumin.

The anti-colorectal cancer mechanisms of HHC have not previously been reported. However, Shao *et al.*^[14]

reported that this compound inhibited the biosynthesis of PGE₂ in LPS-stimulated macrophages. Our study

found that HHC alone significantly decreased the COX-2 protein level compared to vehicle treatment which was similar to CUR treatment alone. The results support our recent report which showed that HHC is significantly responsible for the down-regulation of COX-2 mRNA synthesis, but does not alter the expression of COX-1 in HT-29 human colon cancer cells^[16]. Furthermore, HHC in combination with 5-FU has a higher level of potency against COX-2 mRNA expression when compared with cells treated with 5-FU or HHC alone. We therefore propose that HHC plays a significant role in inhibiting the expression of COX-2 protein in colorectal carcinogenesis, and when combined with 5-FU can lead to an overall decrease in the level of COX-2 protein and a reduction in the formation of ACF in the early stage of colorectal carcinogenesis through attenuation of COX-2 expression. When compared to the vehicle treatment group, this is an important distinction. Furthermore, this study found that the level of COX-1 protein after treatment with HHC alone and in combination with 5-FU was not different from normal rats. Therefore, HHC can be classified as a selective COX-2 inhibitor in contrast to some NSAIDs such as meloxicam, diclofenac, indomethacin which inhibit both COX-2 and COX-1 expression, causing injury to the gastric mucosa^[32,33]. We suggest that HHC treatment alone or in combination with 5-FU would not produce unwanted side effects in the colorectal cancer rat model, and thus HHC administration may be suitable for the long-term treatment and/or prevention of human colorectal cancer. However, we must consider that the relationship between COX-2 and carcinogenesis may involve several pathways, including conversion of procarcinogens into active carcinogens, inhibition of apoptosis, increase in tumor growth and invasiveness, and promotion of angiogenesis^[25-30,34]. Therefore, it is reasonable that COX-2 inhibitors may offer an important and powerful target for cancer prevention and treatment. In the present study, we also investigated whether HHC could induce colon cell apoptosis in DMH-induced colon cancer in rats. We showed that colon cell apoptosis in the DMH-injected group was significantly lower than that in normal rats and was very low in the vehicle-treated group, however, treatment with HHC alone resulted in greater levels of apoptosis. These results suggested that HHC may also suppress colorectal carcinogenesis through its ability to induce apoptosis, possibly by inhibiting COX-2 production. The combined treatment of HHC and 5-FU significantly increased apoptosis when compared to the vehicle group, but not when compared to the groups treated with HHC and 5-FU alone. These findings suggested that HHC combined with 5-FU did not have a synergistic effect on apoptosis induction in colorectal carcinogenesis in the DMH-induced rat model. Therefore, the combined effects of these agents on inhibition of ACF formation may be due to other modes of actions which need to be explored.

In summary, the evidence presented in this *in vivo* study gives new insight into the anti-carcinogenic activity

of HHC in this colorectal cancer model. Our findings indicate that the addition of DMH caused COX-2 over-expression resulting in resistance to apoptosis in colorectal tissues. HHC treatment alone and combined with 5-FU significantly suppressed the growth of colorectal cancer at the initiation step by down-regulating the expression of COX-2 and inducing apoptosis. Although the combined effect of HHC and 5-FU was similar to its progenitor, CUR, the greater bioavailability of HHC makes it more suitable for further pharmaceutical development in the prevention and therapy of colon cancer. Further studies should be encouraged to determine the mechanisms underlining these anti-cancer activities.

COMMENTS

Background

The toxicity of 5-fluorouracil (5-FU) chemotherapy in normal tissues is a major limitation of its treatment of colon cancer. To avoid the toxicity of chemotherapeutic agents, alternative treatments using chemopreventive natural medicine may be an option. The previous study showed that hexahydrocurcumin (HHC), a natural metabolite of curcumin (CUR), enhanced 5-FU in inhibiting the growth of HT-29 human colon cancer cells and down-regulated the expression of cyclooxygenase-2 (COX-2) mRNA *in vitro*. However, the effects of HHC combined with 5-FU *in vivo* have not yet been studied. The present study therefore aimed to investigate the anti-colon carcinogenic effects of HHC in combination with 5-FU on dimethylhydrazine (DMH)-induced colon cancer in rats, with a focus on the expression of the COX-2.

Research frontiers

This study involved testing the anti-colon carcinogenesis effect of HHC combined with the standard chemotherapeutic agent, 5-FU. Areas of this research included: studying the natural substance-HHC as an alternative or an adjunctive cancer therapy; testing the anti-carcinogenic effect against DMH-induced colon cancer in rats; measuring aberrant crypt foci (ACF) which are early preneoplastic lesions of colorectal cancer; measuring COX-2 protein, an important enzyme which promotes/initiates the colon carcinogenesis process; and detecting the apoptosis of colonic cells after these treatments.

Innovations and breakthroughs

In a previous study of 5-FU combined with CUR, it was found that this combined treatment had a synergistic effect on inhibiting the growth of a colorectal cancer cell line. However, the physical properties of CUR are unsuitable for pharmaceutical development. The authors thus synthesized HHC, a major metabolite of CUR, which shows greater bioavailability compared to CUR. In this study, HHC in combination with 5-FU was tested for the treatment of colorectal cancer. The authors found that HHC in combination with 5-FU suppressed the formation of ACF more than HHC or 5-FU treatment alone, which indicates that this combination shows a synergistic effect for anti-carcinogenic activity.

Applications

The results suggest that HHC combined with 5-FU can suppress the growth of colorectal cancer without any side effects. HHC plus 5-FU also showed a synergistic inhibitory effect to reduce ACF formation. Although the combined effect of HHC and 5-FU was similar to its progenitor, CUR, its greater bioavailability in colon cancer makes it more suitable for further pharmaceutical development in the prevention of colon cancer and its treatment.

Terminology

5-FU is a conventional cancer chemotherapeutic drug used to treat human colorectal cancer. Its toxicity to normal cells and long-term exposure to this drug shows higher resistance in cancer cells to treatment. CUR is the major yellow pigment in turmeric which is obtained from the rhizome of *Curcuma longa* L. HHC is one of the natural metabolites of CUR.

Peer review

This study examines the effects of HHC, a relatively low toxic CUR metabolite, on colonic ACF formation in a rat model induced by DMH to produce colorectal cancer. The authors suggest that HHC has similar effects to CUR and reduced ACF numbers by increasing apoptosis, inhibiting COX-2 protein expression but

no expression of COX-1. This is an interesting study that extends previous *in vitro* studies and suggests that HHC effectively reduced preneoplastic changes in an *in vivo* model of colorectal cancer.

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Endoscopic findings in patients with Schatzki rings: Evidence for an association with eosinophilic esophagitis

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Abstract

AIM: To investigate endoscopic findings in patients with Schatzki rings (SRs) with a focus on evidence for eosinophilic esophagitis (EoE).

METHODS: We consecutively approached all adult patients scheduled for elective outpatient upper endoscopy for a variety of indications at the German Diagnostic Clinic, Wiesbaden, Germany between July 2007 and July 2010. All patients with endoscopically diagnosed SRs, defined as thin, symmetrical, mucosal structures located at the esophagogastric junction, were prospectively registered. Additional endoscopic findings, clinical information and histopathological findings with a focus on esophageal eosinophilia (≥ 20 eosinophils/high power field) were recorded. The criteria for active EoE were defined as: (1) eosinophilic tissue infiltration ≥ 20 eosinophils/hpf; (2) symptoms of esophageal dysfunction; and (3) exclusion of other causes of esopha-

geal eosinophilia. Gastroesophageal reflux disease was excluded by proton pump inhibitor treatment prior to endoscopy. The presence of ≥ 20 eosinophils/hpf in esophageal biopsies in patients that did not fulfil the criteria of EoE was defined as esophageal hyper-eosinophilia.

RESULTS: A SR was diagnosed in 171 (3.3%; 128 males, 43 females, mean age 66 ± 12.9 years) of the 5163 patients that underwent upper gastrointestinal-endoscopy. Twenty of the 116 patients (17%) from whom esophageal biopsies were obtained showed histological hyper-eosinophilia (≥ 20 eosinophils/hpf). Nine of these patients (8 males, 1 female, mean age 49 ± 10 years) did not fulfill all diagnostic criteria of EoE, whereas in 11 (9%) patients with ≥ 20 eosinophils/hpf, a definite diagnosis of EoE was made. Three of the 11 patients (27%) with definite EoE had no suspicious endoscopic features of EoE. In contrast, in the 25 patients in whom EoE was suspected by endoscopic features, EoE was only confirmed in 7 (28%) patients. Patients with EoE were younger (mean age 41.5 ± 6.5 vs 50.5 ± 11.5 years, $P = 0.012$), were more likely to have a history of allergies (73% vs 29%, $P = 0.007$) and complained more often of dysphagia (91% vs 34%, $P = 0.004$) and food impaction (36% vs 6%, $P = 0.007$) than patients without EoE. Endoscopically, additional webs were found significantly more often in patients with EoE than in patients without EoE (36% vs 11%, $P = 0.04$). Furthermore, the SR had a tendency to be narrower in patients with EoE than in those without EoE (36% vs 18%, $P = 0.22$). The percentage of males (73% vs 72%, $P = 1.0$) and frequency of heartburn (27% vs 27%, $P = 1.0$) were not significantly different in both groups. The 9 patients with esophageal hyper-eosinophilia that did not fulfil the diagnostic criteria of EoE were younger (mean age 49 ± 10 years vs 58 ± 6 years, $P = 0.0008$) and were more likely to have a history of allergies (78% vs 24%, $P = 0.003$) than patients with < 20 eosinophils/hpf. Predictors of EoE were younger age, presence of dysphagia or food impaction

and a history of allergies.

CONCLUSION: A significant proportion of patients with SRs also have EoE, which may not always be suspected according to other endoscopic features.

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Key words: Schatzki ring; Dysphagia; Esophageal eosinophilia; Eosinophilic esophagitis; Food impaction

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INTRODUCTION

The prevalence of a lower esophageal Schatzki ring (SR) ranges from 4%-15%, depending on the diagnostic method and population investigated. In the majority of cases, it does not cause any symptoms, however, it is also one of the most common causes of intermittent dysphagia and food impaction^[1-3]. The etiology and pathogenesis still remain unclear. Gastroesophageal reflux disease has been suggested as an etiological factor^[4]. However, prospective studies have documented an association with gastroesophageal reflux disease (GERD) in less than two-thirds of patients^[5,6]. Therefore, additional pathogenetic factors should be considered.

More recently, an association of SRs with eosinophilic esophagitis (EoE) has been reported^[7,8], although the causal relationship between the two entities is under discussion^[9-11].

The aim of this study was to obtain additional endoscopic findings and assess the prevalence of EoE in patients with SRs.

MATERIALS AND METHODS

Patients

We consecutively approached all adult patients scheduled for elective outpatient upper endoscopy for a variety of indications at the German Diagnostic Clinic, Wiesbaden between July 2007 and July 2010. We recruited all patients in which we could endoscopically identify a lower esophageal SR. A SR was defined as a thin, symmetric, mucosal structure located at the esophagogastric junction (Figure 1A)^[12]. Data including sex, medical history, medications,

allergies, additional endoscopic findings and esophageal biopsy results were recorded.

After Institutional Review Board (IRB) approval was obtained (approved by the Gesellschaft zur Förderung der Forschung an der Deutschen Klinik für Diagnostik; GFF Approval No. IRB-2012-I), medical records were reviewed to assess symptoms, medical history, medications and endoscopic findings of all patients with SRs in whom esophageal biopsies were obtained to determine the prevalence of EoE. Patients with concomitant eosinophilic infiltration in the stomach or duodenum and patients with known Barrett's esophagus were excluded.

Endoscopy

Endoscopic findings for patients consenting to participate in the study were prospectively recorded in an electronic database (Clinic Win Data, E+L GmbH, Erlangen, Germany). All endoscopies were performed by senior gastroenterologists with a GIF 160 or GIF 180 Olympus upper endoscope (Olympus Corp., Hamburg, Germany) with an outer diameter of 9.5 mm. The internal diameter of the SR was estimated during endoscopy. A narrow ring was defined as being difficult to pass through with the endoscope and a wide ring was easy to pass through with the endoscope. A sliding hiatal hernia was diagnosed when gastric mucosa folds extended for more than 1.5 cm above the diaphragm^[13].

Erosive esophagitis was classified according to the criteria of the Los Angeles classification system^[14,15]. An esophageal web was defined as an eccentric, mucosal narrowing proximal to the esophagogastric junction having a maximum thickness of 1.5 mm^[16]. The diagnosis of a diverticulum was based on the presence of a pouch in the esophagus (Zenker's diverticulum: pouch in the pharyngo-esophageal area; midesophageal diverticulum: pouch in the mid esophagus; epiphrenic diverticulum: pouch just proximal to the diaphragm)^[16]. EoE was assumed with the presence of linear furrows, whitish exudates, trachealization or a small calibre esophagus^[17].

Following the standard procedure in our department, all patients in whom no otherwise obvious cause of their symptoms could be detected (e.g., reflux esophagitis, malignant or peptic strictures) and who had no recent exposure to anticoagulants had esophageal biopsies. This was done using a standard biopsy protocol, which included 2 duodenal biopsies, 2 gastric biopsies and a total of 4 esophageal biopsies (2 distal within 5-8 cm proximal to the esophagogastric junction, and 2 mid > 10 cm proximal to the esophagogastric junction).

Histopathological assessment

Biopsy specimens were submitted for routine processing and pathology evaluation with a request to evaluate for EoE. All histopathological analyses were performed by senior pathologists. The peak count of intraepithelial eosinophils/high power field ($\times 400$ magnifications on the Optiphot-2 microscope, ocular $\times 10$ with Plan $\times 40$ objective; Nikon, Japan) was determined in the area of

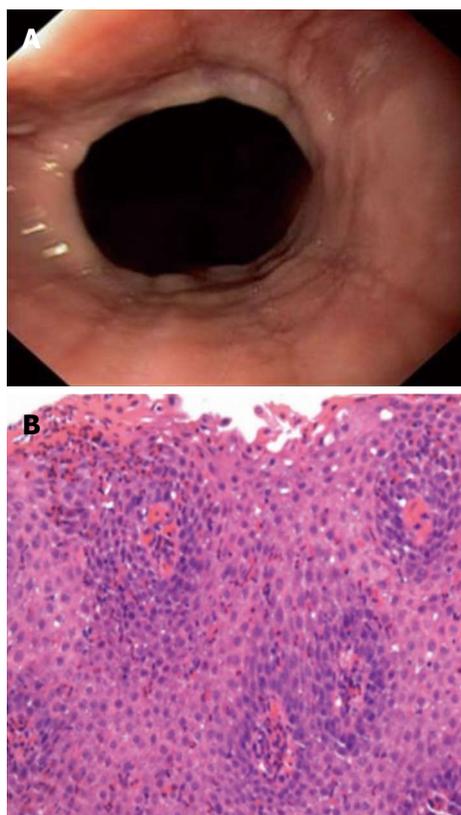


Figure 1 Endoscopic image and histological image of eosinophilic esophagitis. A: Endoscopic image showing a lower esophageal Schatzki ring and linear furrowing of the esophageal mucosa, an endoscopic feature associated with eosinophilic esophagitis; B: Histological image of an esophageal biopsy, showing eosinophilic esophagitis with numerous intraepithelial eosinophils (> 50 eosinophils/high power field, hematoxylin and eosin, $\times 400$).

highest density of eosinophils using the most densely populated hpf. Histological suspicion of EoE was the presence of 20 or more eosinophils/hpf^{10,17}. In biopsy specimens with ≥ 20 eosinophils/hpf, a thorough histopathological review was performed by a second senior pathologist according to further histological features associated with EoE, including a particular affiliation of eosinophils to aggregate in the surface layers of the epithelium, the presence of microabscesses (defined by ≥ 4 eosinophils/cluster), findings of epithelial hyperplasia (basal-zone expansion of 30% and papillary height elongation of $> 70\%$) and lamina propria fibrosis^{18,19} (Figure 1B).

Definition of EoE

Criteria of active EoE were defined as: (1) eosinophilic tissue infiltration ≥ 20 eosinophils/hpf; this cut-off was chosen as the study was designed prior to the publication of the AGA guidelines¹⁷; (2) symptoms of esophageal dysfunction (dysphagia, food impaction and PPI-resistant heartburn); and (3) exclusion of other causes of esophageal eosinophilia. Gastroesophageal reflux disease (GERD) was excluded by proton pump inhibitor treatment prior to endoscopy.

The presence of ≥ 20 eosinophils/hpf in esophageal

Table 1 Demographic and clinical characteristics of all enrolled patients with Schatzki rings n (%)

Variables	Schatzki ring ($n = 116$)
Age, yr (mean \pm SD)	55.2 \pm 4.9
Gender (M/F)	84/32
History of allergy	34 (29)
Dysphagia	47 (41)
History of food impaction	9 (8)
Heartburn	31 (27)
No symptoms of esophageal dysfunction	31 (27)

M/F: Male/female.

biopsies in patients that did not fulfil the criteria of EoE was defined as esophageal hypereosinophilia.

Statistical analysis

Numeric variables are described as the mean \pm SD and the number of observations (n). Categorical variables are described using frequencies (n) and percentages (%). In order to assess whether group differences were compatible with pure chance, we performed exploratory tests. Therefore, all reported P -values are descriptive. Association of age with categorical variables was assessed by the two-sample Wilcoxon test. Association of categorical variables was tested using Fisher's exact test. All statistical analyses were performed using SPSS 15.0 for Windows (SPSS Inc, Chicago, IL, United States).

RESULTS

During the study period, a total of 5163 patients underwent upper gastrointestinal-endoscopy in our department. A SR was diagnosed in 171 (3.3%) patients (128 males, 43 females, mean age 66 ± 12.9 years). Reflux esophagitis was present in 45 patients (who were then excluded from the analysis) and an additional 10 patients were excluded because of the presence of peptic stenosis ($n = 1$), Barrett's esophagus ($n = 6$), and esophageal malignancy ($n = 1$). Two patients were on anticoagulation therapy and therefore a biopsy specimen was not taken.

The clinical and demographic characteristics of the 116 patients enrolled are shown in Table 1.

The endoscopically estimated diameter of the lower esophageal ring was wide in 93 (80%) and narrow in 13 (11%) patients. The most frequent additional endoscopic finding was a sliding hiatal hernia in 95 (82%) patients. Sixteen (14%) of 116 patients showed esophageal webs and 3 (3%) patients had esophageal diverticula. One of the 3 patients with esophageal diverticula showed a Zenker's diverticulum and, in 2 patients, midesophageal diverticula were diagnosed. EoE was assumed in 25 (21%) patients because of the presence of specific endoscopic features.

In 20 (17%; 16 males, 4 females, mean age 39 ± 6 years) of the 116 patients, hypereosinophilia (≥ 20 eosinophils/hpf) could be shown in the esophageal biopsies. The results of the histopathological analysis of these

Table 2 Histopathological features

Patient ID	Age (yr)	Gender	Met the clinical criteria of EoE	Eosinophils/hpf	Further histological findings suspicious of EoE
1	35	M	Yes	25	H
2	57	M	Yes	25	S
3	37	M	Yes	40	H
4	34	M	Yes	60	M1, S, H
5	31	F	Yes	20	None
6	43	M	Yes	50	H
7	44	M	Yes	45	S, H
8	43	F	Yes	45	S, H, F
9	48	M	Yes	55	S, H, F
10	23	F	Yes	35	H
11	35	M	Yes	60	H
12	60	M	No	20	S, H
13	44	M	No	20	H
14	46	M	No	25	S, H, F
15	47	M	No	60	H
16	39	M	No	45	H
17	29	M	No	45	H
18	35	M	No	60	S, H
19	39	M	No	25	H
20	39	F	No	30	H

Histopathological features of the 20 patients with a SR and hypereosinophilia (≥ 20 eosinophils/hpf) in the biopsy specimen of the esophagus. M: Male; F: Female; EoE: Eosinophilic esophagitis; F: Fibrosis of lamina propria; H: Epithelial hyperplasia; M1: Microabscess; S: Superficial layering of eosinophils; hpf: High power field.

specimens are shown in Table 2. Biopsy specimens of the stomach and duodenum of these patients showed no evidence of eosinophilic gastroenteritis, Crohn's disease or infection.

Eleven of these 20 patients met the diagnostic criteria of EoE. Table 3 shows the baseline characteristics of the 11 patients with SRs and EoE. From the 25 patients in whom EoE was assumed because of the endoscopic features, EoE was confirmed in 7 (28%) patients. Three of the 11 patients (27%) with defined EoE had no suspicious endoscopic features of EoE.

When compared with the remainder of the 116 patients, the 11 patients with documented EoE were of younger age, more often showed an allergic predisposition, and complained to a larger degree of dysphagia and food impaction (Table 4). Whereas no gender difference (27% *vs* 28% females, $P = 1.0$) was demonstrated between both groups, EoE was diagnosed in 50% of the patients < 45 years of age *vs* 3% in patients 45 years or older (relative risk 10).

Endoscopically, additional webs were found significantly more often in patients with EoE than in patients without EoE (36% *vs* 11%, $P = 0.04$). Furthermore patients with EoE had a tendency to have narrower SR than patients without EoE (36% *vs* 18%, $P = 0.22$). Nine patients (8 males, 1 female, mean age 49 ± 10 years) showed ≥ 20 eosinophils/hpf in the histological examination, but did not fulfil the diagnostic criteria of EoE because gastroesophageal reflux disease could not be excluded prior to the esophageal biopsy sampling (6 patients) or the patients had no symptoms of esophageal dysfunc-

Table 3 Baseline characteristics

Patient ID	Age (yr)	Gender	Dysphagia	History of food impaction	History of allergy	Endoscopic findings suspicious of EoE
1	35	M	+	-	+	Furrows
2	57	M	+	-	+	None
3	37	M	+	+	-	Trachealization
4	34	M	-	+	+	Furrows, trachealization
5	31	F	+	+	+	Furrows
6	43	M	+	-	-	Whitish exudates
7	44	M	+	-	+	None
8	43	F	+	-	+	None
9	48	M	+	-	+	Furrows
10	23	F	+	-	+	Furrows
11	35	M	+	+	-	Furrows

Baseline characteristics of the 11 patients with a Schatzki ring and eosinophilic esophagitis (EoE). M: Male; F: Female.

Table 4 Demographics, clinical and endoscopic findings *n* (%)

Variable	SR with EoE (<i>n</i> = 11)	SR without EoE (<i>n</i> = 105)	<i>P</i> value
Age (yr, mean \pm SD)	41.5 \pm 6.5	50.5 \pm 11.5	0.012
Gender (M/F)	8/3	76/29	1.0
History of allergy	8 (72.7)	24 (29)	0.007
Dysphagia	10 (90.9)	36 (34.3)	0.004
History of food impaction	4 (36.4)	6 (5.7)	0.007
Heartburn	3 (27.3)	28 (26.7)	1.0
Ring narrow	4 (36.4)	19 (18.1)	0.22
Webs	4 (36.4)	12 (11.4)	0.044

Demographics and clinical and endoscopic findings for patients with a Schatzki ring (SR) with and without eosinophilic esophagitis (EoE). M: Male; F: Female.

tion (3 patients had abdominal pain). Four (44%) of the 9 patients with esophageal hypereosinophilia complained of dysphagia and one of the 4 also had heartburn. One (11%) of the 9 patients had a history of food impaction and another (11%) had heartburn. The endoscopically estimated diameter of the lower esophageal ring was wide in 5 (56%) and narrow in 4 (44%) patients and 3 (33%) patients exhibited additional esophageal webs. EoE was assumed in 4 (44%) of those patients because of the endoscopic features.

Patients with esophageal hypereosinophilia were younger (mean age 49 ± 10 years *vs* 58 ± 6 years, $P = 0.0008$), were more likely to have a history of allergies (78% *vs* 24%, $P = 0.003$) and were more frequently found to have additional webs (33% *vs* 9%, $P = 0.009$) and more narrow SRs (44% *vs* 16%, $P = 0.05$) than patients with < 20 eosinophils/hpf. Furthermore, patients with esophageal hypereosinophilia had a tendency to complain less often of heartburn (11% *vs* 27%, $P = 0.23$). The number of males (89% *vs* 71%, $P = 0.4$), frequency of dysphagia (44% *vs* 33%, $P = 0.5$) and rate of food impaction (11% *vs* 5%, $P = 0.4$) were not significantly different in both groups.

Three of the 6 patients in whom GERD could not be excluded prior to biopsy sampling had relief of their symptoms after proton pump inhibitor treatment in standard doses for 4 wk following the endoscopy.

DISCUSSION

In this study, the prevalence of a lower esophageal SR was 3.3%, which is in the lower range of that reported in the literature^[20]. SRs are seen in up to 14% of routine barium radiographs^[2], however, similar to the presented data, symptomatic rings are less common and endoscopic examinations seem to be less accurate in diagnosing a ring^[21,22]. This might be because endoscopic visualization of mucosal rings depends on proper distension of the esophagogastric region beyond the caliber of the ring, which is often not accomplished. This is especially true for the detection of wider rings^[23].

SRs are frequently associated with erosive reflux esophagitis. Histologically, this might be reflected by increased tissue eosinophilia^[24]. Therefore, those patients were excluded from further analysis. In 17.1% of the patients in whom esophageal biopsies were obtained, an esophageal hypereosinophilia with ≥ 20 eosinophils/hpf was demonstrated. However, there also was significant overlap with EoE in more than half of those patients.

As was demonstrated in a previous study with a different cohort of patients, a SR is frequently associated with other esophageal disorders^[25]. In the current investigation, erosive esophagitis was found in a quarter of the patients. Because of the frequent association, gastroesophageal reflux has been discussed by some authors as the main etiological factor for the development of a SR ring^[4,25], while others have pointed out that GERD is only present in less than two-thirds of these patients and, therefore, might not be the only cause for the development of the ring^[5,6]. However, inflammation, as a main pathogenic factor in the development of a SR, might be supported by the fact that, in the present study, esophageal hypereosinophilia with ≥ 20 eosinophils/hpf was a common finding in patients in whom esophageal biopsies were obtained, but only half of them met the diagnostic criteria for EoE according to the AGA guidelines^[17].

In 6 of the 9 patients with esophageal hypereosinophilia, gastroesophageal reflux disease could not be excluded prior to the esophageal biopsy sampling and 3 patients complained of abdominal pain instead of symptoms of esophageal dysfunction. Because of this, they could not be diagnosed with EoE. Although some of these patients might have GERD, it is possible that some of these patients had early EoE. Particularly with regard to the fact that in 3 patients with an esophageal hypereosinophilia there was a surface layering of eosinophils, and in one a lamina propria fibrosis could be demonstrated, the findings were more typical of EoE than GERD^[19]. Therefore, it could be assumed that the prevalence of EoE might actually be higher than 9% in the presenting group of patients with SRs. The association

between SRs and EoE in children was first described by Nurko *et al*^[7]. In a further prospective study that evaluated the etiology of esophageal bolus obstruction in adults, SRs were diagnosed in 9 of 37 patients, five (55.5%) of whom also exhibited EoE^[9]. In contrast, such an association was not found in the investigation of Sgouros *et al*^[9]. These discrepancies were explained by differences in life style and the age distribution of the populations under investigation^[10]. Especially the latter argument could be supported by the results of Mackenzie *et al*^[8]. Although it remains under debate whether the association between SRs and EoE is merely a coincidental finding of two common diseases or whether they share a common pathophysiology, in our opinion these data support the latter.

In the last decade, EoE has been increasingly recognized as a cause of dysphagia and food impaction. It is a chronic, immune/allergen mediated clinic-pathological disease of the esophagus characterized by dense eosinophilic inflammation^[8]. The prevalence of EoE in an asymptomatic European population has been described as 0.4% and as 6.5% in a US population undergoing upper endoscopy for a variety of indications^[26,27].

However, similar to the SR, the etiology of EoE is not completely understood and the connection between GERD and SRs, as well as the connection between GERD and EoE is still under debate^[22,28]. Nevertheless, it is important to be aware of this association because biopsies should be taken from the esophagus in patients with symptoms of esophageal dysfunction despite the presence of a SR. This may be obvious in cases that present with endoscopic features suspicious of EoE, such as linear furrows, trachealization, white plaques or a narrow caliber esophagus^[18,29], but EoE may be present even in the absence of such features. Similar to other studies^[30,31], the current investigation demonstrated that one-third of the patients do not present endoscopic features suspicious of EoE. Thus, the presence of EoE would have been missed in those patients if no biopsies had been obtained. We therefore suggest that routine biopsies should be taken from the esophagus in all symptomatic patients even in the presence of a SR, because EoE might be additionally present, even in the absence of other suggestive endoscopic features.

On the other hand, among the 25 patients that had endoscopic features suggestive of EoE, the diagnosis was confirmed by means of biopsy in only 28%. Therefore, endoscopic findings might not be reliable for supporting a diagnosis of EoE.

In the present study, the patients with SRs and EoE presented with symptoms of dysphagia, food impactions, and heartburn, all of which have been described as leading symptoms in both clinical entities^[3,18,32]. Similar to previous observations of EoE, patients with SRs and EoE were younger and had a history of allergies more often than patients with SRs alone^[18,29,30]. However, EoE was diagnosed in patients with SRs older than 50 years, in whom EoE was of unclear significance. It could be spec-

ulated that these patients had a late onset of the disease or that it represents a late diagnosis of a long-standing disease. The latter explanation might be more reasonable, due to the fact that EoE in adults is chronic and often indolent, especially when patients adapt their chewing habits over time^[33].

In contrast to other studies of EoE^[29,34], there was no gender preference in cases with SR and EoE, in comparison to those with SRs alone. This observation, however, might be due to a male preponderance for patients with SRs in the current investigation.

Endoscopically, patients with EoE had a tendency to have narrower rings than patients without EoE. Additional webs could be found significantly more often in patients with EoE than in patients without EoE. Although, 5%-15% of patients presenting with dysphagia are found to have esophageal webs^[16], the etiology is often unknown. At least one study also showed a possible association with EoE^[35], which was not confirmed by others^[28,36]. However, in our opinion, narrow SRs and esophageal webs should raise the suspicion of EoE.

This study has its limitation in that 24 h esophageal pH monitoring was not available; therefore, we cannot exclude the possibility that some of the patients with esophageal hypereosinophilia might have had GERD instead of EoE. We attempted to overcome this limitation by administering PPI before the endoscopy.

In summary, the current data strongly suggest that the association of SRs and EoE in adults is not a chance finding but is a rather frequent coincidence that should be considered whenever one is confronted with a patient exhibiting a SR. Endoscopic findings might not be reliable for supporting a diagnosis of EoE. Therefore, it appears advisable to obtain esophageal biopsies, not only in the presence of typical endoscopic features of EoE, but also whenever a SR is detected in symptomatic patients, especially when a ring is narrow or patients are younger and have a history of allergies.

COMMENTS

Background

The Schatzki ring (SR) is the most common cause of episodic dysphagia to solid food, but its etiology and pathogenesis remain unknown. The most common theory as to its origin suggests inflammation, with gastroesophageal reflux disease (GERD) as the main cause of inflammation. However, an association with GERD could only be documented in less than two-thirds of the patients, suggesting that additional pathogenetic factors must be considered.

Research frontiers

Eosinophilic esophagitis (EoE) is an emerging inflammatory esophageal disorder that can cause fibrosis with thickening of the esophageal mucosa, submucosa and muscularis. Therefore, EoE as a pathogenic factor for the development of SRs would support the "inflammation theory". However, the causal relationship between SRs and EoE in adults is controversial.

Innovations and breakthroughs

Recent reports have demonstrated an association of SRs with EoE in children. The present study shows that a significant proportion of adult patients with SRs also exhibit EoE and that endoscopic findings are not reliable for supporting a diagnosis of EoE. Furthermore, predictors of an association of SRs with EoE were younger age, presence of dysphagia or food impaction, narrow rings and a history of allergies.

Applications

The study results suggest that esophageal biopsies should be obtained, not only in the presence of typical endoscopic signs for EoE, but also whenever a SR is detected in symptomatic patients, especially if the ring is narrow and if the patient is younger and has a history of allergies.

Peer review

This paper enrolled one hundred seventy-one patients with endoscopically diagnosed SRs who were prospectively registered and followed. EoE was diagnosed in 9% of the patients with SRs, some of whom did not present with typical endoscopic features of EoE. Therefore esophageal biopsies are warranted in patients presenting with SRs, not only in the presence of endoscopic findings suggestive of EoE, but also whenever a SR is detected.

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Sex-dimorphic adverse drug reactions to immune suppressive agents in inflammatory bowel disease

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Abstract

AIM: To analyze sex differences in adverse drug reactions (ADR) to the immune suppressive medication in inflammatory bowel disease (IBD) patients.

METHODS: All IBD patients attending the IBD outpatient clinic of a referral hospital were identified through the electronic diagnosis registration system. The electronic medical records of IBD patients were reviewed and the files of those patients who have used immune suppressive therapy for IBD, i.e., thiopurines, methotrexate, cyclosporine, tacrolimus and anti-tumor necrosis factor agents (anti-TNF); infliximab (IFX), adalimumab (ADA) and/or certolizumab, were further analyzed. The reported ADR to immune suppressive drugs were noted. The general definition of ADR used in clinical practice comprised the occurrence of the ADR in the temporal relationship with its disappearance upon discontinuation of the medication. Patients for whom the required information on drug use and ADR was not

available in the electronic medical record and patients with only one registered contact and no further follow-up at the outpatient clinic were excluded. The difference in the incidence and type of ADR between male and female IBD patients were analyzed statistically by χ^2 test.

RESULTS: In total, 1009 IBD patients were identified in the electronic diagnosis registration system. Out of these 1009 patients, 843 patients were eligible for further analysis. There were 386 males (46%), mean age 42 years (range: 16-87 years) with a mean duration of the disease of 14 years (range: 0-54 years); 578 patients with Crohn's disease, 244 with ulcerative colitis and 21 with unclassified colitis. Seventy percent (586 pts) of patients used any kind of immune suppressive agents at a certain point of the disease course, the majority of the patients (546 pts, 65%) used thiopurines, 176 pts (21%) methotrexate, 46 pts (5%) cyclosporine and one patient tacrolimus. One third (240 pts, 28%) of patients were treated with anti-TNF, the majority of patients (227 pts, 27%) used IFX, 99 (12%) used ADA and five patients certolizumab. There were no differences between male and female patients in the use of immune suppressive agents. With regards to ADR, no differences between males and females were observed in the incidence of ADR to thiopurines, methotrexate and cyclosporine. Among 77 pts who developed ADR to one or more anti-TNF agents, significantly more females (54 pts, 39% of all anti-TNF treated women) than males (23 pts, 23% of all anti-TNF treated men) experienced ADR to an anti-TNF agent [$P = 0.011$; odds ratio (OR) 2.2, 95%CI 1.2-3.8]. The most frequent ADR to both anti-TNF agents, IFX and ADA, were allergic reactions (15% of all IFX users and 7% of all patients treated with ADA) and for both agents a significantly higher rate of allergic reactions in females compared with males was observed. As a result of ADR, 36 patients (15% of all patients using anti-TNF) stopped the treatment, with significantly

higher stopping rate among females (27 females, 19% vs 9 males, 9%, $P = 0.024$).

CONCLUSION: Treatment with anti-TNF antibodies is accompanied by sexual dimorphic profile of ADR with female patients being more at risk for allergic reactions and subsequent discontinuation of the treatment.

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Key words: Adverse drug reactions; Sexual dimorphism; Infliximab; Adalimumab; Inflammatory bowel disease

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INTRODUCTION

The existence of a sex dimorphic profile of adverse drug reactions (ADR) has been increasingly recognized in recent years. Several studies on various therapeutics pointed to differences between sexes in the incidence as well as character of ADR. In general, females seem to be more at risk for ADR to various medication, 70% of drug users with ADR in a large cohort of 2367 patients being women^[1]. In addition to this generally increased risk of ADR, female patients also differ from males in terms of types of ADR to a range of medication such as antiarrhythmics, antipsychotics, anti-retroviral drugs, and analgesics^[2,3].

A limited number of small size studies performed in the field of auto-immune diseases and transplant medicine suggested existence of a sexual dimorphic profile of ADR to immunosuppressive medication, but this has not been studied in depth. Male sex has been reported as a risk factor for nodular regenerative hyperplasia in inflammatory bowel disease (IBD) patients treated with azathioprine^[4]. In rheumatoid arthritis patients' population, males have been shown to be more at risk than females for methotrexate-associated interstitial pneumonia^[5] and to bacterial pneumonia complicating treatment with infliximab (IFX)^[6]. For females, a higher incidence of azathioprine-related alopecia has been reported in transplant recipients^[7]. In a pediatric population of Crohn's disease (CD) patients, female sex was one of the risk factors for infusion reactions to anti-tumor necrosis factor (anti-TNF) treatment^[8] and in an adult population of ankylosing spondylitis patients, females were more at

risk of discontinuation of anti-TNF agents^[9].

The sexual dimorphism of the immune responses is a generally accepted concept that has been studied predominantly in the context of the female predominance in autoimmune disorders^[10] with the most important factor determining this dimorphism being the immunomodulatory properties of sex hormones. Considering these differences between the two sexes in basic immune reactions, further modulation of immune response by the immunosuppressive medication might have sex-specific consequences, including the quantitative and qualitative differences in ADR to these agents.

The limitations resulting from ADR for further therapeutic strategy in patients with chronic inflammatory conditions such as IBD are important. The ADR occurring in up to 20% of IBD patients using immune suppressive and anti-TNF agents^[11,12] represent an important factor leading to the modulation or discontinuation of effective treatment. The thorough understanding of the underlying mechanism of ADR to these drugs, including the sex-related differences would help optimizing the therapy in these patients.

Therefore, in the present study we aimed to specifically determine the difference between male and female IBD patients in the occurrence and type of ADR to commonly used immunosuppressive agents, including 'classical' immune suppressive medication, i.e., thiopurines and methotrexate, as well as anti-TNF agents.

MATERIALS AND METHODS

Patients

All IBD patients attending the outpatient clinic of the Department of Gastroenterology and Hepatology of the Erasmus MC as of January 2009 were identified through the electronic diagnosis registration system. The medical records were reviewed with emphasis on details of drug treatment. Reported ADR to immune suppressive medication used for IBD were noted. Patients for whom the required information on drug use and ADR was not available in the electronic medical record and patients with only one registered contact and no further follow-up at the outpatient clinic were excluded.

Definition of ADR

All ADRs designated as such by the treating physician in the medical record were registered. The general definition of ADR used in clinical practice comprised the occurrence of the ADR in the temporal relationship with its disappearance upon discontinuation of the medication. In case of doubt about other concomitant factors contributing to the ADR a positive re-challenge was considered to be necessary for the event to be definitely categorized as ADR.

The ADR to immunosuppressive agents were divided into the following categories according to the type of symptom/event: gastro-intestinal, arthralgia and/or myalgia, cutaneous, infectious, malignancy, myelosuppression, hepatotoxicity, or pancreatitis. In case of anti-TNF

Table 1 Demographic characteristics and the use of medication (*n* = 843)

	Males ¹ <i>n</i> (%)	Females ¹	<i>P</i> value ²
Males/females (%males)	386 (46)	457	
Age (mean, range), yr	43 (16-87)	42 (18-87)	0.138
Duration of the disease (mean, range)	14 (0-48)	14 (0-54)	0.168
CD/UC/unclassified	233 (40)/141 (58) /12 (57)	345/103/9	< 0.0001
Immunosuppressive agents	265 (45)	321	0.652
Thiopurines	247 (45)	299	0.665
Methotrexate	73 (42)	103	0.203
Cyclosporine	26 (57)	20	0.170
Tacrolimus	0	1	
Anti-TNF	101 (42)	139	0.193
Infliximab	93 (41)	134	0.102
Adalimumab	39 (41)	60	0.328
Certolizumab	1 (20)	4	0.401

¹For each categorical variable number and percentage of males within the group is displayed; ²*P* values for test of the sex-related differences; two-sided χ^2 test for categorical variables and *t*-test for continuous variables. Anti-TNF: Anti-tumor necrosis factor agents; CD: Crohn's disease; UC: Ulcerative colitis.

agents, additional categories of allergic reactions, lupus-like syndrome, and injection-site reactions were used.

Gastro-intestinal ADR comprised abdominal pain recognized by the patient as different from the IBD-related pain, diarrhea, nausea, and vomiting. Cutaneous ADR were defined as any kind of reported skin abnormality that occurred in temporal relationship with the treatment and resolved after cessation of the medication. Remittent or opportunistic infections occurring during the immunosuppressive treatment were noted as infectious ADR. For malignancies, any malignancy that was revealed during the use of the treatment was categorized as ADR.

Myelosuppression was defined as leucopenia (leucocytes count < $4.0 \times 10^9/L$), and/or anemia (hemoglobin level < 8.5 mmol/L for males and < 7.5 mmol/L for females) and/or thrombocytopenia (thrombocytes count < $1.5 \times 10^{11}/L$). To categorize abnormal liver tests as hepatotoxic ADR, the increase of liver tests above 2 times upper normal value and absence of other causes, i.e., viral or autoimmune were required. Drug-induced pancreatitis was defined as a new abdominal pain and hyperamylasemia occurring during the treatment.

Any of the following symptoms occurring during or within one day after infusion alone or in combination were considered as allergic reactions: skin reactions, dyspnoea, chest pain, low blood pressure, angioedema, fever and/or chills. Dyspnoea, skin abnormalities and arthralgia/myalgia occurring later than two days after infusion were categorized separately as potential delayed allergic reactions. Any motoric or sensoric loss, paresthesia and/or seizures were categorized as neurological ADR. Lupus-like syndrome diagnosis was characterized as the combination of arthritis and/or flu-like symptoms or fever and presence of anti-nuclear and or anti-double strand DNA antibodies. Injection site reactions (appli-

cable for adalimumab-ADA) were defined as pain or local skin reaction after injection.

Non-specific ADR which could not be categorized according to these criteria were analyzed together and are further referred as others.

Demographic characteristics and the use of medication

In total, 1009 IBD patients were identified in the electronic diagnosis registration system. Out of these 1009 patients, 843 patients were eligible for analysis according to the exclusion criteria stated in the methods part. There were 386 males (46%), mean age 42 years (range: 16-87 years) with a mean duration of the disease of 14 years (range: 0-54 years); 578 patients with CD, 244 with ulcerative colitis and 21 with unclassified colitis. There were no differences between male and female patients with regard to age and disease duration; significantly more males suffered from ulcerative colitis (141 pts, 58% of all ulcerative colitis patients).

Seventy percent (586 pts) of patients used any kind of immunosuppressive agents during the disease course, the majority of the patients (546 pts, 65%) used thiopurines, 176 pts (21%) methotrexate, 46 pts (5%) cyclosporine and one patient tacrolimus. No differences between male and female patients were observed with regard to the frequency of use of immunosuppressive agents in general or of particular agent.

One third (240 pts, 28%) of patients were treated with anti-TNF, the majority of patients (227 pts, 27%) used IFX, 99 (12%) ADA and five patients certolizumab. There were no sex-related differences in the use of anti-TNF agents (Table 1).

Statistical analysis

The sex-related differences in categorical variable were analyzed statistically using two-sided χ^2 testing, for continuous variables a two-sided independent *t*-test was used. *P*-values < 0.05 were considered significant. The analysis was performed using SPSS PASW 17 software.

RESULTS

ADR to immune suppressive agents

In total 278 patients experienced ADR to immunosuppressive agents; of which 155 patients to thiopurines (28% of all thiopurine-treated patients), 44 to methotrexate (25%), 2 (4%) to cyclosporine, and 77 pts (32%) developed ADR to one or more anti-TNF agents. As the age may represent an important factor influencing the development of ADR, a subanalysis was performed to compare the mean age between the groups of patients with and without ADR to immune suppressive agents and to anti-TNF agents. The mean age between the patients with and without ADR did not differ neither in the groups with ADR to immune suppressive agents (mean \pm SEM, 41 ± 1 years in the group with ADR *vs* 40 ± 0.7 years in the group without ADR; *P* = 0.76) nor in the group with ADR to anti-TNF (mean \pm SEM, 37 ± 1.5 years in the group with ADR *vs* 40 ± 1 year in the group

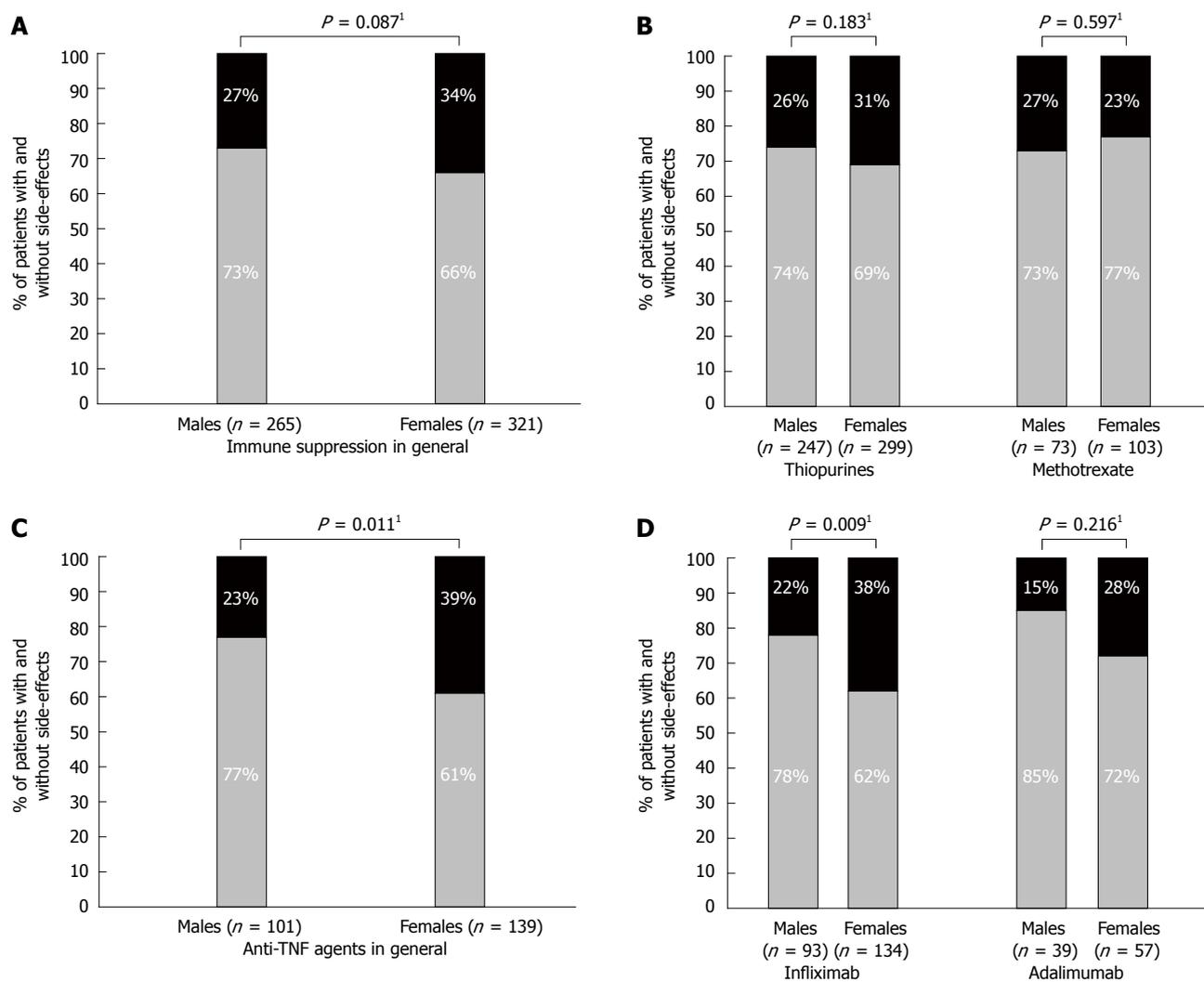


Figure 1 Relative percentages of male and female patients experiencing adverse drug reactions to “classical” immune suppressive agents (thiopurines, methotrexate, cyclosporine and tacrolimus) and anti-tumor necrosis factor agents. A: Adverse drug reactions (ADR) to immune suppressive agents in general; B: ADR to respective immunosuppressive agents; C: ADR to anti-tumor necrosis factor agents (anti-TNF) in general; D: ADR to respective anti-TNF agents. ¹Two-sided χ^2 test. Black bars: ADR present; Gray bars: No ADR.

without ADR to anti-TNF; $P = 0.79$).

Overall, there were no significant differences in the frequencies of the ADR to immune suppressive agents between males and females. In total, 27% of males (71 pts) experienced ADR to any kind of immune suppressive agent compared to 34% of females (108 pts; $P = 0.087$), (Figure 1A).

Among thiopurines users, 26% of males (63 pts) and 31% of females (92 pts) suffered from ADR to thiopurines ($P = 0.183$; Figure 1B). The most frequent ADR to thiopurines were myelosuppression, hepatotoxicity (both in 33 pts, 6%) and gastro-intestinal ADR (23 pts, 4%), (Table 2). No differences between male and female patients were observed with regard to specific type of ADR to thiopurines.

In the group of patients treated with methotrexate, 27% (20 pts) of males and 23% (24 pts) of females experienced ADR ($P = 0.597$; Figure 1B). The most frequent ADR to methotrexate were hepatotoxicity and gastro-intestinal ADR (both in 10 pts, 6%) (Table 2).

Out of 46 pts treated with cyclosporine, two female

patients experienced an ADR, one developed pseudo-membranous colitis following treatment but she was also treated with systemic steroids. The second patient had a cutaneous reaction to cyclosporine.

Among 77 pts who developed ADR to one or more anti-TNF agents, significantly more females (54 pts, 39% of all anti-TNF treated women) than males (23 pts, 23% of all anti-TNF treated men) experienced ADR to an anti-TNF agent [$P = 0.011$; odds ratio (OR) 2.2, 95%CI 1.2-3.8] (Figure 1C). In the subanalysis of respective anti-TNF agents, significantly more females (51 pts, 38% of all IFX-treated women) than males (20 pts, 22% of all IFX-treated men) suffered from ADR to IFX ($P = 0.009$; OR 2.2, 95%CI 1.2-4.1) (Figure 1D). Relatively more females than males experienced ADR to ADA (16 females; 28% vs 6 males; 15%), this difference was not significant though ($P = 0.216$) Figure 1D.

The most frequent ADR to both IFX and ADA were allergic reactions (15% of all IFX users and 7% of all patients treated with ADA) and for both agents a significantly higher rate of allergic reactions in females com-

Table 2 Categories of adverse drug reactions to immunosuppressive agents in general and stratified by sex *n* (%)

	Thiopurines	Thiopurines ^{1,2}	Methotrexate	Methotrexate ^{1,2}
Myelosuppression	33 (6)	12 (36)	NA	NA
Hepatotoxicity	33 (6)	19 (58)	10 (6)	6 (60)
Pancreatitis	10 (2)	2 (20)	NA	NA
Gastro-intestinal side-effects	23 (4)	8 (35)	10 (6)	6 (60)
Arthralgia and/or myalgia	11 (2)	5 (45)	2 (1)	1 (50)
Cutaneous side-effects	10 (2)	4 (40)	3 (2)	3 (100)
Infectious	6 (1)	3 (50)	2 (1)	1 (50)
Others ³	23 (4)	7 (30)	13 (7)	2 (15)
Not specified ⁴	6 (1)	3 (50)	4 (2)	1 (25)

¹No significant difference observed between males and females for none of the ADRs as tested with two-sided χ^2 test² Number and percentage of males in the group of patients with adverse drug reactions (ADR) to thiopurines; ³Number of all the people treated with the medicine; ⁴Headache, paresthesia, hair loss, fatigue, emotional instability, malaise; specific for methotrexate-injection site reaction. NA: Not available.

Table 3 Types of adverse drug reactions to respective anti-tumor necrosis factor agents in general and stratified by sex *n* (%)

	Infliximab ¹	Infliximab ^{2,3}	<i>P</i> value ²	Adalimumab	Adalimumab ^{2,3}	<i>P</i> value ²
Allergic reaction	33 (15)	7 (21)	0.045	7 (7)	0	0.039
Cutaneous SE	9 (4)	5 (56)	0.492	3 (3)	3 (100)	0.064
Neurological SE	4 (1.8)	0	0.146	2 (2)	0	0.512
Dyspnoea	4 (1.8)	0	0.146	1 (1)	0	1.00
Arthralgia and/or myalgia	11 (5)	4 (45)	1.00	3 (3)	1 (33)	1.00
Injection site reactions ⁴	NA	NA		3 (3)	1 (33)	1.00
Infectious SE	0	0		3 (3)	1 (33)	1.00
Malignancy	1 ⁶ (0.4)	16 (100)	0.41	0	0	
Lupus-like	1 (0.4)	0		0	0	
Others ⁵	8 (3.5)	2 (25)	0.476	0	0	

¹Number of all the people treated with the medicine; ²Two-sided χ^2 test to compare the differences in the frequencies of specific adverse drug reaction between males and females; ³Number of males; ⁴In case of adalimumab; ⁵Abdominal pain, hair loss, fatigue, malaise, emotional instability; ⁶Testis carcinoma. NA: Not available.

pared with males was observed (Table 3). There were no other sex-specific ADR observed to anti-TNF agents.

Of 77 patients experiencing ADR to an anti-TNF agent, 36 pts stopped the treatment (47%; overall discontinuation rate 15%), 27 pts (35%) switched to another anti-TNF agent, and 14 pts (18%) continued the treatment. As a result of ADR, 27 females (19% of all anti-TNF-treated women) stopped the treatment compared with 9 males (9% of all anti-TNF-treated men; $P = 0.024$). Furthermore, significantly higher proportion of females (21 pts, 15% of all anti-TNF-treated women) switched to another anti-TNF agents compared with males (6 pts, 6% of all anti-TNF-treated men; $P = 0.026$) significantly more frequently than males.

In the subanalysis of the influence of disease type and the use of immune suppression as a co-medication with anti-TNF treatment, no differences were found between the group of patients with ADR to anti-TNF compared with patients without ADR. Overall, 138 patients (58%) were on the combo therapy. Out of these 138 patients, 43 patients (31%) developed ADR to anti-TNF *vs* 34 patients (33%) with ADR from the group not using combo therapy ($P = 0.78$).

DISCUSSION

In this large retrospective study, we studied sex differences in the frequency and types of ADR to immune suppressive medication in IBD patients. In contrast to thiopurines and methotrexate with similar rates of ADR in both sexes, we observed a sex dimorphic profile of ADR to anti-TNF agents with higher frequency of ADR among female IBD patients compared with male patients. With regard to particular types of ADRs, females experienced more often allergic reactions to the most frequently used anti-TNF agents, IFX and ADA. In addition, these ADR have led to discontinuation of treatment more frequently in females than males, thus substantially limiting the long term use of anti-TNF agents by female patients.

The landmark randomized controlled trials on IFX and ADA efficacy and safety did not reveal sex dimorphic profile of ADR to anti-TNF agents^[13-17]. However, the design of these studies with rather short follow-up might underestimate the overall incidence of ADR and immune-mediated ADR occurring at long term in particular. This would subsequently limit the sample size to study specific risk factors for the development of ADR.

Another source of information on ADR, the safety registry with inclusion of patients being at the physicians' discretion are difficult to interpret with regard to sex differences in ADR incidence due to the possible selection bias and also a rather short follow-up^[18]. Interestingly, in a large retrospective, real-life study on long-term safety of IFX for the treatment of IBD, female sex was shown to be an independent risk factor for the development of hypersensitivity reactions and dermatological ADR to IFX^[12]. In addition, one small size study with pediatric CD patients determined female sex as one of the risk factors for infusion reactions^[8]. Thus, our results, in line with previous reports, suggest that female IBD patients are at specific risk for hypersensitivity reactions to monoclonal anti-TNF antibodies.

For each drug group with sex dimorphic ADR profile specific considerations for underlying mechanisms are applicable. The basic pharmacokinetic differences between the genders were at first considered to cause the predominance of ADR to some drugs in females, but over the past years, it became evident that sex hormones interacted with the particular drugs' metabolism and mechanisms of action^[3]. The sex hormones greatly influence the immune responses which might also account for the sex differences in ADR to immune system modulating therapy. However, in the present study, only biological anti-TNF agents and no other immunosuppressive treatment showed a specific ADR sex dimorphic profile. In addition, the particular ADR presenting more frequently in females were hypersensitivity reactions, suggesting a female-specific immunogenic potential related to biological therapy.

ADR to monoclonal anti-TNF antibodies have been shown to be related to the development of antibodies against these agents. Anti-infliximab as well anti-adalimumab antibodies are found in the sera of patients with loss of response and/or ADR to IFX or ADA^[19-23], suggesting thus the humoral immune response as underlying mechanism of IFX- and ADA- related immunogenicity. Interestingly, some studies analyzing the sex differences in the humoral response to vaccinations showed higher antibody titers in females^[24,25] and more local and systemic adverse reactions to influenza and rubella vaccines have been observed in women compared with men^[26,27]. The underlying mechanism of these sex differences in humoral immune response in general is not elucidated thus far, but taken all these observational data together, it is tempting to speculate that the use of monoclonal antibodies against TNF- α might result in higher anti-anti-TNF antibodies formation rate in female patients which in turn would lead to the female-specific higher incidence of immune-mediated ADR.

This immunogenic potential of anti-TNF agents resulting in ADR has important consequences for the management of IBD patients using these drugs. In this study, up to 50% of patients experiencing ADR discontinued the treatment, with the overall discontinuation rate of all patients using anti-TNF agents being 15%. There

are thus considerable potential limitations of long term use of these otherwise effective drugs. Understanding the underlying mechanisms, one of which might be female-specific immunogenicity would subsequently help to identify patients at risk and modify the therapeutic strategy accordingly.

To our knowledge, this is the first report studying the sex differences in ADR profile to immunosuppressive medication in a large cohort of patients with immune-mediated disease. The main limitation of our study is its retrospective design in which reporting bias of ADR is inevitable due to the lack of a standardized protocol that would ensure a meticulous screening of every treated patient in a prospective study design. This might particularly affect a study dealing with sex-related differences due to the psychosocial specificities of the two sexes in reporting ADRs. On the other hand, we analyzed sex-related ADRs profile to several immunosuppressive drugs in this large cohort. We found the sex dimorphism specifically applying only for anti-TNF agents and no other medication which would be the case if this reporting bias ensuing from retrospective design was substantially to modify the findings.

In conclusion, female IBD patients are at increased risk of hypersensitivity reactions to anti-TNF agents compared with males. These ADR have important clinical consequences as they lead to the discontinuation of the treatment in half of the patients experiencing these reactions. Further research, with a specific consideration of sex dimorphism of the immunogenic potential of anti-TNF agents is warranted in order to improve the clinical management of patients at risk for ADR.

COMMENTS

Background

Adverse drug reactions (ADR) to immune suppressive agents used for inflammatory bowel disease (IBD) limit the treatment in up to 20% of patients. For various drugs, sex dimorphic profile of ADR has been documented but this has not been studied in depth in case of immune suppressive medication.

Research frontiers

Sex has great impact on the various physiological and pathophysiological processes including pharmacokinetics and pharmacodynamics. The immune responses differ between men and women and therefore, it is presumable that the response to immune suppressive treatment may have specific profile in male and female patients. This could specifically apply for the ADR that are immune-mediated, such as the reactions to biological therapy. With the current situation of immunogenicity of biologicals being the main limitation of this otherwise effective therapy, identifying patients with high immunogenic potential is one of the possible strategies to enhance the overall success of anti-tumor necrosis factor agents (anti-TNF) therapy.

Innovations and breakthroughs

In the present study, authors show that female IBD patients are more at risk for allergic reactions to infliximab and adalimumab compared with male patients. This sex-dimorphic profile of ADR seems to be specific to biological therapy and not a general feature of immune suppressive medication since no sex-specific ADR to thiopurines and methotrexate were observed. This increased risk for ADR to biological has been reported previously in small sample size studies of patients with various immune-mediated conditions but has not been studied as a particular phenomenon in IBD patients.

Applications

The described phenomenon of increased immunogenic potential of anti-TNF

agents in females compared to males has important consequences for the management of IBD patients using these drugs. In this study, up to 50% of patients experiencing ADR to anti-TNF discontinued the treatment, with the overall discontinuation rate of all patients using anti-TNF agents being 15%. There are thus considerable potential limitations of long term use of these otherwise effective drugs. Understanding the underlying mechanisms, one of which might be female-specific immunogenicity would subsequently help to identify patients at risk and modify the therapeutic strategy accordingly.

Peer review

This retrospective study in a cohort of 843 patients is aimed to specifically determine the difference between male and female IBD patients in the occurrence and type of ADR related to immunosuppressive agents (thiopurines, methotrexate, cyclosporine, tacrolimus), and anti-TNF agents (infliximab, adalimumab, certolizumab). The results indicate that IBD women are at increased risk for allergic reactions in the course of anti-TNF treatment.

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Diagnosis of intestinal tuberculosis using a monoclonal antibody to *Mycobacterium tuberculosis*

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Abstract

AIM: To investigate the utility of immunohistochemical (IHC) staining with an antibody to *Mycobacterium tuberculosis* (*M. tuberculosis*) for the diagnosis of intestinal tuberculosis (TB).

METHODS: We retrospectively identified 10 patients (4 males and 6 females; mean age = 65.1 ± 13.6 years) with intestinal TB. Clinical characteristics, including age, gender, underlying disease, and symptoms were obtained. Chest radiograph and laboratory tests, including sputum Ziehl-Neelsen (ZN) staining, *M. tuberculosis* culture, and sputum polymerase chain reaction (PCR)

for tubercle bacilli DNA, as well as Tuberculin skin test (TST) and QuantiFERON-TB gold test (QFT), were examined. Colonoscopic records recorded on the basis of Sato's classification were also reviewed, in addition to data from intestinal biopsies examined for histopathological findings, including hematoxylin and eosin staining, and ZN staining, as well as *M. tuberculosis* culture, and PCR for tubercle bacilli DNA. For the present study, archived formalin-fixed paraffin-embedded (FFPE) intestinal tissue samples were immunohistochemically stained using a commercially available species-specific monoclonal antibody to the 38-kDa antigen of the *M. tuberculosis* complex. These sections were also stained with the pan-macrophage marker CD68 antibody.

RESULTS: From the clinical data, we found that no patients were immunocompromised, and that the main symptoms were diarrhea and weight loss. Three patients displayed active pulmonary TB, six patients (60%) had a positive TST, and 4 patients (40%) had a positive QFT. Colonoscopic findings revealed that all patients had type 1 findings (linear ulcers in a circumferential arrangement or linear ulcers arranged circumferentially with mucosa showing multiple nodules), all of which were located in the right hemicolon and/or terminal ileum. Seven patients (70%) had concomitant healed lesions in the ileocecal area. No acid-fast bacilli were detected with ZN staining of the intestinal tissue samples, and both *M. tuberculosis* culture and PCR for tubercle bacilli DNA were negative in all samples. The histopathological data revealed that tuberculous granulomas were present in 4 cases (40%). IHC staining in archived FFPE samples with anti-*M. tuberculosis* monoclonal antibody revealed positive findings in 4 patients (40%); the same patients in which granulomas were detected by hematoxylin and eosin staining. *M. tuberculosis* antigens were found to be mostly intracellular, granular in pattern, and primarily located in the CD68⁺ macrophages of the granulomas.

CONCLUSION: IHC staining with a monoclonal antibody to *M. tuberculosis* may be an efficient and simple diagnostic tool in addition to classic examination methods for the diagnosis of intestinal TB.

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Key words: Colonoscopy; Intestinal tuberculosis; Immunohistochemistry; Monoclonal antibody; *Mycobacterium tuberculosis*

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INTRODUCTION

Tuberculosis (TB), a chronic granulomatous infectious disease caused by *Mycobacterium tuberculosis* (*M. tuberculosis*), is still a significant cause of morbidity and mortality worldwide. Due to the increasing prevalence of human immunodeficiency virus (HIV) infection and immunosuppressive therapy for various diseases, the global incidence of TB has increased^[1].

The gastrointestinal tract is a common site of extrapulmonary TB. The ileocecal region is frequently involved in most patients diagnosed with intestinal TB, although the diagnosis of intestinal TB is often difficult because of its diverse clinical manifestations and very low positivity using current diagnostic tests including Ziehl-Neelsen (ZN) staining and *M. tuberculosis* culture from intestinal tissue samples^[2]. More recently, detection of tubercle bacilli DNA by polymerase chain reaction (PCR) has been developed as a diagnostic tool with excellent sensitivity and specificity in respiratory specimens. However, diagnosis by PCR in clinical settings still requires validation^[3]. Therefore, diagnosis is generally made on the basis of the classical histopathological demonstration of a caseating epithelioid cell granuloma, which is suggestive of TB. However, it may be difficult to differentiate intestinal TB from Crohn's disease based on this technique due to the fact that intestinal TB and CD have similar clinical, colonoscopic, and pathological findings. Although it is well known that caseating granulomas are a feature of TB, and non-caseating granulomas are that of CD, the prevalence of caseation is low in clinical settings for intestinal tuberculous granulo-

mas^[4,5].

The present study was conducted to investigate the utility of immunohistochemical (IHC) staining with a species-specific monoclonal antibody to the 38-kDa antigen of the *M. tuberculosis* complex to diagnose intestinal TB in archived formalin-fixed paraffin-embedded (FFPE) intestinal tissue sections of suspected intestinal TB patients.

MATERIALS AND METHODS

Patients

We retrospectively identified 10 patients (4 males and 6 females; mean age, 65.1 ± 13.6 years) with intestinal TB between 1996 and 2011. All cases were obtained from the archives of the Department of Infectious, Respiratory, and Digestive Medicine at the University of the Ryukyus Hospital, Okinawa, Japan. The diagnosis of intestinal TB was made by at least one of the following criteria: (1) a positive culture of *M. tuberculosis* from the intestinal tissue; (2) histopathological demonstration of acid-fast bacilli (AFB) in the intestinal tissue; (3) histopathological demonstration of a caseating epithelioid cell granuloma in the intestinal tissue; (4) detection of tubercle bacilli DNA by PCR from the intestinal tissue; and (5) typical endoscopic features together with a favorable response to a trial of antituberculous therapy. These patients were all treated with a full course of anti-tuberculosis therapy (rifampicin, isoniazid, ethambutol, pyrazinamide) following diagnosis. The clinical and colonoscopic records of these patients were obtained, as well as archived FFPE intestinal tissue sections. This study was approved by the Ethics Committee of our institute.

Colonoscopy and histopathology

Colonoscopy was performed with standard colonoscopes (Olympus, Tokyo, Japan). All patients diagnosed with intestinal TB were examined from the rectum to terminal ileum after lavage bowel preparation with a polyethylene glycol electrolyte solution. Colonoscopic findings were recorded on the basis of Sato's classification^[6]. Open ulcers or erosions were classified into 4 types: type 1 (linear ulcers in a circumferential arrangement or linear ulcers arranged circumferentially with mucosa showing multiple nodules), type 2 (round or irregular-shaped isolated small ulcers arranged circumferentially without nodules), type 3 (multiple erosions restricted to the colon), and type 4 (small aphthous ulcers or erosions restricted to the ileum). Healed lesions in the ileocecal area were also recorded, including the patulous ileocecal valve (PV), pseudodiverticular deformity (PD), and atrophic mucosal area (AMA) with multiple ulcer scars^[6]. During colonoscopy, biopsy specimens were obtained in a routine fashion using standard forceps. The specimens were prepared for ZN staining, tuberculous culture, PCR for tubercle bacilli DNA, and hematoxylin and eosin (HE) staining.

Table 1 Clinical, laboratory and bacteriologic findings in patients with intestinal tuberculosis

Case	Age/gender	Underlying disease	Symptoms	Chest radiograph	Sputum ZN stain	Sputum culture	Sputum PCR	TST	QFT
1	38/F	Epilepsy	Fever	Active TB	+	-	+	+	ND
2	66/F	Lumber neuralgia	Diarrhea	Normal	-	-	-	+	ND
3	81/F	Cecal cancer	Diarrhea, weight loss	Active TB	+	-	-	ND	ND
4	72/M	Post-herpes neuralgia	Abdominal pain	Normal	-	-	-	+	+
5	43/M	Diabetes mellitus	Diarrhea	Normal	-	-	-	-	ND
6	76/M	Gout	Diarrhea	Normal	-	-	-	+	+
7	58/M	Ulcerative colitis	Diarrhea	Normal	-	-	-	-	-
8	74/F	Gastric ulcer	Weight-loss	Active TB	+	-	-	+	+
9	74/F	Colonic polyp	None	Normal	-	-	-	ND	+
10	70/F	Rectal cancer	Diarrhea	Inactive TB	-	-	-	+	ND

TB: Tuberculosis; ZN: Ziehl-Neelsen; PCR: Polymerase chain reaction; TST: Tuberculin skin test; ND: Not determined; QFT: QuantiFERON-TB gold test; M: Male; F: Female.

IHC staining

IHC staining was performed using the IgG1 type mouse monoclonal antibody against the 38-kDa antigen of the *M. tuberculosis* complex (Vector Laboratories, Burlingame, CA, United States). 5 µm thick sections were prepared from formalin-fixed, paraffin-embedded tissue. IHC was carried out using the VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA, United States) as described elsewhere^[7-11]. Briefly, after deparaffinization and rehydration, the sections were exposed to antigen retrieval (Target retrieval solution pH 6.0, DakoCytomation, CA, United States) in high temperature water (98 °C) for 30 min, and then cooled for 20 min at room temperature. Endogenous peroxidase activity was inhibited by incubating the sections with hydrogen peroxide for 20 min. To prevent non-specific binding, these sections were incubated in normal mouse serum for 20 min. Primary antibody (anti-*M. tuberculosis* mouse monoclonal antibody in 1:80 dilutions) was applied to the sections overnight. This step was followed by washing and 40-min incubation with a biotinylated secondary antibody. These sections were then subjected to an avidin biotin-peroxidase complex for 40 min. Visualization was performed using ImmPACT DAB (Vector Laboratories, Burlingame, CA, United States), which was applied for 10 s. Sections were counter-stained with hematoxylin. A negative control in which the primary antibody was substituted with antibody diluent was used. IHC expression of the *M. tuberculosis* antigen was evaluated under light microscopy for the distribution of stain in the cytoplasm of epithelioid histiocytes and multinucleated giant cells. Additional IHC staining was performed to evaluate the relationship between *M. tuberculosis* antigen and macrophage distribution in the colonic specimens. These paraffin sections were stained with anti-*M. tuberculosis* rabbit antibody (Abcam, Cambridge, MA) at a 1:200 dilution, and the pan-macrophage marker CD68 antibody (Leica Microsystem, Buckinghamshire, United Kingdom) at a 1:80 dilution using the streptavidin-biotin peroxidase method as described previously.

RESULTS

Clinical features

Clinical features of the 10 patients are summarized in Table 1. No patients were immunocompromised, including immunosuppressive medication use and HIV infection. Primary symptoms were diarrhea and weight loss. Although no patients had respiratory symptoms, chest radiograph and sputum ZN staining in 3 patients revealed active pulmonary TB. Six patients (60%) had a positive Tuberculin skin test (TST) and four patients (40%) had a positive QuantiFERON-TB gold test (QFT), of which three patients had both a positive TST and QFT test.

Colonoscopic, bacteriological, and histopathological findings

Colonoscopic, bacteriological, and histopathological findings of the 10 patients are summarized in Table 2. All patients had type 1 findings (linear ulcers in a circumferential arrangement or linear ulcers arranged circumferentially with mucosa showing multiple nodules), all of which were located in the right hemicolon and/or terminal ileum (Figure 1A). Seven patients (70%) had concomitant healed lesions, including PV, PD, or AMA, in the ileocecal area (Figure 1B). No AFB was detected with ZN staining of the intestinal tissue samples. Tuberculous culture and PCR for tubercle bacilli DNA were negative in all intestinal tissue samples.

In the histopathological studies, tuberculous granulomas were identified in 4 cases (40%) (Figure 2A). IHC staining with anti-*M. tuberculosis* monoclonal antibody was positive in these same 4 patients (40%). The mycobacterial antigens were mainly detected as granular cytoplasmic staining in the epithelioid cells and giant cells within granulomas (Figure 2B). All samples in which granulomas were detected by HE staining were positive for TB by IHC staining. As for the relationship between *M. tuberculosis* antigens and macrophage distribution in the colonic consecutive specimens, the mycobacterial

Table 2 Colonoscopic, bacteriologic and histopathological findings in patients with intestinal tuberculosis

Case	Type	Colonoscopic or macroscopic findings			Histopathological findings		
		Location	PV, PD, AMA	AFB in ZN staining	Culture/PCR	Granuloma	TB by IHC staining
1	1	TI, C, A	PV, AMA	-	-	-	-
2	1	TI, C, A	PV, AMA	-	-	+	+
3	1	TI	-	-	-	+	+
4	1	C, A	PV, PD, AMA	-	-	-	-
5	1	TI, C, A, T, D, S	PV, PD, AMA	-	-	-	-
6	1	TI	-	-	-	-	-
7	1	TI, C, A, T, D, S	PV, PD, AMA	-	-	-	-
8	1	TI, C	PV, AMA	-	-	+	+
9	1	TI, C, A, S	PD, AMA	-	-	-	-
10	1	TI	-	-	-	+	+

TI: Terminal ileum; C: Cecum; A: Ascending colon; T: Transverse colon; D: Descending colon; S: Sigmoid colon; PV: Patulous ileocecal valve; PD: Pseudodiverticular deformity; AMA: Atrophic mucosal area; AFB: Acid-fast bacilli; ZN: Ziehl-Neelsen; PCR: Polymerase chain reaction; IHC: Immunohistochemical; TB: Tuberculosis.

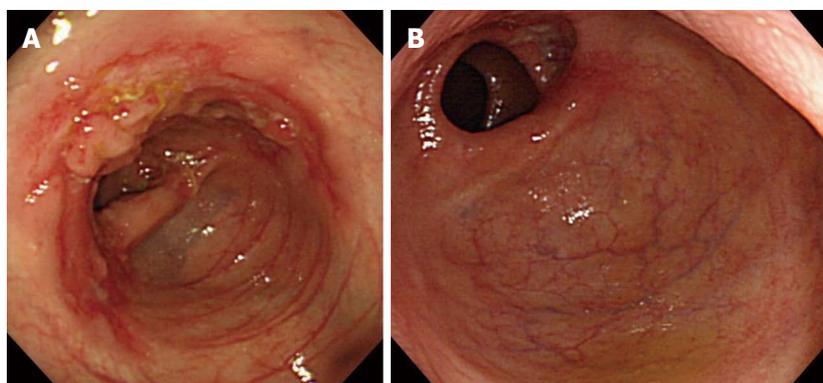


Figure 1 Typical colonoscopic views of intestinal tuberculosis. A: Colonoscopy shows a circumferential ulcer with edematous flared nodules in the ascending colon (patient 1); B: Colonoscopy shows a whitish mucosal area with an absence of the normal vascular pattern of healed ulcer scars in the ascending colon. Note the concomitant active ulcers in the proximal colon (patient 2).

antigens were seen predominantly as coarse granular immunopositive material in CD68⁺ macrophage cytoplasm (Figure 2C, D).

Clinical course and outcome

All patients were suspected to have intestinal TB on the basis of clinicopathologic findings. The regimen for TB was combined chemotherapy containing isoniazid, rifampicin, and ethambutol for six months and pyrazinamide for two months. All patients responded to antituberculous therapy and follow-up colonoscopies showed improvement of the colonic lesions.

DISCUSSION

This study has highlighted several important considerations. Most patients in this study had abdominal symptoms, and although no patients had respiratory symptoms, 4 (40%) had concomitant active or inactive pulmonary TB, consistent with prior reports in which pulmonary TB was apparent in less than 25% of patients with intestinal TB^[2,12]. Based on these findings, we should be aware of the possibility of tuberculous involvement in multiple organs despite apparent symp-

toms.

Although the TST has long been used as a reliable diagnostic examination, the recently developed QFT has been increasingly applied. There is controversy as to whether or not the QFT is effective for the diagnosis of extrapulmonary TB. Kim *et al.*^[13] reported that in a prospective study of 128 patients, QFT was a limited but useful aid in combination with the TST in the diagnosis of intestinal TB. In agreement with their findings, the QFT and TST had a good agreement in our study.

In this study, the vast majority of cases were colonoscopically diagnosed with TB. Similar to previous studies^[6,14], all patients in our study had a type 1 appearance among colonoscopic findings. Although this type has been established as a reliable colonoscopic feature, recent studies have emphasized that healed lesions in the ileocecal area, including PV, PD, and AMA with multiple ulcer scars, can coexist with active tuberculous inflammation. Sato *et al.*^[6] reported that 91% of patients in their study have AMA lesions, and so concluded that the AMA was the most frequently recognized endoscopic manifestation of intestinal TB. Our data, in which 60% of patients had these lesions, correlates strongly with their findings.

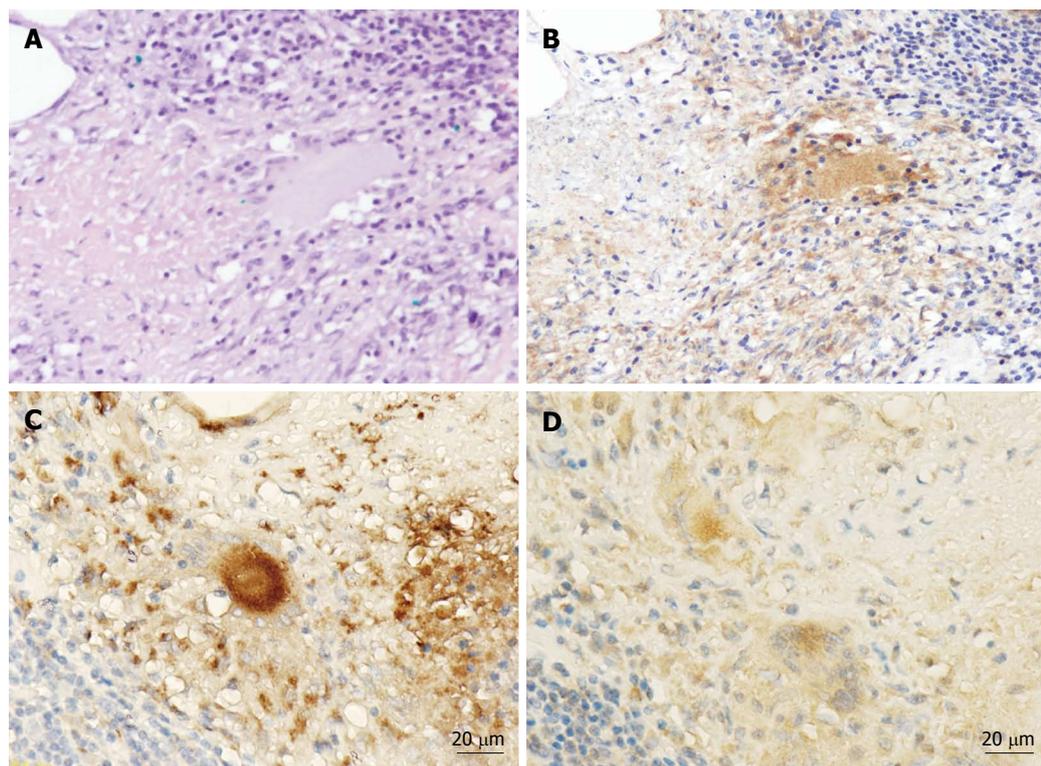


Figure 2 Histopathological views of tuberculous granuloma and localization of the mycobacterial antigen in the colonic lesion (patient 8). A: A granuloma, surrounded by inflammatory lymphocytes, is present in the lamina propria (hematoxylin and eosin, × 200); B: Immunohistochemical staining view of the colonic specimen (× 200). Note that the mycobacterial antigens (brownish granular matter) are present in the cytoplasm of epithelioid histiocytes and multinucleated giant cells in the granuloma; C: Cells stained with the pan-macrophage marker CD68 antibody are present in the granuloma (× 400); D: The mycobacterial antigens (brownish granular matter) are present in the cytoplasm of the macrophages in the granuloma (× 400).

Numerous studies have compared the diagnostic methods of intestinal TB^[15]. A study by Sekar *et al.*^[16] evaluated the role of PCR in the laboratory diagnosis of different forms of extrapulmonary TB in comparison with conventional bacteriologic techniques. They found an 18%, 22%, and 63% sensitivity for smear, culture, and PCR, respectively. A recent study clearly demonstrated that using real-time PCR technology with fluorescence resonance energy transfer probes has much higher sensitivity for the detection of tubercle bacilli DNA in tissue biopsy samples and FFPE surgically resected tissues of the gastrointestinal tract than traditional PCR^[17]. Our negative AFB and PCR tests may be due to a small biopsy sample size.

There have been several studies on IHC staining for the diagnosis of TB in which the most used polyclonal antibodies^[18,19] resulted in false-positive reactions. There are only two studies that have evaluated IHC staining with a species-specific monoclonal antibody to 38-kDa antigen of *M. tuberculosis* complex in archival FFPE tissue sections of intestinal TB. Goel *et al.*^[7] demonstrated that 2/2 samples tested had positive IHC staining (100%), whereas Ince *et al.*^[8] demonstrated IHC positivity in 6/8 (75%) intestinal tuberculous granulomas. Our results revealed 4 positive samples using IHC staining out of 4 tuberculous granulomas, whereas ZN staining for AFB was negative in all of these sections. Our results dem-

onstrating that granulomas detected with HE staining are all positive with IHC have confirmed these previous findings. It is important to note that this was a retrospective study on previously diagnosed TB; hence we cannot determine the false positivity rate of IHC staining for intestinal TB.

Granular cytoplasmic staining of *M. tuberculosis* in IHC staining is considered to be due to fragments or debris of the bacilli^[7]. Low positivity of AFB could be due to the fact that only the intact bacilli take up the stain. The fact that fragments or debris of *M. tuberculosis* can be detected easily using IHC staining may be a great advantage. To the best of our knowledge, this is the first report that demonstrates that *M. tuberculosis* antigens are located as coarse granular immunopositive material in the cytoplasm of CD68⁺ macrophages within human intestinal TB. This finding may help to uncover the unknown relationship between *M. tuberculosis* and macrophages^[20].

Distinguishing intestinal TB from CD is still challenging^[21-25], and the treatments of TB and CD are quite different. Corticosteroids, immunosuppressive and anti-tumor necrosis factor (TNF) agents are widely applied in the treatment of CD, whereas they may be harmful in TB. For example, anti-TNF agents can induce reactivation of TB; therefore differentiating TB from CD is extremely important. Histopathological features en-

countered frequently in intestinal TB include granulomas that are confluent, large ($> 200 \mu\text{m}$), and multiple in number (> 5 per section)^[26]. The classical histological picture of tuberculous granulomatous inflammation is not a diagnostic problem in tissue samples; however, when sections show non-caseous epithelioid granulomas mimicking TB as is the case in CD, it creates a diagnostic dilemma. In the present study, we did not conduct any experiments using intestinal samples of CD; however Ince *et al*^[8] concluded that positive IHC staining with species-specific antibodies to TB can rule out the diagnosis of CD with high sensitivity and specificity. We plan to conduct a large-scale study to confirm these findings in the future.

In conclusion, IHC staining using a monoclonal antibody to *M. tuberculosis* antigen, which is a novel translational implication, can potentially be an efficient and simple diagnostic tool to compliment classic clinicopathological examinations for the diagnosis of intestinal TB.

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COMMENTS

Background

The diagnosis of intestinal tuberculosis (TB) is often difficult because of its diverse clinical manifestations and very low positivity using current diagnostics, including acid-fast bacilli in Ziehl-Neelsen staining and culture of *Mycobacterium tuberculosis* (*M. tuberculosis*) from intestinal tissue samples.

Research frontiers

Detection of *M. tuberculosis* DNA by polymerase chain reaction has been established as a diagnostic tool with excellent sensitivity and specificity in pulmonary TB. However, the diagnosis of intestinal TB still requires validation.

Innovations and breakthroughs

In this study, the authors have shown that immunohistochemical (IHC) staining using a monoclonal antibody to *M. tuberculosis* antigen in archived formalin-fixed paraffin-embedded intestinal tissue samples, which is a novel translational implication, can be an efficient and simple diagnostic tool to compliment classic clinicopathological examinations for the diagnosis of intestinal TB.

Applications

Although further studies are needed, IHC staining may be beneficial to differentiate TB from Crohn's disease (CD) with similar microscopic features, including the presence of granulomas.

Terminology

Monoclonal antibodies are monospecific antibodies which have monovalent affinity. IHC staining is widely applied to investigate the distribution of antigens in biological tissues by using specific antibodies.

Peer review

In this manuscript, the authors have clearly illustrated the potential advantage for the diagnosis of intestinal tuberculous granuloma. The importance of the research and its significance show that this topic is an important issue. The differentiation between TB and CD is very important in gastroenterology.

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-449 C>G polymorphism of *NFKB1* gene, coding nuclear factor-kappa-B, is associated with the susceptibility to ulcerative colitis

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Abstract

AIM: To clarify the association between a polymorphism -449 C>G (rs72696119) in 5'-UTR of *NFKB1* with ulcerative colitis (UC).

METHODS: The studied population comprised 639 subjects, including patients with UC (UC cases, $n = 174$) and subjects without UC (controls, $n = 465$). We employed polymerase chain reaction-single strand conformation polymorphism to detect the gene polymor-

phism.

RESULTS: The rs72696119 G allele frequencies in controls and UC cases were 33.4% and 38.5%, respectively ($P = 0.10$). Genotype frequency of the GG homozygote in UC cases was significantly higher than that in controls ($P = 0.017$), and the GG homozygote was significantly associated with susceptibility to UC [odds ratio (OR), 1.88; 95%CI, 1.13-3.14]. In male subjects, the GG homozygote was associated with an increased risk for UC (OR, 3.10; 95%CI, 1.47-6.54; $P = 0.0053$), whereas this association was not found in female subjects. In addition, the GG homozygote was significantly associated with the risk of non-continuous disease (OR, 2.06; 95%CI, 1.12-3.79; $P = 0.029$), not having total colitis (OR, 2.40; 95%CI, 1.09-3.80, $P = 0.040$), disease which developed before 20 years of age (OR, 2.80; 95%CI, 1.07-7.32, $P = 0.041$), no hospitalization (OR, 2.28; 95%CI, 1.29-4.05; $P = 0.0090$) and with a maximum of 8 or less on the UCDAI score (OR, 2.45; 95%CI, 1.23-4.93; $P = 0.022$).

CONCLUSION: Our results provide evidence that *NFKB1* polymorphism rs72696119 was significantly associated with the development of UC. This polymorphism influences the susceptibility to and pathophysiological features of UC.

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Key words: Genetic polymorphism; *NFKB1*; Ulcerative colitis

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INTRODUCTION

Ulcerative colitis (UC) is precipitated by a complex interaction of environmental, genetic, and immunoregulatory factors^[1,2]. UC affects the colon and rectum and typically involves the innermost lining mucosa, manifesting as continuous areas of inflammation, with no segments of normal mucosa^[3]. The pathogenesis of UC is only partially understood. Recently, the important role of innate immune response in the pathogenesis of UC has been reported^[4]. In addition, some genes are associated with UC itself^[5]. We have previously investigated the association between genetic polymorphisms in several genes and susceptibility to UC^[6-9].

One of the linkage regions for inflammatory bowel diseases (IBD) has been mapped to chromosome 4q^[10]. In this region, the *NFKB1*, encoding 2 subunits (p50 and p105) of nuclear factor κ B (NF- κ B), is located (4q24)^[11]. NF- κ B is a pleiotropic transcription factor involved in diverse immunologic processes including regulation of the intestinal immune system^[12]. Dysregulation of NF- κ B has been demonstrated in different inflammatory disorders, including UC^[13]. Recently, many studies have reported the association between polymorphism rs28362491 (-94 ins/del ATTG of *NFKB1*) and various inflammatory diseases^[14]. However, these studies have not always led to the same conclusions. Furthermore, a genetic variation, rs72696119 (-449 C>G in 5'-UTR of *NFKB1*), has been identified. We previously reported a close association between *NFKB1* polymorphisms (rs28362491 and rs72696119) and aberrant gene methylation in gastric mucosa^[15].

In this study, we attempted to clarify the association between the *NFKB1* polymorphism, rs72696119 (-449 C>G), and susceptibility to UC.

MATERIALS AND METHODS

Clinical samples

The studied population comprised 639 subjects, including patients with UC (UC cases, $n = 174$), who were enrolled in Fujita Health University Hospital, and subjects without UC (controls, $n = 465$). The diagnosis of UC was based on standard clinical, endoscopic, radiological, and histological criteria^[16]. The control subjects had no

lower abdominal symptoms, diarrhea or hematochezia. Genomic DNA was isolated from peripheral blood using the FlexiGene DNA Kit (QIAGEN GmbH, Hilden, Germany).

The Ethics Committees of Fujita Health University and Kanazawa Medical University approved the protocol, and written informed consent was obtained from all the participating subjects.

Classification

According to their clinical courses, UC cases were classified into continuous disease and non-continuous disease (relapsing and only one episode)^[17]. UC patients were also classified as having total colitis or not having total colitis (left sided, distal colitis and proctitis) according to the location and extension of the inflammatory lesions judged by endoscopic findings.

Genotyping of polymorphisms

The polymorphism was genotyped by polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) as previously reported^[15,18]. To detect *NFKB1* rs72696119 C>G, using the primer pairs (449F: 5'-cgtgtgtccgtctgtctgtatgctc-3' and 449R: 5'-cgtgtgtg-cacttctctctcttct-3'), was carried out in a volume of 20 μ L containing 0.1 μ g of genomic DNA. The DNA was denatured at 95 °C for 3 min, followed by 35 cycles at 95 °C for 30 s, 57 °C for 40 s, and 72 °C for 45 s, with final extension at 72 °C for 5 min. SSCP was carried out at 6 °C using a GenePhor DNA separation system with GeneGel Excel 12.5/24 (Amersham Biosciences Corp., United States), after which the denatured single strand DNA bands were detected using a DNA Silver Staining Kit (Amersham Biosciences Corp.).

Statistical analysis

Patient age was expressed as mean \pm SD. Mean age between the 2 groups was compared by Student's *t*-test. Allelic and genotype frequencies were calculated by direct counting. The allele counts and the distribution of genotypes were compared between the cases and the controls by a 2 \times 2 table using Fisher's exact test. Furthermore, the strength of the association between allele frequencies and the disease was assessed by calculating the odds ratio (OR) and 95%CI. For all analyses, the level of significance was set at $P < 0.05$.

RESULTS

Characteristics of subjects and the frequencies of genotypes

As shown in Figure 1, single strand DNA was clearly separated by SSCP. *NFKB1* rs2505901 was in Hardy-Weinberg equilibrium ($P = 0.26$). The mean age of the controls was significantly higher than that of UC cases (Table 1). The minor allele frequencies of rs72696119 were 33.4% and 38.5% in controls and UC cases, re-

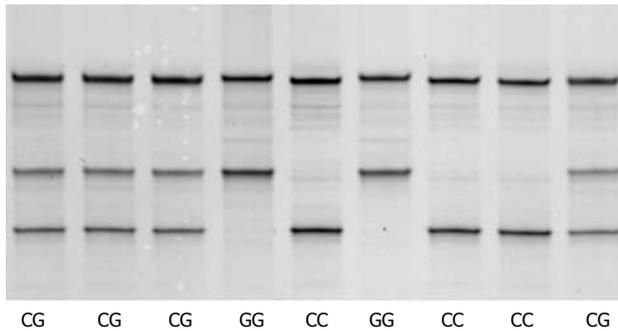


Figure 1 Polymerase chain reaction-single strand conformation polymorphism images using clinical samples. Single strand DNA was clearly separated by single strand conformation polymorphism.

Table 1 Characteristics of the subjects and allelic frequency

	Controls	UC cases	P value
Number of sample	465	174	
Age (mean \pm SD)	50.6 \pm 17.3	40.3 \pm 13.9	< 0.0001
(age of onset)		(33.0 \pm 13.4)	
Male:female	253:212	98:76	NS
rs72696119 C>G			
C/C	197	68	
C/G	225	78	
G/G	43	28	0.017
G allele frequency	33.40%	38.50%	0.10

UC: Ulcerative colitis; NS: Not significant.

spectively ($P = 0.10$). The genotype frequencies of the rs72696119GG homozygote was significantly higher in UC cases than in controls ($P = 0.017$).

Association between rs72696119 and UC

The rs72696119GG homozygote was significantly associated with increased risk for UC (OR, 1.88; 95%CI, 1.13-3.14; $P = 0.017$, Table 2). This association was stronger in male subjects (OR, 3.10; 95%CI, 1.47-6.54; $P = 0.0053$), whereas it was not observed in female subjects.

Association between rs72696119 and phenotypes of UC

The rs72696119 was associated with UC cases with an onset age below 20 years (OR, 2.80; 95%CI, 1.11-7.14; $P = 0.041$, Table 3). In addition, the GG homozygote was significantly associated with non-continuous disease (OR, 2.06; 95%CI, 1.13-3.77; $P = 0.029$), not having total colitis (OR, 2.04; 95%CI, 1.10-3.78; $P = 0.040$), no hospitalization (OR, 2.28; 95%CI, 1.29-4.05; $P = 0.0090$), and with a maximum of 8 or less on the UCDAI score (OR, 2.45; 95%CI, 1.23-4.93; $P = 0.022$). This polymorphism was not associated with response to steroid treatment.

DISCUSSION

In the current study, we evaluated the association between the polymorphism rs72696119 (-449C>G) in

Table 2 Association between rs72696119 and ulcerative colitis

Overall	Genotype (n)			GG vs others OR (95%CI)	P value
	C/C	C/G	G/G		
Controls (465)	197	225	43	Ref.	-
UC cases (174)	68	78	28	1.88 (1.13-3.14)	0.017
Male					
Controls (253)	102	136	15	Ref.	-
UC cases (98)	40	42	16	3.10 (1.47-6.54)	0.0053
Female					
Controls (212)	95	89	28	Ref.	-
UC cases (76)	28	36	12	1.23 (0.592-2.57)	0.57

UC: Ulcerative colitis; OR: Odds ratio.

Table 3 Association between rs72696119 and phenotype of ulcerative colitis

	Genotype (n)			GG vs others OR (95%CI)	P value
	CC	CG	GG		
Controls (465)	197	225	43	Ref.	-
Age of onset					
≤ 20 (27)	12	9	6	2.80 (1.07-7.32)	0.041
$21 \leq$ (133)	50	66	17	1.44 (0.791-2.62)	0.25
Clinical type					
Not continuous (98)	34	47	17	2.06 (1.13-3.77)	0.029
Continuous (71)	32	30	9	1.43 (0.662-3.07)	0.39
Extension					
Not total colitis (93)	31	46	16	2.04 (1.09-3.80)	0.040
Total colitis (78)	35	32	11	1.61 (0.792-3.28)	0.22
Max UCDAI score					
≤ 8 (60)	20	28	12	2.45 (1.23-4.93)	0.022
$9 \leq$ (106)	44	49	13	1.37 (0.716-2.63)	0.37
Hospitalization					
None (106)	41	45	20	2.28 (1.29-4.05)	0.0090
One time \leq (60)	25	30	5	0.892 (0.350-2.28)	1.00
Response to treatment					
Steroid-dependent (34)	13	17	4	1.31 (0.440-3.89)	0.54
Steroid-refractory (46)	18	23	5	1.20 (0.449-3.19)	0.79

OR: Odds ratio.

5'-UTR of *NFKB1* and the risk for developing UC. The rs72696119 GG homozygote was significantly associated with increased risk for UC, especially in male subjects. In addition, this genotype was associated with younger age at onset, non-continuous disease, not having total colitis, no hospitalization and with a UCDAI score below 8. These results suggest that this genotype may be associated with UC of comparatively mild or moderate severity. In our study, sample selection may have affected the outcome, as our controls included patients who came to hospital in order to have treatment for complaints other than diarrhea, bloody feces and lower abdominal discomfort, and were not completely healthy subjects. Moreover, the effect of type II error cannot be excluded in relatively small sample sizes. Another limitation of this study was that mean age was different between the controls and UC cases. However, it seems that this was not an obstacle in the analysis, as UC developed at a

relatively young age.

To the best of our knowledge, there have been no reports on the distribution of rs72696119 in Japanese subjects, including HapMap-JPT. In a previous study, we demonstrated that rs72696119 has a strong allelic association with rs28362491^[15]. It has been reported that the rs28362491 ATTG deletion variant in the promoter region destroys a transcription factor binding site, resulting in lower expression of NF- κ B^[19]. Therefore, NF- κ B expression is considered to be low in rs72696119 GG variants, as well as rs28362491 del/del variants. Due to their important role in inflammation, the lower expression of NF- κ B protein seems to suppress inflammation. However, several studies have shown that the rs28362491 ATTG deletion variant is associated with increased risk for the development of inflammatory or auto-immune diseases^[19,20]. Our results also indicated that the rs72696119 GG homozygote was associated with an increased risk for UC in Japanese.

NF- κ B names a number of different transcription factors that are homo- or heterodimers of p65, p50, p105, C-rel and relB^[21]. *NFKB1* encodes both the subunits p105 and p50 of the transcription factor NF- κ B by alternative splicing^[22]. NF- κ B is involved in both inflammatory and anti-inflammatory processes^[23]. The role of NF- κ B in inflammation is determined by subunit type. As part of the p65/p50 NF- κ B transcription factor complex, it is pro-inflammatory, controlling transcription of pro-inflammatory cytokines^[24]. Conversely, p50 has anti-inflammatory properties in the p50 homodimer by repressing transcription^[25]. The relative abundance of p65/p50 heterodimers and p50 homodimers may determine the magnitude of inflammation by balancing the pro-inflammatory and anti-inflammatory response^[21]. In fact, p50 deficient mice have an increased sensitivity to lipopolysaccharide (LPS) and have increased LPS-induced inflammation^[26,27]. In subjects with the del/del genotype, decreased p50 synthesis may lead to decreased repressive homodimers and increased active heterodimers of the NF- κ B complex. This balance may influence the susceptibility to inflammatory diseases, including UC.

The significant association between the rs28362491 ATTG deletion allele and UC was first reported by Karban *et al.*^[19]. Borm *et al.*^[28] also reported the same results. However, several studies did not find a significant association between this allele and UC^[29-32]. On the other hand, there have been no reports on the association between the *NFKB1* polymorphism and UC in Japan. In our study, the rs72696119 G allele, in linkage disequilibrium with the rs28362491 ATTG deletion allele, was significantly associated with susceptibility to UC using a recessive genetic model. In addition, this genotype was associated with patients who developed UC at a relatively young age, similar to Borm's report^[28]. These contrasting observations may be explained by differences in the genotypic composition of populations in different coun-

tries with different racial groups. Another explanation is that it is possible that the results may be controlled by the composition of the phenotypes in UC cases, as our results indicated that the *NFKB1* polymorphism was more closely associated with specific phenotypes of UC. Furthermore, the influence of rs72696119 has not yet been investigated. The association between rs28362491 and rs72696119 has not been described in the HapMap project. More studies will be necessary to clarify the influence of rs72696119 on susceptibility to UC.

It is difficult to evaluate the severity of UC at any one point, because it fluctuates with clinical period and medications. Thus, we assessed the association between rs72696119 and the severity of UC, when the cases with a history of hospitalization or with a maximum of 9 or more on the UCDAI score were considered to be severe cases. Our results suggested that this genotype might be associated with UC of comparatively mild or moderate severity. Moreover, a strong significant association between the rs72696119 GG homozygote and UC was found in male subjects. It is unclear why this genotype was associated with specific phenotypes and male UC cases. UC is a multifactorial disorder with both genetic and environmental etiological factors, and is considered a complex genetic disorder predicted to involve multiple genes of relatively low penetrance^[33]. In fact, Fisher *et al.*^[34] reported that several regions of male-specific linkage were found in the susceptibility to IBD. It may be no surprise that *NFKB1* polymorphism is more closely associated with specific phenotypes of UC. Further studies will be necessary in order to clarify how the *NFKB1* polymorphism influences susceptibility to UC.

In conclusion, the GG homozygote of rs72696119, which is located in *NFKB1* 5'-UTR and is in linkage disequilibrium with rs28362491, is significantly associated with susceptibility to UC, especially in Japanese male subjects. This genotype is associated with UC of mild or moderate severity.

COMMENTS

Background

The incidence of ulcerative colitis (UC) is currently rising in Japan although the pathogenesis of UC is only partially understood. Recently, variations in some genes have been associated with the development of UC.

Research frontiers

One of the linkage regions for inflammatory bowel diseases maps to chromosome 4q. In this region, the *NFKB1*, encoding 2 subunits (p50 and p105) of nuclear factor (NF)- κ B, is located (4q24). A certain genetic variation, rs72696119, has been identified at position -449 C>G in 5'-UTR of *NFKB1*. In this study, the authors demonstrate that rs72696119 GG genotype is associated with the development of UC in Japan.

Innovations and breakthroughs

Many studies have reported the association between polymorphism rs28362491 (-94 ins/del ATTG of *NFKB1*) and various inflammatory diseases. However, these studies have not always led to the same conclusions. In addition, there have been no reports investigating an association between rs72696119 and inflammatory diseases. This is the first study to report that rs72696119 is asso-

ciated with the development of UC in Japan.

Applications

The authors assessed how genetic variation contributes to the development of UC using a case-control study (174 cases and 465 controls). The genotype analysis was performed by polymerase chain reaction-single strand conformation polymorphism.

Terminology

NF- κ B activation is known to regulate cellular growth responses, including apoptosis, and is required for the induction of inflammatory and tissue-repair genes. NF- κ B names a number of different transcription factors that are homo- or heterodimers of p65, p50, p105, C-rel and relB. Subunits p105 and p50 of NF- κ B are encoded by *NFKB1*. A p65/p50 heterodimer is pro-inflammatory, and p50 has anti-inflammatory properties.

Peer review

It is suitable for acceptance to this journal. Authors compared the GG homozygote in UC patients between total colitis and not total colitis.

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Treatment strategies using adefovir dipivoxil for individuals with lamivudine-resistant chronic hepatitis B

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Abstract

AIM: To investigate retrospectively the long-term efficacy of various treatment strategies using adefovir dipivoxil (adefovir) in patients with lamivudine-resistant chronic hepatitis B.

METHODS: We included 154 consecutive patients in two treatment groups: the "add-on" group ($n = 79$), in which adefovir was added to ongoing lamivudine treatment due to lamivudine resistance, and the "switch/combination" group ($n = 75$), in which lamivudine was first switched to adefovir and then re-added later as

needed. The "switch/combination" group was then divided into two subgroups depending on whether participants followed (group A, $n = 30$) or violated (group B, $n = 45$) a proposed treatment strategy that determined whether to add lamivudine based on the serum hepatitis B virus (HBV) DNA levels (< 60 IU/mL or not) after 6 mo of treatment (roadmap concept).

RESULTS: The cumulative probability of virologic response (HBV DNA < 60 IU/mL) was higher in group A than in the "add-on" group and in group B ($P < 0.001$). In contrast, the cumulative probability of virologic breakthrough was lower in the "add-on" group than in group B ($P = 0.002$). Furthermore, the risk of virologic breakthrough in the multivariate analysis was significantly lower in the "add-on" group than in group A (hazard ratio = 0.096; 95%CI, 0.015-0.629; $P = 0.015$).

CONCLUSION: The selective combination of adefovir with lamivudine based upon early treatment responses increased the odds of virologic breakthrough relative to the use of uniform combination therapy from the beginning of treatment.

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Key words: Chronic hepatitis B; Lamivudine-resistant; Adefovir; Combination therapy; Roadmap concept

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INTRODUCTION

The aims of chronic hepatitis B (CHB) treatment are to achieve the sustained suppression of hepatitis B virus (HBV) replication and to prevent progression to cirrhosis, liver failure, and hepatocellular carcinoma^[1]. Lamivudine is a safe and effective nucleoside analog that has been approved by the United States Food and Drug Administration as the first oral therapy for CHB. Lamivudine suppresses HBV replication by inhibiting the activity of HBV DNA polymerase, which leads to the normalization of serum alanine aminotransferase (ALT) levels, hepatitis B e antigen (HBeAg) seroconversion, and histological improvement^[2-5]. However, some CHB patients who are treated with lamivudine over a long period of time develop resistance to the drug at a rate reaching approximately 70% after five years of administration^[6]. This resistance leads to virologic breakthrough, re-elevation of the serum ALT levels, hepatic failure, and even death in some patients^[3,4,7]. Despite the introduction of more potent antiviral agents for the treatment of CHB, lamivudine is still frequently used due to its lower cost.

Adefovir dipivoxil (adefovir) is a nucleotide analog that is effective against both wild-type and lamivudine-resistant forms of HBV^[8-13]. However, adefovir resistance mutations develop more frequently in lamivudine-resistant patients than in treatment-naïve patients^[14-17]. Several recent studies have reported that the combination of adefovir with lamivudine results in a lower incidence of resistance to adefovir and has higher efficacy than adefovir monotherapy in patients with lamivudine resistance^[18-21]. In reality, however, adefovir is frequently initiated as a monotherapy due to the high cost of combination therapy, and lamivudine is sometimes added later if needed. It has not yet been determined when and how to assess the appropriateness of the virologic response to adefovir monotherapy so that lamivudine can be added at the optimal time. In such situations, healthcare providers may consult the roadmap concept proposed by Keeffe *et al.*^[22]. The roadmap concept uses the treatment response at 6 mo to individualize ongoing antiviral management to minimize resistance and improve long-term efficacy. Although this concept is primarily based on the results of previous studies on treatment-naïve CHB patients^[23-25], a recent study suggested that the roadmap concept might be useful as a means of increasing the therapeutic efficacy during adefovir monotherapy in patients with lamivudine-resistant HBeAg-positive CHB^[26].

To date, however, no studies have investigated whether treatment strategies based on the roadmap con-

cept (first initiating adefovir monotherapy and then adding lamivudine based on early treatment response) are as effective as *ab initio* combination therapy in patients with lamivudine-resistant CHB.

In this study, we retrospectively compared the long-term efficacies of various treatment strategies using adefovir in patients with lamivudine-resistant CHB and analyzed factors associated with treatment efficacy. In particular, we focused on whether the long-term efficacies differed between patients treated in accordance with the roadmap concept and those treated with combination therapy from the start of treatment.

MATERIALS AND METHODS

Study sample

We screened the medical records of 255 consecutive patients who were prescribed adefovir 10 mg daily for the treatment of lamivudine-resistant CHB and followed up for more than 12 mo at Korea University Anam Hospital from May 2004 to February 2010. Of these 255 patients, 14 were excluded from the study because they had already been prescribed adefovir at another hospital. 20 patients were excluded because they received other drugs (entecavir, remofovir, or clevudine) prior to adefovir treatment. We also excluded another 67 patients with serum HBV DNA levels at month six that were below the detection limit (< 0.5 pg/mL) of the hybridization method and that were not measured with more sensitive quantitative techniques. The data were collected for the remaining 154 patients and analyzed retrospectively. Patients were divided into two groups depending on how adefovir was initiated: a “switch/combination” group and an “add-on” group. For the “switch/combination” group ($n = 75$), lamivudine was first switched to adefovir, and lamivudine was re-added later if necessary in cases of primary non-response and inadequate response ($n = 31$) or virologic breakthrough ($n = 6$). For the “add-on” group ($n = 79$), adefovir was added to ongoing lamivudine treatment due to lamivudine resistance.

Methods

Clinical information (including age, gender, duration of prior lamivudine treatment, body mass index, presence of liver cirrhosis, and Child-Pugh score) was obtained by reviewing the patient medical records. Data were also collected from laboratory tests that were performed prior to adefovir administration and every three months thereafter. These tests included routine complete blood counts; biochemical tests to measure the serum levels of ALT, aspartate aminotransferase, alkaline phosphatase, gamma glutamyl transpeptidase, albumin, total bilirubin, blood urea nitrogen, creatinine, and phosphorus; assays to determine the prothrombin time; and serologic studies to determine the HBeAg, anti-HBeAg antibody (anti-HBe), and HBV DNA levels.

The diagnosis of liver cirrhosis was based on liver

biopsy features or on clinical, laboratory, radiologic and endoscopic data. Lamivudine resistance mutations (rtM204V/I or rtL180M) were detected using direct sequencing assays or restriction fragment mass polymorphisms at baseline in all patients included in our study. In the same manner, testing for adefovir resistance mutations was also performed in patients who exhibited virologic breakthrough during adefovir treatment. Prior to July 2007, the quantitative analyses of serum HBV DNA levels were conducted using a polymerase chain reaction (PCR) assay (COBAS Amplicor HBV Monitor, Roche Diagnostics, Indianapolis, IN, United States), which has a lower detection limit of 60 IU/mL; thereafter, a real-time PCR assay (COBAS TaqMan HBV test, Roche Diagnostics, Indianapolis, IN, United States) with a lower detection limit of 20 IU/mL was used.

Definition

Virologic response was defined as a decrease in HBV DNA to undetectable levels (HBV DNA < 60 IU/mL). Biochemical response was defined as a decrease in the serum ALT level to within the normal range. HBeAg seroclearance was defined as the loss of HBeAg from the serum with a decrease in serum HBV DNA to undetectable levels (HBV DNA < 60 IU/mL) in patients who had been seropositive for HBeAg before the initiation of adefovir. We defined virologic breakthrough as a confirmed increase in HBV DNA levels of more than 1 log₁₀ IU/mL relative to the lowest HBV DNA levels observed during treatment. Biochemical breakthrough was defined as an increase in the serum ALT level above the upper limit of normal on at least two occasions after achieving normalization with treatment. Primary non-response was defined as a decrease of less than 2 log₁₀ IU/mL in the HBV DNA level from baseline after six months of treatment. Inadequate response was defined as HBV DNA levels of 2000 IU/mL or greater after six months of treatment.

We based our treatment strategy for the “switch/combination” group on the roadmap concept, in which clinicians decide whether to add lamivudine based on the early treatment response after six months of adefovir monotherapy. However, if serum HBV DNA levels at six months were lower than 60 IU/mL, patients were regarded as having early treatment response and continued to receive adefovir monotherapy. However, if serum HBV DNA levels at six months were 60 IU/mL or higher, the response was considered inadequate, and lamivudine was added to the treatment regimen. The “switch/combination” group was then divided into subgroups A and B, in which the treatment strategy based on the roadmap concept was either satisfied or not satisfied, respectively.

Statistical analysis

The serum HBV DNA levels were logarithmically trans-

formed for analysis. To evaluate differences in clinical aspects prior to adefovir treatment between the “switch/combination” group and the “add-on” group, continuous variables were compared using the two-sample *t* test, and categorical data were compared using the χ^2 test. The cumulative probabilities of virologic and biochemical responses, HBeAg seroclearance, and virologic and biochemical breakthroughs were evaluated using Kaplan-Meier analysis. The log-rank test was used to evaluate differences between the two groups. We identified independent factors associated with treatment outcomes using the Cox proportional hazard model. Variables with *P* values < 0.1 in the univariate analysis were included in the multivariate analysis. *P* values < 0.05 were considered statistically significant. All data were analyzed using the Statistical Package for the Social Sciences (SPSS) for Windows (version 18, SPSS Inc., Chicago, IL, United States).

RESULTS

Baseline characteristics of the patients prior to adefovir treatment

Of the 154 patients included in the study, 109 (70.8%) were male, and 45 (29.2%) were female. The mean (\pm SD) age of the entire sample was 45.5 \pm 11.8 years. 110 patients (71.4%) were seropositive for HBeAg, and 65 (42.2%) had liver cirrhosis. The mean levels of serum HBV DNA and ALT were 6.85 \pm 1.01 log₁₀ IU/mL and 200.1 \pm 179.3 IU/L, respectively. The mean duration of prior lamivudine treatment before the initiation of adefovir was 28.5 \pm 14.6 mo, and we conducted follow-up after the initiation of adefovir for an average of 33.8 \pm 14.1 mo (median, 33; range 12-66). The “switch/combination” group included 75 patients, and the “add-on” group included 79 patients. The median durations of follow-up in the “switch/combination” group and the “add-on” group were 42 mo (range, 12-66) and 24 mo (range, 12-36), respectively. At baseline, there were no significant differences in the clinical and laboratory features between groups except for age and the presence of liver cirrhosis (Table 1).

Treatment efficacy in the “switch/combination” group and the “add-on” group

After the initiation of adefovir, virologic response was achieved in 51 of 75 (68.0%) patients in the “switch/combination” group during the follow-up period. In the “add-on” group, 51 of 79 (64.6%) patients achieved virologic response. There were no significant differences in the virologic response rate between the two groups (*P* = 0.249), with cumulative probabilities at the first and third years of 41.3% and 68.2% in the “switch/combination” group and 48.1% and 73.5% in the “add-on” group, respectively. In the multivariate analysis, however, virologic response was found to be significantly more common in the “add-on” group [hazard ratio (HR) = 1.646; 95%CI,

Table 1 Baseline characteristics of patients with lamivudine-resistant chronic hepatitis B

	"Switch/ combination" group ² (n = 75)	"Add-on" group ³ (n = 79)	P value ¹
Age (yr)	43.3 ± 10.2	47.6 ± 12.9	0.022
Male gender	56 (74.7)	53 (67.1)	0.301
BMI (kg/m ²)	24.1 ± 5.1	23.8 ± 3.2	0.583
Positive for HBeAg	57 (76.0)	53 (67.1)	0.221
Positive for anti-HBe	19 (25.3)	25 (31.6)	0.386
HBV DNA (log ₁₀ IU/mL)	6.75 ± 0.93	6.94 ± 1.08	0.233
AST (IU/L)	148.7 ± 134.5	141.1 ± 148.3	0.742
ALT (IU/L)	230.1 ± 213.4	199.5 ± 191.8	0.351
Albumin (g/dL)	4.4 ± 0.5	4.3 ± 0.6	0.166
ALP (IU/L)	83.4 ± 37.6	76.8 ± 27.6	0.211
GGT (IU/L)	60.5 ± 42.9	67.1 ± 71.1	0.494
Total bilirubin (mg/dL)	1.2 ± 0.9	1.4 ± 1.9	0.425
Prothrombin time (INR)	1.16 ± 0.29	1.15 ± 0.19	0.825
Hemoglobin (g/dL)	14.5 ± 1.8	14.2 ± 1.7	0.345
WBC (/mm ³)	5279 ± 1771	4972 ± 1374	0.233
Platelet (× 10 ³ /mm ³)	156 ± 68	145 ± 69	0.283
BUN (mg/dL)	12.5 ± 3.1	13.1 ± 4.4	0.325
Creatinine (mg/dL)	0.97 ± 0.15	0.92 ± 0.20	0.110
Duration of prior lamivudine treatment (mo)	28.7 ± 13.6	28.3 ± 15.5	0.886
Cirrhosis	25 (33.3)	40 (50.6)	0.030

All values are expressed as mean ± SD or number of patients (%). ¹P values were calculated using the two-sample *t* test for continuous variables and the χ^2 test for categorical variables; ²Lamivudine was first switched to adefovir dipivoxil (adefovir), and then lamivudine was re-added later as needed in cases of primary non-response, inadequate response or virologic breakthrough; ³Adefovir was added to ongoing lamivudine treatment due to lamivudine resistance. BMI: Body mass index; HBeAg: Hepatitis B e Antigen; Anti-Hbe: Antibody to HBeAg; HBV DNA: Hepatitis B virus DNA; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GGT: γ glutamyl transpeptidase; INR: International normalized ratio; WBC: White blood cell; BUN: Blood urea nitrogen.

1.080-2.510; *P* = 0.021] and less frequent in patients with inadequate response (serum HBV DNA \geq 2,000 IU/mL at 6 mo) (HR = 0.121; 95%CI, 0.069-0.212; *P* < 0.001) (Table 2).

Biochemical response was achieved in 143 of 154 patients (the "switch/combination" group *vs* the "add-on" group: 92.0% *vs* 93.7%, respectively). There was no significant difference in the cumulative probability of biochemical response between the two groups (*P* = 0.190). In the multivariate analysis, inadequate response was the only independent factor associated with biochemical response (HR = 0.574; 95%CI, 0.402-0.820; *P* = 0.002).

Of our 110 patients who were HBeAg-positive at baseline, HBeAg seroclearance was achieved in 28 of 57 (49.1%) in the "switch/combination" group and in 23 of 53 (43.4%) in the "add-on" group. The incidence of HBeAg seroclearance did not differ significantly between the two groups (*P* = 0.326). The cumulative probabilities of seroclearance during the first and third years were 17.5% and 44.8% in the "switch/combination" group and 13.2% and 62.4% in the "add-on" group, respectively. Inadequate response was the only independent factor that was correlated with HBeAg seroclearance (HR

Table 2 Multivariate analysis for factors of virologic response (undetectable in polymerase chain reaction assay)

Variables	HR	95%CI	P value ¹
Male gender	0.958	0.621-1.477	0.846
HBeAg (+)	0.668	0.415-1.077	0.098
HBV DNA (log ₁₀ IU/mL)	0.810	0.644-1.018	0.071
AST (IU/L)	1.000	0.998-1.002	0.955
ALT (IU/L)	1.000	0.999-1.002	0.606
Duration of prior lamivudine treatment (mo)	1.011	0.998-1.024	0.112
Inadequate response ²	0.121	0.069-0.212	< 0.001
Treatment group ³	1.646	1.080-2.510	0.021
"Add-on" <i>vs</i> "switch/combination"			

¹P-values were calculated with the Cox's proportional hazard model; Variables with a *P* < 0.1 in the univariate analysis were included in the multivariate analysis; ²Inadequate response was defined as serum HBV DNA levels of 2000 IU/mL or greater at 6 mo of treatment; ³Patients were divided into two treatment groups: the "add-on" group, in which adefovir dipivoxil (adefovir) was added to ongoing lamivudine treatment due to lamivudine resistance, and the "switch/combination" group, in which lamivudine was first switched to adefovir and then re-added later as needed. HBeAg: Hepatitis B e Antigen; HBV DNA: Hepatitis B virus DNA; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HR: Hazard ratio.

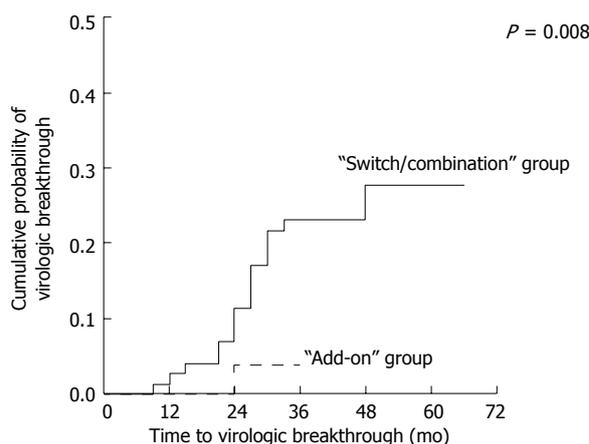


Figure 1 Cumulative probabilities of virologic breakthrough in the two treatment groups.

= 0.214; 95%CI, 0.109-0.419; *P* < 0.001).

Virologic and biochemical breakthroughs during adefovir treatment were observed in 19 patients [17 of 75 (22.7%) in the "switch/combination" group and 2 of 79 (2.5%) in the "add-on" group] and 16 patients (20.0% *vs* 1.3%), respectively. Both breakthroughs were more common in the "switch/combination" group than in the "add-on" group, according to the Kaplan-Meier analysis (*P* < 0.05). The cumulative probabilities of virologic breakthrough at the first and third years were 2.7% and 23.2% in the "switch/combination" group and 0% and 3.8% in the "add-on" group (Figure 1). In the multivariate analysis, the risk of virologic breakthrough was significantly lower in the "add-on" group (HR = 0.130; 95%CI, 0.028-0.599; *P* = 0.009) and significantly higher in patients with inadequate response (HR = 5.251;

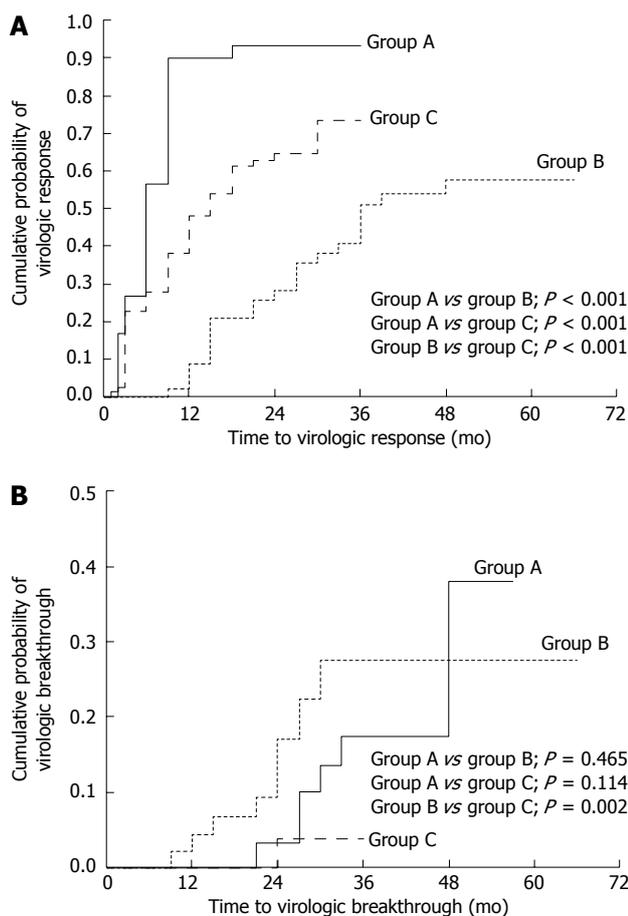


Figure 2 A comparison of the cumulative probabilities of virologic response and viral breakthrough according to different treatment strategies using adefovir dipivoxil in patients with lamivudine-resistant chronic hepatitis B. Groups A and B represent the patients who followed or violated the roadmap treatment strategy, respectively; Group C stands for those treated by adding adefovir dipivoxil in addition to ongoing lamivudine therapy. The roadmap treatment strategy was supposed to initiate adefovir monotherapy first and then add lamivudine when serum HBV DNA levels were ≥ 60 IU/mL at six months of treatment. A: There were significant differences in the cumulative probabilities of virologic responses between any two treatment groups ($P < 0.001$); B: The cumulative probability of virologic breakthrough was significantly lower in group C than in group B ($P = 0.002$) but not lower than group A ($P = 0.114$). However, there was a significant difference between groups C and A in the multivariate Cox's regression model (Table 3).

95%CI, 1.684-16.371; $P = 0.004$).

Treatment efficacy in the "switch/combination" group according to compliance with the treatment strategy based on the roadmap concept

As mentioned above, we used a roadmap concept-based treatment strategy, which recommends the addition of lamivudine based on an early treatment response after six months of adefovir monotherapy (< 60 IU/mL), for patients in the "switch/combination" group. group A included patients who were treated in accordance with the roadmap strategy. group B was made up of patients for whom the roadmap-dictated treatment strategy was not followed. Thirty patients were included in group A (27 maintained adefovir monotherapy, and 3 received

	HR	95%CI	P value ¹
HBeAg (+)	3.251	0.400-26.403	0.270
Albumin (g/dL)	0.552	0.194-1.573	0.266
ALP (IU/L)	1.004	0.994-1.014	0.427
PT (INR)	1.269	0.186-8.648	0.808
Platelet ($\times 10^3/\text{mm}^3$)	0.997	0.986-1.007	0.519
Cirrhosis	1.016	0.243-4.448	0.983
Duration of prior lamivudine treatment (mo)	0.99	0.945-1.038	0.691
Inadequate response ²	6.57	-1.517-28.458	0.012
Treatment groups ³			0.029
Group B vs group A	0.668	0.149-2.984	0.597
Group C vs group A	0.096	0.015-0.629	0.015

¹ P -values were calculated with Cox's proportional hazard model; Variables with a $P < 0.1$ in the univariate analysis were included in the multivariate analysis; ²Inadequate response was defined as serum HBV DNA levels of 2000 IU/mL or greater at 6 mo of treatment; ³Treatment groups were divided into three groups according to the timing of the drug combination; groups A and B represent the patients who followed or violated the roadmap treatment strategy, respectively; group C included those participants who were treated by adding adefovir dipivoxil (adefovir) to ongoing lamivudine therapy. Roadmap treatment strategy was supposed to initiate adefovir monotherapy first and then add lamivudine later when serum HBV DNA levels were detectable (≥ 60 IU/mL) at six months of treatment. HBeAg: Hepatitis B e antigen; ALP: Alkaline phosphatase; PT: Prothrombin time; INR: International normalized ratio; HR: Hazard ratio.

concomitant lamivudine), and 45 patients were included in group B.

Patients in "switch/combination" group A were more likely to experience virologic response than those in group B ($P < 0.001$) or those in the "add-on" group (group C) ($P < 0.001$) (Figure 2A). In contrast, the cumulative probability of virologic breakthrough was significantly lower in the "add-on" group (0% at the first year, 3.8% at the third year) than in "switch/combination" group B (4.4% at the first year, 27.5% at the third year) ($P = 0.002$) but was not lower than in "switch/combination" group A (0% at the first year, 17.4% at the third year, 38.0% at the fifth year) ($P = 0.114$) (Figure 2B). In the multivariate analysis, however, the risk of virologic breakthrough was significantly lower in the "add-on" group than in "switch/combination" group A (HR = 0.096; 95%CI, 0.015-0.629; $P = 0.015$) (Table 3).

In addition, patients with undetectable (< 60 IU/mL) serum HBV DNA levels after six months of adefovir monotherapy had cumulative probabilities of virologic breakthrough of 15.7% and 36.7% at three and five years of treatment, respectively, with continued adefovir monotherapy based on the roadmap concept strategy.

Mutations conferring adefovir resistance

Testing for mutations conferring adefovir resistance was performed in patients with primary non-response, inadequate response or virologic breakthrough during adefovir treatment. 28 (37.3%) and 6 (7.6%) patients were tested for genotypic resistance to adefovir in the "switch/

Table 4 Patterns of genotypic resistance to adefovir dipivoxil in patients with lamivudine-resistant chronic hepatitis B

Mutation pattern	"Switch/combination" group ¹ (n = 75)	"Add-on" group ² (n = 79)
rtA181V	2	0
rtA181T	3	1
rtA181V + rtA181T	0	1
rtN236T	3	0
rtA181V + rtN236T	1	0
rtA181T + rtN236T	1	0
Total, n (%)	10 (13.3)	2 (2.5)

rtA181V, alanine to valine substitution at rt181; rtA181T, alanine to threonine substitution at rt181; rtN236T, asparagine to threonine substitution at rt236. ¹Lamivudine was first switched to adefovir dipivoxil (adefovir), and then lamivudine was re-added later as needed in case of primary non-response, inadequate response, or virologic breakthrough; ²Adefovir was added to ongoing lamivudine treatment due to lamivudine resistance.

combination" group and the "add-on" group, respectively. Among these patients, adefovir resistance mutations were detected in 10 and 2 patients in the "switch/combination" and "add-on" groups, respectively (Table 4). Adefovir resistance mutations were only identified in 6 (40%) of 15 patients with virologic breakthrough (5 of 13 in the "switch/combination" group and 1 of 2 in the "add-on" group).

Nephrotoxicity due to adefovir

After initiating adefovir therapy, increased serum creatinine levels (> 1.2 mg/dL and an increase of over 20% from baseline) were observed more than once in 9 of 154 patients (5.8%). Of 4 patients with elevated serum creatinine levels (> 1.2 mg/dL) at baseline, 2 showed a greater than 20% increase from baseline.

9 of 154 patients (5.8%) had hypophosphatemia at least once during adefovir treatment, and 5 experienced the condition at least twice. Of these 5 patients, 3 recovered from hypophosphatemia after taking oral KH₂PO₄. In 3 patients (1.9%), increased serum creatinine and hypophosphatemia were both observed, and one of the participants showed severe hypophosphatemia, with levels less than 1.5 mg/dL.

DISCUSSION

Despite the high incidence of resistance, lamivudine is still prescribed for the treatment of CHB because of its low cost relative to other available drugs. Therefore, the treatment of lamivudine-resistant CHB will remain a clinical challenge in the field of hepatology for the foreseeable future.

In South Korea, we are currently able to use adefovir or entecavir to treat patients with lamivudine resistance. Approximately 40% of patients with lamivudine resistance develop resistance to adefovir monotherapy after four years of treatment^[21]; patients receiving entecavir monotherapy exhibited resistance at a rate of 35.9% af-

ter three years^[27]. By contrast, when adefovir was added to ongoing lamivudine therapy, the rate of resistance to adefovir was just 0%-2% at two years and 4.4% at four years^[18,19,21]. In addition, as time passed, the proportion of patients who maintained undetectable serum HBV DNA levels and serum ALT levels within the normal range was greater in the combination therapy group than in the adefovir monotherapy group^[21]. Therefore, when lamivudine resistance was detected, we prescribed combination therapy as soon as possible. In patients already receiving adefovir monotherapy, we added lamivudine only when an incomplete response was observed.

In the present study, we found that the cumulative probability of virologic breakthrough at three years was just 3.8% in the "add-on" group compared with 23.2% in the "switch/combination" group. Upon multivariate analysis, we confirmed that the probability of virologic response (serum HBV DNA < 60 IU/mL) was significantly higher and that the risk of virologic breakthrough was significantly lower in the "add-on" group than in the "switch/combination" group. These results suggest that adding adefovir to lamivudine may be the most appropriate treatment currently available for patients with lamivudine resistance in Korea. However, many patients do not have access to combination therapy because of its cost. Therefore, from a cost-effectiveness point of view, a treatment strategy based on the roadmap concept is worth considering.

The roadmap concept was originally proposed for treatment-naïve patients after researchers realized that early virologic suppression during antiviral treatment was closely related to long-term therapeutic efficacy and the development of viral resistance to a drug^[22]. However, a recent study of the outcomes of adefovir rescue monotherapy suggested that the roadmap concept might also be applicable in patients with lamivudine resistance^[26]. Our study also found that early treatment responses (at six months) in patients with lamivudine-resistant CHB were strongly correlated with long-term therapeutic outcomes, such as virologic response, biochemical response and HBeAg seroclearance.

In this study, there was a significant difference in the rate of treatment response between the "switch/combination" and "add-on" groups. However, this result could not be interpreted to indicate that the add-on combination treatment was superior to a stepwise combination treatment based on the roadmap concept because the "switch/combination" group included many patients whose treatment was not based on the roadmap concept. To address this question, we divided the "switch/combination" group into two sub-groups depending on whether the participants received treatment based on the roadmap concept, and then we compared the treatment outcomes in these subgroups with those in the "add-on" group. We found that the cumulative probability of virologic response was significantly better in the subgroup whose treatment conformed to the roadmap concept

(group A) than in the “add-on” group. However, the multivariate analysis showed that the risk of virologic breakthrough was significantly higher in group A than in the “add-on” group, with three-year cumulative probabilities of 17.4% and 3.8%, respectively. Even if the serum HBV DNA became undetectable (< 60 IU/mL) following six months of adefovir monotherapy, the cumulative probability of virologic breakthrough reached 15.7% and 36.7% at three and five years of treatment, respectively, when adefovir monotherapy was continued based on the roadmap strategy. These results suggest that the roadmap concept-based treatment strategy, which initiates adefovir first and adds lamivudine back to the treatment regimen depending on the treatment response at six months, should not be recommended for patients with lamivudine-resistant CHB. Of course, because the longest follow-up duration in our “add-on” group was only 36 mo, the resistance rate in the “add-on” group might increase as time passes. However, that possibility appears to be very low, given that the resistance rate at 48 mo in the “add-on” group was just 4% in two previous studies^[18,21].

Adefovir resistance mutations were identified in only 40% of patients with virologic breakthrough in this study. This finding might be explained by the following: First, it is possible that virologic breakthrough developed following low drug compliance among patients without mutations. Second, lamivudine-resistant HBV may be decreased by the use of adefovir; at the same time, however, the wild-type HBV, which does not respond well to adefovir, might exhibit increased growth. These two processes may lead to virologic breakthrough. Third, these findings might be due to methodological problems regarding the analysis of genetic variations.

In this study, HBV genotyping was not performed because almost all patients with CHB (> 95%) in Korea are infected with HBV genotype C^[28-31]. In addition, previous studies have not shown any relationship between HBV genotype and response to nucleos(t)ide analogs^[32]. Therefore, the routine genotyping of HBV appears to be uninformative in predicting therapeutic outcome, and genotyping might be meaningless for Korean patients with CHB.

Nephrotoxicity is a well-known side-effect of adefovir. In the present study, we investigated two of the primary nephrotoxic side-effects that can be caused by adefovir. Serum creatinine levels were increased in 5.8% of patients after initiating adefovir in this study. This figure is similar to that reported in other studies^[13,17-19]. Some case reports have found that adefovir can induce Fanconi syndrome or hypophosphatemic osteomalacia^[33-35]. In this study, 5.8% of patients showed hypophosphatemia, and some of them required the administration of oral KH₂PO₄. Therefore, clinicians must monitor these side effects more thoroughly in case patients require further treatment. Although the mechanism of nephrotoxicity due to adefovir has not been clearly determined, it is thought to occur mainly in the proximal tubule of the

kidney. As a nucleotide analog, adefovir might serve as a substrate for DNA polymerase γ , which is responsible for the replication of mitochondrial DNA (mtDNA). Thus, adefovir is thought to inhibit mtDNA replication and impair cellular oxidative respiration^[36,37]. In addition, the over-expression of renal tubular transporters, which increases the intracellular concentration of the drug, is implicated in adefovir nephrotoxicity^[38-40].

In conclusion, our study reaffirmed that the *ab initio* combination of adefovir with lamivudine was more effective than switching to adefovir monotherapy in a stepwise fashion in patients with lamivudine-resistant CHB. Although early treatment response is closely related to long-term efficacy, stepwise combination therapy based on the roadmap concept carries a higher risk of virologic breakthrough than initiating combination therapy from the beginning of treatment.

COMMENTS

Background

Lamivudine is still being used in many patients with chronic hepatitis B (CHB) due to its lower cost, despite its higher risk of promoting viral resistance. Adefovir dipivoxil (adefovir) has been used to treat lamivudine-resistant CHB. It is known that the combination of adefovir with lamivudine results in a lower rate of resistance than adefovir monotherapy in patients with lamivudine-resistant CHB. Due to the high cost of combination therapy, however, adefovir is frequently initiated as a monotherapy, with lamivudine added back to the treatment regimen later as needed.

Research frontiers

A recent study suggested the potential usefulness of response-guided adefovir monotherapy based on the roadmap concept in patients with lamivudine-resistant CHB. However, no study has compared the effectiveness of response-guided stepwise combination therapy with that of *ab initio* combination therapy.

Innovations and breakthroughs

This study aimed to compare the long-term efficacies of various treatment strategies including adefovir in patients with lamivudine-resistant CHB, with a focus on whether long-term efficacy differs between patients treated in accordance with the roadmap concept and those treated with combination therapy from the start of treatment. The results suggest that, although an early virologic response helps to predict long-term efficacy, the treatment strategy conforming to the roadmap concept carries a higher risk of virologic breakthrough than does initiating combination therapy from the beginning of treatment.

Applications

For patients with lamivudine-resistant CHB, adefovir should be used *ab initio* in combination with ongoing treatment with lamivudine rather than switching to adefovir monotherapy with the later addition of lamivudine.

Terminology

The roadmap concept: The roadmap concept is a treatment strategy that is used to individualize ongoing antiviral management based on early treatment responses to minimize resistance and improve long-term efficacy.

Peer review

The authors present the results for 154 lamivudine-resistant CHB patients treated with adefovir and the long-term efficacies of various treatment strategies. The study is well designed, and the manuscript is well written.

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Tenofovir rescue therapy for chronic hepatitis B patients after multiple treatment failures

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Abstract

AIM: To evaluate the efficacy and safety of tenofovir disoproxil fumarate (TDF) for chronic hepatitis B (CHB) patients after multiple failures.

METHODS: A total of 29 CHB patients who had a suboptimal response or developed resistance to two or more previous nucleoside/nucleotide analogue (NA) treatments were included. Study subjects were treated with TDF alone ($n = 13$) or in combination with lamivudine (LAM, $n = 12$) or entecavir (ETV, $n = 4$) for ≥ 6 mo. Complete virologic response (CVR) was defined as an achievement of serum hepatitis B virus (HBV) DNA level ≤ 60 IU/mL by real-time polymerase chain reaction method during treatment. Safety assessment was based on serum creatinine and phosphorus level. Eleven patients had histories of LAM and adefovir dipivoxil (ADV) treatment and 18 patients were exposed to LAM, ADV, and ETV. Twenty-seven patients (93.1%) were hepatitis B e antigen (HBeAg) positive and the mean

value of the baseline serum HBV DNA level was $5.5 \log$ IU/mL $\pm 1.7 \log$ IU/mL. The median treatment duration was 16 mo (range 7 to 29 mo).

RESULTS: All the patients had been treated with LAM and developed genotypic and phenotypic resistance to it. Resistance to ADV was present in 7 patients and 10 subjects had a resistance to ETV. One patient had a resistance to both ADV and ETV. The cumulative probabilities of CVR at 12 and 24 mo of TDF containing treatment regimen calculated by the Kaplan Meier method were 86.2% and 96.6%, respectively. Although one patient failed to achieve CVR, serum HBV DNA level decreased by $3.9 \log$ IU/mL from the baseline and the last serum HBV DNA level during treatment was 85 IU/mL, achieving near CVR. No patients in this study showed viral breakthrough or primary non-response during the follow-up period. The cumulative probability of HBeAg clearance in the 27 HBeAg positive patients was 7.4%, 12%, and 27% at 6, 12, and 18 mo of treatment, respectively. Treatment efficacy of TDF containing regimen was not statistically different according to the presence of specific HBV mutations. History of prior exposure to specific antiviral agents did not make a difference to treatment outcome. Treatment efficacy of TDF was not affected by combination therapy with LAM or ETV. No patient developed renal toxicity and no cases of hypophosphatemia associated with TDF therapy were observed. There were no other adverse events related to TDF therapy observed in the study subjects.

CONCLUSION: TDF can be an effective and safe rescue therapy in CHB patients after multiple NA therapy failures.

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Key words: Tenofovir; Chronic hepatitis B; Treatment failure

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INTRODUCTION

Nucleoside/nucleotide analogues (NAs), which inhibit reverse transcription of hepatitis B virus (HBV) polymerase, are an important class of drugs that changed the treatment paradigm and prognosis of chronic hepatitis B (CHB). Oral NA therapy has advantages over interferon therapy due to its potent antiviral effects, good tolerance, lower side-effect profile, and convenience^[1,2]. However, the development of antiviral resistance to the drug is the major limitation of NA therapy and frequently leads to treatment failure. The development of drug resistance begins with mutations in the polymerase gene, followed by viral breakthrough, biochemical breakthrough, clinical deterioration, and even progressive liver failure. Favorable effects obtained by NA therapy are lost in patients who developed antiviral resistance. Today, an increasing number of patients experience multiple NA treatment failures, especially when they are sequentially treated with drugs that have similar characteristics^[3]. To achieve sustained suppression of HBV replication and remission of liver disease, successful management of CHB patients who developed treatment failure due to antiviral resistance or incomplete inhibition of viral replication is critical.

Tenofovir disoproxil fumarate (TDF) is an oral NA with the most potent activity against HBV and a high genetic barrier that has been approved in the United States and Europe for the treatment of CHB since 2009. In treatment-naïve patients, viral resistance to TDF was not detected after up to 144 wk of therapy, and persistent viremia through week 144 was only 0.8%, mostly due to poor compliance^[4]. TDF has also shown efficacy in NA experienced patients with various results. TDF monotherapy in patients with prior failure or resistance to two or more NAs showed a 79% viral suppression rate at 23 mo of treatment^[5]. In another study, TDF rescue therapy following lamivudine (LAM) and adefovir dipivoxil (ADV) treatment failure achieved a viral suppression rate of only 46% at 48 wk and 64% at 96 wk of treatment^[6]. However, experience with TDF in Asian countries, including Korea, is limited because this drug has not yet been approved for the treatment of CHB in this region of the world. A single report of six South Korean patients with prior LAM and ADV treatment failure stated that complete virologic suppression was achieved in all patients at

12 mo with TDF plus LAM therapy^[7].

Many CHB patients in Korea have undergone sequential treatment with LAM, ADV, and/or entecavir (ETV) at 1 mg to manage antiviral resistance or insufficient suppression of HBV DNA and they have begun to emerge as an important and difficult issue for clinicians^[8]. However, the efficacy of TDF treatment in such patients with previous treatment failures using multiple NAs, including ETV, is not well known. Multiple treatment failures of NAs in CHB patients are not limited to Korea; it is considered to be a global problem. In this study, we evaluated the efficacy and safety of a TDF containing treatment regimen in CHB patients after the failure of multiple NA therapies.

MATERIALS AND METHODS

Subjects

CHB patients with failures of two or more previous NA therapies were treated with TDF containing regimens for at least 6 mo. The TDF containing regimens included TDF monotherapy (300 mg/d), or combination therapy with LAM (100 mg/d) or ETV (1 mg/d). Failures of previous NA therapies included suboptimal viral suppression (serum HBV DNA level > 2000 IU/mL despite continued therapy for more than 1 year) or the development of resistance. Exclusion criteria were coinfection with human immunodeficiency virus or hepatitis C virus, and history of underlying renal problems. Since TDF is not approved for use with CHB patients in Korea, informed consent was obtained from all subjects. The study protocol was approved by the Institutional Review Board of Samsung Medical Center (IRB file number: 2011-06-027) and was conducted in accordance with the principles of the Declaration of Helsinki.

Laboratory and clinical assessment

Serum HBV DNA, hepatitis B e antigen (HBeAg), anti-hepatitis B e antibody, alanine aminotransferase, creatinine, and phosphorus levels were recorded every 3 mo. All data were collected from medical records and analyzed retrospectively.

Definition of treatment response

The mean reduction of HBV DNA levels was assessed during treatment. Complete virologic response (CVR) was defined as a decrease of serum HBV DNA \leq 60 IU/mL. The primary non-response was defined as a decrease in serum HBV DNA of less than 2 log IU/mL at 24 wk of therapy. Viral breakthrough (BT) was defined as an increase of HBV DNA > 1 log IU/mL from nadir during TDF treatment. HBeAg clearance included HBeAg loss or seroconversion to anti-HBe.

Safety assessment

The safety assessment was based on the development of renal toxicity or hypophosphatemia. Renal toxicity was defined when the estimated glomerular filtration

Table 1 Baseline clinical characteristics of the subjects (*n* = 29)

Characteristics	
Age, yr, median (range)	56 (22-63)
Male, <i>n</i> (%)	21 (72.4)
HBeAg positive, <i>n</i> (%)	27 (93.1)
Serum HBV DNA, log IU/mL, mean ± SD	5.5 ± 1.72
ALT, U/L, median (range)	47 (12-763)
Prior exposed NAs	
LAM + ADV, <i>n</i> (%)	11 (37.9)
LAM + ADV + ETV, <i>n</i> (%)	18 (62.1)
Treatment duration or prior NAs, mo, median (range)	66 (28-125)
Treatment regimen	
TDF/TDF+ LAM/TDF + ETV, <i>n</i> (%)	13 (44.8)/12 (41.4)/4 (13.8)
Treatment duration of TDF, mo, median (range)	16 (7-29)
Serum creatinine, mg/dL, median (range)	0.93 (0.6-1.16)
Serum phosphorus, mEq/dL, median (range)	3.3 (2.8-4.4)

ADV: Adefovir dipivoxil; ALT: Alanine aminotransferase; ETV: Entecavir; HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; LAM: Lamivudine; NA: Nucleoside/nucleotide analogue; TDF: Tenofovir disoproxil fumarate.

rate (eGFR) decreased by more than 20% from baseline. Calculation of eGFR was performed with this formula: $eGFR = (140 - \text{age}) \times \text{weight (kg)} (\times 0.85 \text{ if female}) / (72 \times \text{serum creatinine})$. Investigation of other adverse events was performed by review of medical records.

Virologic assay

Serum HBV DNA level was determined by real-time polymerase chain reaction (PCR) assay, using the COBAS TaqMan HBV quantitative test (Roche Molecular System Inc., Branchburg, NJ, United States), which has a lower limit of detection at 9 IU/mL. To identify mutations associated with resistance in the gene encoding HBV polymerase, PCR amplification and direct sequencing was performed in a single reference laboratory as previously described^[9].

Statistical analysis

To describe continuous variables with normal distributions, the mean ± SD was used. Continuous variables without normal distributions were expressed as the median with range. Cumulative probability of CVR during the treatment period was calculated using the Kaplan-Meier method and comparison between groups was performed with a log-rank test. *P* values less than 0.05 were considered statistically significant. All data were analyzed using SPSS (version 15.0; Chicago, IL, United States).

RESULTS

Baseline characteristics of the study subjects

A total of 29 patients were included in the study. De-

tailed demographics of the patients are presented in Table 1. The median age was 56 years (range 22 to 63 years) and 72.4% were male. Twenty-seven patients were HBeAg positive (93.1%) and the mean baseline serum HBV DNA level was 5.5 log IU/mL ± 1.7 log IU/mL. Eleven patients (37.9%) had been exposed to LAM and ADV, and 18 (62.1%) subjects had been additionally exposed to ETV. TDF treatment alone was used in 13 subjects (44.8%), with LAM in 12 (41.4%), or with ETV in 4 (13.8%). The median treatment duration of the TDF containing regimen was 16 mo (range 7 to 29 mo).

More detailed information about prior NA therapy history and genotypic resistance profiles is described in Table 2. All patients had been treated with LAM at first, and then developed a resistance to it. In LAM and ADV experienced patients, ADV was used as a sequential or add-on therapy in an attempt to suppress LAM resistant strains; however, the patients developed viral BT with or without genotypic resistance to ADV, or showed suboptimal viral response. In LAM, ADV, and ETV experienced patients, patients were moved to an 1 mg ETV regimen, due to the failure of sequential or add-on ADV therapy. 10 out of these 18 patients were confirmed to have genotypic resistance to ETV (Table 2).

Virologic response

The cumulative probability of CVR during the treatment period is presented as a Kaplan-Meier curve (Figure 1). The probability of CVR was 86.2% at 12 mo and 96.6% at 24 mo of treatment. In this study, only one patient did not achieve CVR with TDF treatment. The detailed clinical course of this patient is given in Figure 2. This patient underwent sequential therapy with LAM, ADV, and ETV at 1 mg; however, the development of resistance and suboptimal viral suppression led to treatment failure. TDF monotherapy was introduced and maintained for 24 mo. Even though the patient did not reach CVR, the patient's serum HBV DNA decreased to 85 IU/mL with a reduction of 3.9 log IU/mL, therefore becoming near CVR. No patient in this study developed primary non-response or viral BT during follow-up.

To define whether there is any difference in the rates of CVR according to prior exposures to antiviral agents, genotypic resistance profile, or TDF monotherapy *vs* combination therapy with LAM or ETV, the CVR rates were compared according to these variables using a log-rank test. There were no significant differences between patients with prior exposure to LAM and ADV *vs* LAM, ADV, and ETV (*P* = 0.93). Genotypic resistance to ADV (rtA181V/T or rtN236T) and resistance to ETV did not affect CVR rates (*P* = 0.99 and 0.14, respectively). TDF monotherapy, or in combination with other NAs, was not related to the achievement of CVR (*P* = 0.19).

Serologic response

Cumulative probability of HBeAg clearance was calculated with the Kaplan-Meier method. The rate of HBeAg clearance in the 27 HBeAg positive patients was 7.4%,

Table 2 Summary of prior nucleotide analogue treatment regimens and genotypic resistance analysis

Treatment history	LAM resistance	ADV resistance	ETV resistance	Outcome	n
LAM→ADV	rtM204V/I ± rtL180M	None		No VR	1
LAM→LAM + ADV	± rtV173L	rtA181V/T		Viral BT	1
		None	None	No VR	6
LAM→ADV→LAM + ADV		rtN236T,rtN238A		Viral BT	1
		rtA181V/T		No VR	2
			rtT184L + rtI169T		1
			rtT184S		2
		None	rtS202G	Viral BT	3
LAM→ADV→ETV			rtS202G + rtV207I		1
	rtM204V/I ± rtL180M		rtS202G + rtT184A		1
			None		5
		rtA181V/T	None	No VR	2
		None			1
LAM→LAM+ADV→ETV		rtN238H	rtS202G	Viral BT	1
		None	rtS202G + rtV207I		1

ADV: Adefovir dipivoxil; BT: Breakthrough; ETV: Entecavir; LAM: Lamivudine; VR: Virologic response.

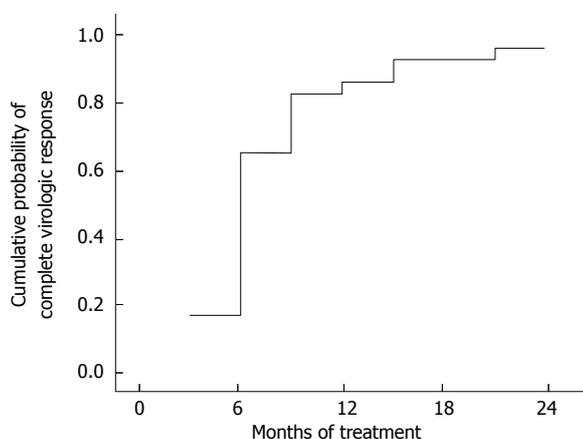


Figure 1 Probability of complete virologic response during the treatment period.

12%, and 27% at 6, 12, and 18 mo of treatment, respectively.

Safety analysis

No patient developed renal toxicity, defined as a decrease of eGFR more than 20% from baseline. No cases of hypophosphatemia associated with TDF therapy were observed. There were no other adverse events related to TDF therapy observed in the subjects.

DISCUSSION

The management of CHB has improved markedly over the last decade, primarily due to the availability of oral NA therapy. LAM was the first NA approved for CHB in 1998 and has been used extensively for treatment since that time. However, resistance to LAM occurs frequently and is observed in up to 80% of patients treated for 5 years^[10]. When sequential ADV monotherapy was introduced to these patients, ADV resistance was found in up to 21% of the patients after 1 to 2 years^[11-13]. Sequential

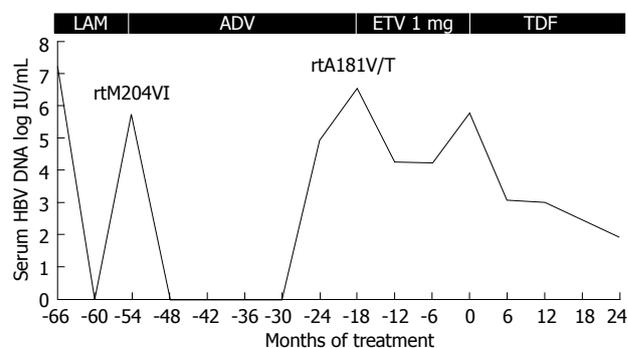


Figure 2 Detailed clinical course of a patient who did not achieve complete virologic response. The patient had undergone sequential therapy with lamivudine (LAM), adefovir (ADV), and entecavir (ETV) at 1 mg and developed sequential resistance and suboptimal viral suppression leading to treatment failure. Although tenofovir disoproxil fumarate (TDF) monotherapy was not able to result in complete virologic response (CVR), serum hepatitis B virus DNA levels were decreased to 85 IU/mL with a reduction of 3.9 IU/mL, accomplishing near CVR.

therapy with ETV, a newer generation NA, has been attempted to counteract LAM resistance. Despite good treatment efficacy and low levels of ETV resistance in treatment naive patients, rescue therapy in LAM resistance patients revealed that the treatment was less effective and had a higher rate of resistance^[14,15]. Sequential therapies with multiple NAs in this manner can promote selection for multidrug-resistant strains of HBV and frequently leads to viral BT or inadequate viral response^[3]. As a result, increased numbers of CHB patients with NA (mostly LAM and ADV, or ETV) treatment failures are becoming a global problem.

TDF is an oral NA that has been used for the treatment of human immunodeficiency virus infection and is approved for the treatment of CHB in the United States and Europe^[16]. Owing to its potent antiviral activity and high genetic barrier to the development of resistance for up to 3 years as determined in phase 4 clinical trials^[4,17], TDF is now recommended as a first-line therapy for HBV infected patients in recently published guide-

lines^[1,18].

The efficacy of TDF in the treatment of prior NA refractory HBV infection has also been evaluated. TDF showed an excellent antiviral activity on LAM resistant virus independently of the resistance mutation profile *in vitro*^[19]. In patients with LAM resistant CHB, treatment with TDF was well tolerated without significant adverse events such as renal toxicity. Treatment resulted in a median decline of 4.5 log copies/mL in HBV DNA levels after median treatment duration of 12 mo^[20]. TDF alone, or combined with LAM, exerted a greater viral reduction than ADV for LAM-resistant HBV infection without developing phenotypic resistance, and showed a high antiviral efficacy in patients with LAM resistance and an inadequate response during therapy with ADV^[21-23]. TDF plus LAM therapy improved the Child-Pugh score in decompensated liver cirrhosis patients with multiple NA treatment failures^[7].

This current study included 29 patients with a prior history of treatment failure with two or more NAs. Treatment with a TDF containing regimen in these subjects resulted in 86.2% of CVR at 12 mo and 96.6% at 24 mo of treatment. A distinctive feature of our study is that we included 18 patients who had failed treatment with ETV at 1 mg in addition to LAM and ADV. A retrospective multicenter study conducted by van Bömmel *et al.*^[5] included only 3 patients who had failed ETV therapy; two who had been only ETV experienced and one who had been treated with sequential LAM and ETV at 1 mg. ETV has an extremely high anti-HBV suppressive effect and very low chance of resistance emergence at only 1.2% after 5 years in treatment-naïve subjects^[24]. In this study, among the 18 patients who failed multiple NA treatments including LAM, ADV, and ETV, 17 patients achieved CVR and one patient showed a viral reduction of 3.9 log IU/mL, nearly reaching CVR. Genotypic resistance to ETV did not affect the probability of CVR. Based on these results, it can be suggested that the strong antiviral activity of TDF is also valid in ETV failed CHB patients.

Patterson *et al.*^[6] reported 64% of CVR rate at 96 wk of TDF rescue therapy in CHB patients following failures of both LAM and ADV treatment. 21 of the 60 patients had baseline ADV resistance (14 patients with the rtA181T/V mutation and 7 patients with the rtN236T mutation), and the authors concluded that the viral response was independent of mutations conferring ADV resistance. However, in another study, the presence of ADV resistance was considered to decrease the efficacy of TDF. During the observation period, the probability of achieving HBV DNA levels below 400 copies/mL was 52% for patients with ADV resistant variants and 100% for those without^[5]. In the current study, 6 patients had genotypic resistance to ADV. Although one patient with the rtA181V/T mutant strain did not reach CVR, resistance to ADV did not influence CVR during the treatment period. The effect of ADV resistance on the antiviral efficacy of TDF cannot be concluded from the results of this study because of the small sample size,

and is an area that should be explored in further research. However, considering that *in vitro* cross resistance of ADV and TDF has been described previously^[25-27], the possibility of altered response to TDF in CHB patients with genotypic resistance to ADV should be considered.

The ultimate goal of CHB therapy is to prevent cirrhosis, hepatic failure, hepatocellular carcinoma, and death. This goal can be achieved by the complete and sustained suppression of HBV replication. Therefore, the primary aim of treatment in chronic HBV infection is to suppress HBV replication to an undetectable level^[1,18,28], leading to decreased infectivity and pathogenicity of the virus and resulting in reduced hepatic necroinflammation^[29]. Viral BT resulting from resistance or insufficient suppression of HBV DNA due to the use of a less potent drug can result in failure to completely suppress HBV DNA. After the availability of ETV and TDF, NAs with potent antiviral activity and a high genetic barrier, insufficient viral suppression or resistance in treatment naïve patients is not common. However, in the era when these drugs with a high genetic barrier were not available, the majority of patients started therapy with LAM and subsequently received ADV and/or ETV to manage LAM resistance. Many of these patients developed resistance to ADV or ETV and remained viremic despite prolonged treatment^[11-15,22]. Multiple failures of NA therapies are a growing and global problem, especially in some parts of Asia, where the use of LAM remains common due to the high prevalence of CHB and generics are available at a low cost^[30]. In the present study, we have demonstrated the high efficacy of a TDF containing regimen in patients with sequential resistance to multiple NAs, and that TDF containing regimens are effective and safe rescue therapies for CHB patients after multiple failures with NAs.

Treatment efficacy, evaluated as CVR, did not differ in patients treated with TDF alone or in patients treated with combination therapy with LAM or ETV. Generally, combination therapy of drugs that are not in the same cross-resistance group is recommended for the management of resistant strains^[1,3,18]. However, since TDF monotherapy without other NAs was also effective without viral BT after approximately 2 years of follow-up^[5], it is questionable as to whether combination therapy with this strong antiviral agent is really necessary when considering the cost and potential adverse effects of combination therapy. As such, further studies are necessary to explore these results.

This study has some limitations. The number of subjects included was not large enough ($n = 29$) and the follow-up period was relatively short (median 16 mo). Nevertheless, the patients in this study represent the most difficult-to-treat population in the management of CHB. Further studies with larger sample sizes are necessary to remedy these shortcomings and to elucidate the long-term outcome of TDF treatment. Although the genotype of HBV could be a factor affecting the treatment efficacy of antiviral agents, we did not perform an analysis of the

genotype. However, previous studies have documented that almost all HBV infected patients in Korea have genotype C; constituting 98%-100% of HBV infected patients^[31-33]. Therefore these results could be applicable to our study patients, and can represent the genotype C HBV infected patients.

In conclusion, a TDF containing treatment regimen suppressed HBV DNA in CHB patients with multiple treatment failures of NA therapy, regardless of genotypic resistance or treatment regimen. TDF can be an effective and safe rescue therapy for these patients.

COMMENTS

Background

The excellent treatment efficacy of tenofovir disoproxil fumarate (TDF) in treatment naive chronic hepatitis B (CHB) has been documented in various clinical studies. However, data on its role in CHB patients with prior treatment failure to other oral anti-viral agent is insufficient.

Research frontiers

TDF has shown efficacy in nucleoside/nucleotide analogue (NA) experienced patients with various results outside of Asia. A single report including six South Korean patients with prior treatment failure to lamivudine (LAM) and adefovir stated that a complete virologic suppression was achieved in all patients at 12 mo with a TDF containing regimen, and this has been the only study of its type conducted in Asian country.

Innovations and breakthroughs

Many CHB patients in Asian countries develop sequential antiviral resistance to oral anti-viral agents or develop insufficient suppression of hepatitis B virus (HBV) DNA, which has begun to emerge as an important and difficult issue for clinicians. In this study, the authors documented the efficacy and safety of a TDF rescue therapy in Asian CHB patients after failure of multiple NA therapies. The efficacy was not affected by genotypic resistance or prior exposure to a specific agent.

Applications

After documenting the efficacy and safety of TDF regimen in CHB patients with multiple NA treatment failure, the authors suggest the possibility of using TDF as a rescue therapy for CHB patients, even those with prior entecavir therapy failure.

Peer review

The authors evaluated the efficacy and safety of a TDF treatment for CHB patients after failures of multiple NA therapies, and showed that TDF successfully suppressed HBV DNA levels in these patients. The study is very important, as the authors stated in the manuscript, in some countries where LAM treatment is still common due to financial problems.

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Interleukin-28 and hepatitis C virus genotype-4: Treatment-induced clearance and liver fibrosis

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Abstract

AIM: To investigate the association between *interleukin-28B* (*IL28B*) genotype and response to treatment and hepatic fibrosis in patients with hepatitis C virus (HCV) genotype 4.

METHODS: Two hundred and one HCV-genotype 4 patients were included. All patients were treated with Peginterferon alpha2a/Ribavirin for 48 wk. End of treatment response (ETR) was defined as loss of detectable serum HCV RNA at the end of treatment. Sustained viral response (SVR) was defined as loss of detectable serum HCV RNA at the end of 24 wk follow up. Genotyping of *IL28B* rs12979860 was performed using the TaqMan assay. We used logistic regression to estimate

the adjusted odds ratio (aOR) and 95%CI.

RESULTS: The study included 201 HCV-genotype 4 patients. The majority of patients were men (89.6%), with a median age of 47 years, inter-quartile range (40-51). Approximately 62.5% of patients had ETR, and 49.6% had SVR. Individuals who achieved SVR were more likely to be younger ($\chi^2 = 4.91, P = 0.027$), and less likely to have fibrosis ($\chi^2 = 15.54, P < 0.0001$), or inflammation ($\chi^2 = 7.58, P = 0.006$). The genotype distribution of rs12979860 was 36.2%, 49.0% and 14.8% for genotypes CC, CT, and TT, respectively. In these participants, rs12979860 genotype distribution did not differ by gender ($P = 0.466$), pretreatment viral load ($P = 0.600$), inflammation ($P = 0.435$), or fibrosis ($P = 0.291$). The frequencies of *IL28B* rs12979860 genotypes were TT (14.8%), CT (49.0%), and CC (36.2%). Compared to rs12979860 genotype TT, aORs (95%CI) for ETR and SVR were: CC genotype, [17.55 (5.34-57.69) and 5.92 (2.09-16.76), respectively]; CT genotype, [5.15 (1.80-14.78) and 2.48 (0.94-6.52), respectively]. In the current study, the patients who did not achieve ETR or SVR had a lower prevalence of rs12979860 CC (17.4% and 23.3%, respectively) than individuals who had ETR or SVR (47.9% and 47.2%, respectively). Individuals with rs12979860 CC genotype had approximately 6 times the odds of SVR compared to individuals with TT genotype (aOR = 5.92; 95%CI: 2.09-16.76). Similarly, patients with CT genotype had SVR more often than patients with TT genotype (aOR = 2.48; 95%CI: 0.94-6.52). Carrying at least one copy of the C allele (genotypes CT and CC) had almost 8 times the probability of ETR compared to those with genotype rs12979860 TT (aOR = 7.87; 95%CI: 2.84-21.82), and approximately 3 times the odds of SVR compared to those with genotype rs12979860 TT (aOR = 3.46; 95%CI: 1.37-8.74). In addition, data were consistent with a significant gene-dose relationship (aOR = 4.05/allele; 95%CI: 2.27-7.22). The association between rs12979860 genotype and SVR was

similar among those who achieved and those who did not achieve SVR.

CONCLUSION: In HCV-genotype 4 patients, rs12979860 is a sensitive predictor of viral clearance, independent of viral load, age, gender or fibrosis, with no similar relation to severity of fibrosis.

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Key words: Genotype 4; Hepatic fibrosis; Hepatitis C virus; *Interleukin-28B*; rs12979860

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INTRODUCTION

Hepatitis C virus genotype 4 (HCV-G4) is the most common type of HCV in the Middle East and Africa, particularly in Egypt, which has the highest prevalence of HCV worldwide (15%), and HCV-G4 represents 90% of all HCV cases^[1]. Despite the development of new direct antiviral agents such as protease inhibitors, which have improved response in HCV genotypes^[1,2], unfortunately G4 has emerged as a resistant genotype to new treatment strategies. This raises the importance, both for patient care and the economic approach, of improving the prediction of response to standard pegylated interferon alfa (PEG-IFN) and ribavirin therapy.

Although the mechanisms leading to clearance of acute HCV infection are not completely understood, both innate and adaptive immune responses have been suggested to play crucial roles in viral eradication and response to treatment^[3]. Besides immune responses, other host factors have also been associated with treatment-induced viral clearance. Higher rates of viral resolution have been reported in women compared with men; however, other factors such as age, race, or route of transmission were not significantly associated with viral resolution^[4].

Recently, another host factor, a genetic variation in the *interleukin-28B* (*IL28B*) gene, was found to predict spontaneous clearance of HCV infection^[5]. A single nucleotide polymorphism (SNP) rs12979860 located 3 kb upstream of *IL28B*, the gene that encodes IFN- λ 3, has been strongly associated with resolution of HCV infection^[6]. Patients with C/C genotype were more likely

to eradicate HCV than those with T/T genotype^[7,8]. The same SNP has also been associated with treatment-induced viral clearance^[9,10]. Patients with the C/C genotype were twice as likely to achieve an sustained viral response (SVR) compared to patients with other *IL28B* SNP genotypes^[6]. These findings jointly suggest a role for IFN- λ in the innate control of HCV. Of note, the T/T genotype was shown to be more common among those with African ancestry^[5].

However, the association between *IL28B* genotype and response to treatment among individuals infected with HCV-G4 still needs further investigation among the ethnic group living in the Middle East. Therefore, we conducted the present study to assess the extent of the association between *IL28B* genotype and response to treatment in HCV-G4 and severity of liver fibrosis.

MATERIALS AND METHODS

Subjects

The study included 201 Egyptian patients with chronic HCV genotype-4 who were followed in the Hamad Medical Corporation outpatient clinic in the State of Qatar. Patients received HCV treatment between 2007 and 2010. This was a retrospective-prospective cohort study in which all patients who were treated between 2007 and 2010 and had finished their follow up to week 72 were invited to participate (retrospective aspect). In addition, all patients who were currently on treatment and had not completed 72 wk of follow up (thus their outcome was not known yet) were invited to participate in the study (prospective aspect) and were followed until week 72 to determine their outcome.

All patients provided written informed consent in accordance with the Declaration of Helsinki of 1979, and the ethics research committee of the Hamad Medical Corporation provided ethical approval.

Chronic HCV infection was diagnosed by a sustained increase in alanine aminotransferase (ALT), positive anti-HCV serology and active virus replication shown by the detection of HCV-RNA and histological pattern of chronic active hepatitis. Patients were excluded from treatment if they had: active alcohol consumption over 80 g/d, concurrent hepatic B virus, immunodeficiency viruses, autoimmune hepatitis, hemochromatosis, Wilson disease, or were on antiviral or corticosteroid therapy.

All patients were treated with 180 μ g of Peginterferon-2a (Pegasys[®], Hoffmann-La Roche, Basel, Switzerland) subcutaneously once weekly and Ribavirin (COPE-GUS[®]; Hoffmann-La Roche) 1000 mg (body weight \leq 75 kg) or 1200 mg (body weight \geq 75 mg) orally for 48 wk. End of treatment response (ETR) was defined as loss of detectable serum HCV RNA at the end of treatment (48 wk). SVR was defined as loss of detectable serum HCV RNA at the end of follow up (72 wk).

Laboratory assays

Viral assays: Testing for anti-HCV was carried out us-

Table 1 Demographic and clinical characteristics of the study participants

	End of treatment response		Sustained viral response	
	No	Yes	No	Yes
Age, median (IQR)	48.0 (40.5-53.0)	46.0 (40.0-51.0)	48.0 (43.5-53.0)	46.0 (38.0-51.0)
HCV log ₁₀ viral load, median (IQR)	5.9 (5.1-6.4)	5.5 (3.8-6.3)	5.8 (5.2-6.5)	5.4 (3.6-6.1)
Male (%)	93.1	87.5	91.3	88.1
rs12979860 (%)				
TT	30.0	5.1	23.3	7.6
CT	52.9	47.0	53.4	45.2
CC	17.1	47.9	23.3	47.2

HCV: Hepatitis C virus; IQR: Inter-quartile range.

ing a commercial ELISA kit (AxSYM 3.0; Abbott Laboratories, Chicago, IL, United States). All patients were HCV-G4 as detected by the Inno-LiPA HCV II assay (Innogenetics Inc., Alapharetta, GA, United States). Serum HCV RNA level monitoring was by Amplicor (version 2.0; Hoffmann-La Roche) with a minimum detection limit of 50 IU/mL.

Liver histology: The necro-inflammatory and fibrosis scores were assigned based on the Scheuer scoring system from 0 to 4. The patients were further subdivided into mild fibrosis (stages I and II) and severe fibrosis (stages III and IV).

***IL28B* genotype assay:** Genomic DNA was extracted from EDTA whole-blood samples using the QiaAmp DNA Blood Mini Kit # 51166 (Qiagen GmbH, Hilden, Germany). DNA concentration was measured using a Nanodrop Spectrophotometer to assess the quantity of the product. Polymorphisms of the studied SNP were carried out by the 5' nuclease assay using the TaqMan MGB probe. The reaction was performed using an ABI 7500 (Applied Biosystems, Foster City, CA, United States) in the Biomedical Labs-Health Sciences Department at Qatar University, Doha, Qatar. The primers and the TaqMan MGB probes of the SNP were provided by the Custom assay-on demand™ service (Applied Biosystems). The 5' nuclease assay was performed using 10-30 ng genomic DNA, 1 × TaqMan Universal polymerase chain reaction Master Mix (Applied Biosystems), and 1 × primer/probe mix using the correct conditions for amplifications according to the manufacturer's instructions. Negative controls as well as non-template controls were included in each run.

Statistical analysis

In the bivariate analyses we used the χ^2 test for categorical variables and ANOVA for continuous variables to compare the relevant characteristics by treatment response status. We conducted multivariable logistic regression to calculate adjusted odds ratio (aOR) and 95%CI of the association between treatment response and *IL28B* genotype. Only variables that were significantly associated with treatment response or *IL28B* genotype were included in the multivariable models. To test whether

the association between *IL28B* genotype and response to treatment was modified by gender, grade, viral load, inflammation or fibrosis, we employed logistic regression models with an interaction term (cross product) for *IL28B* genotype and the modifier of interest included. ORs were computed using the homozygous minor allele as the reference group. All analyses were performed using SAS program version 9.2 (SAS Institute, Cary, NC, United States).

RESULTS

Descriptive data

Table 1 presents the characteristics of the study population. The study included 201 patients. The majority of participants were males (89.6%). Median age was 47 years (inter-quartile range 40-51). We observed Rapid Virological response (RVR), ETR, and SVR in 52.5%, 62.5% and 54.2% of patients, respectively. The mean, median and range of viral load (log 10) over the duration of therapy among responders and non-responders, were (4.9, 2.2, 0.0-6.9 and 5.5, 3.9, 2.1-6.9) for RVR, (3.6, 1.0, 0.0-4.23 and 6.0, 4.9, 2.5-6.5) for ETR, respectively. There was no difference in gender, or pretreatment HCV viral load by RVR, ETR or SVR status. RVR was not associated with age, liver inflammation or fibrosis. Those who had ETR were less likely to have fibrosis ($\chi^2 = 12.54$, $P < 0.001$), or inflammation ($\chi^2 = 5.17$, $P = 0.023$). Only 62.5% of patients had an ETR and 49.6% had SVR. There was no difference in gender, or pretreatment HCV viral load by ETR or SVR status. In addition, individuals who achieved SVR were more likely to be younger ($\chi^2 = 4.91$, $P = 0.027$), and less likely to have fibrosis ($\chi^2 = 15.54$, $P < 0.0001$), or inflammation ($\chi^2 = 7.58$, $P = 0.006$).

The genotype distribution of rs12979860 was 36.2%, 49.0% and 14.8% for genotypes CC, CT, and TT, respectively. Among our participants, rs12979860 genotype distribution did not differ by gender ($P = 0.466$), pretreatment viral load ($P = 0.600$), inflammation ($P = 0.435$), or fibrosis ($P = 0.291$). In our study, the patients who did not achieve ETR or SVR had a lower prevalence of rs12979860 CC (17.4% and 23.3%, respectively) than individuals who had ETR or SVR (47.9% and 47.2%, respectively). Genotype distributions by HCV status are presented in Figure 1.

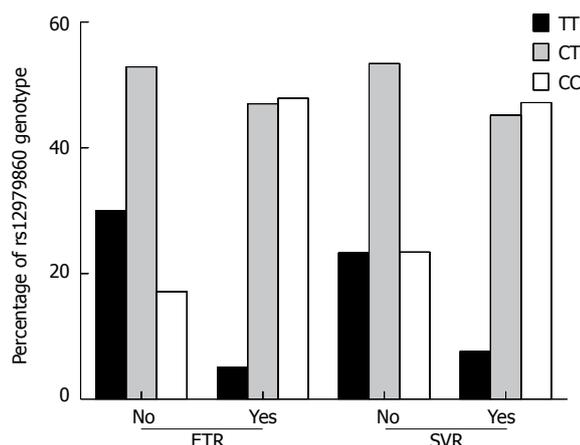


Figure 1 Genotype frequency in percentage of rs12979860 of the *interleukin-28B* gene under treatment with and without end of treatment response and with and without sustained virological response in chronic hepatitis C-genotype 4 patients. ETR: End of treatment response; SVR: Sustained viral response.

End of treatment response

The data revealed that adjusting for stage, patients with rs12979860 *CC* and *CT* genotypes had ETR more often than patients with *TT* genotype [aOR (95%CI) = 17.55 (5.34-57.69) and 5.15 (1.80-14.78), respectively]. Patients carrying at least one copy of the C allele (genotypes *CT* and *CC*) had almost 8 times the probability of ETR compared to those with genotype rs12979860 *TT* (aOR = 7.87; 95%CI: 2.84-21.82) (Table 2). In addition, data were consistent with a significant gene-dose relationship (aOR = 4.05/allele; 95%CI: 2.27-7.22). No significant interactions were detected by gender, fibrosis, inflammation, or pretreatment ALT levels ($P > 0.05$).

Sustained viral response

Individuals with rs12979860 *CC* genotype had approximately 6 times the odds of SVR compared to individuals with *TT* genotype (aOR = 5.92; 95%CI: 2.09-16.76). Similarly, patients with *CT* genotype had SVR more often than those with *TT* genotype (aOR = 2.48; 95%CI: 0.94-6.52). Carrying at least one copy of the C allele (genotypes *CT* and *CC*) had approximately 3 times the odds of SVR compared to those with genotype rs12979860 *TT* (aOR = 3.46; 95%CI: 1.37-8.74) (Table 2). Of note, we observed a significant gene-dose relationship (aOR = 2.42/allele; 95%CI: 1.47-3.99). We did not reveal any significant interactions by gender, fibrosis, inflammation, or pretreatment ALT levels ($P > 0.05$).

There were no significant interactions between the rs12979860 genotype and RVR indicating that the association between the rs12979860 genotype and SVR was similar among those who achieved RVR and those who did not achieve RVR.

DISCUSSION

Recent studies have suggested that SNPs within or ad-

Table 2 Adjusted associations between *interleukin-28B* genotype and response to treatment

	End of treatment response		Sustained viral response	
	OR (95%CI)	P value	OR (95%CI)	P value
rs12979860				
TT	Ref. genotype		Ref. genotype	
CT	5.15 (1.80-14.78)	0.002	2.48 (0.94-6.52)	0.065
CC	17.55 (5.34-57.69)	< 0.0001	5.92 (2.09-16.76)	< 0.001

OR: Odds ratio.

acent to *IL28B* are the most promising, host-related predictors of treatment-induced clearance in HCV genotype-1 patients^[11,12], however, in studies of individuals infected with genotypes 2 and 3, the association between *IL28B* genotype and outcome is less pronounced, including rs12979860 with treatment-induced clearance^[13,14]. The impact of genetic *IL28B* variability on viral elimination during therapy in G4 is still limited. Similar to HCV genotype-1, in the present study, we demonstrated that *CC* genotypes of rs12979860 significantly determined SVR in patients with HCV G4. Not only that, even carrying at least one copy of the C allele increased sensitivity to PEG-IFN/Ribavirin therapy, which was 3 times that of rs12979860-negative hosts. The mechanism and explanation behind the association between genetic variations in the *IL28B* gene and spontaneous clearance may be related to the host innate immune response. *IL28B* encodes IFN- λ 3, which is involved in viral control, including HCV. In vitro, IFN- α induces expression of IFN- λ genes, which inhibit HCV replication^[15]. *IL28A* and *IL29* are three closely related cytokine genes that encode proteins known as type III IFNs at chromosomal region 19q13. The three cytokines are induced by viral infection and have antiviral activity. In addition, an experimental genetic variant regulating *IL28* expression is important for PEG-IFN TLR-mediated antiviral protection.

HCV treatment should not be withheld based solely on *IL28B* genotype, as patients with the *TT* genotype can achieve SVR, as it was found that the association between rs12979860 genotype and SVR was similar between the patients regardless of their RVR status.

In contrast to Ge *et al*^[6] who reported higher viral load with the C allele, in our participants, rs12979860 genotype distribution did not differ by pretreatment viral load.

Rao *et al*^[16] reported a significant genetic polymorphism among women who responded to treatment, but Montes-Cano *et al*^[17] found that rs12979860 genotype distribution did not differ by gender in G4 patients. This can be explained simply, by the difference in tested SNPs, while Rao *et al*^[16] reported this gender-related difference with the rs8099917 *TT* genotype, in our study and in the study by Montes-Cano, we studied rs12979860.

The association between *IL28B* polymorphisms and liver fibrosis progression is still controversial^[18]. While, Barreiro *et al*^[19] reported that *IL28B* *CC* carriers might experience a more rapid progression of HCV-related

liver fibrosis, Marabita *et al.*^[20] reported that *IL28B* polymorphisms had no impact on fibrosis progression. Some investigators found that the unfavorable rs12979860 T/T gene pattern was associated with worse liver fibrosis^[21], while others did not replicate this finding. In G4 patients, and similar to Asselah *et al.*^[22], no significant differences and associations for genotype and alleles frequencies were detected between mild and severe fibrosis. Host genetic factors associated with the risk of liver disease progression in hepatitis C-G4 needs further investigation.

Few reports have studied the relation between *IL28B* and G4, particularly among patients in our area, where Egyptians represent the majority of cases with different ethnic and G4-subtypes^[23]. The overall frequency of the protective C allele in our study was 85%, which was higher than that found by Kurbanov *et al.*^[24], who reported a frequency of 67% among the Egyptians studied. When the diploid genotype was considered, the overall frequency of the protective C/C genotype was 45%, which was close to our finding (36.2%). With regard to the association between genetic polymorphisms and response to treatment, Asselah *et al.*^[22] reported that SNP rs12979860 was strongly associated with SVR in patients infected with HCV-G4 in 164 HCV-G4 patients who were from different ethnic groups (Egyptian, European, and Sub-Saharan African). Although Egyptians represented 67% (75/112) of the patients with G4 in the study by De Nicola *et al.*^[25], the results were very similar to ours concerning *IL28B* genotype distribution and distribution of the C allele among the patients who achieved SVR. Our study re-enforces the importance of SNP rs12979860 of *IL28B* in G4, regardless of viral clearance by week 4 and confirmed the absence of its correlation with hepatic fibrosis.

This study specifically examined the relationship between *IL28B* rs12979860 CC genotype, rapid viral clearance, treatment response and severity of liver disease in patients with chronic HCV-G4 infection which included a large sample of patients from the same ethnic group.

In conclusion, in patients with HCV-G4, the *IL28B* single nucleotide polymorphism (rs12979860) is a sensitive predictor of viral clearance, independent of baseline viral load, age, gender or fibrosis. This polymorphism does not have a similar relation with rapid viral clearance or severity of hepatic fibrosis.

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COMMENTS

Background

Genotype 4 (G4) has emerged as a resistant genotype to new treatment strategies. This raises the importance, both for patient care and the economic approach, of improving the prediction of response to standard pegylated interferon

alfa and ribavirin therapy. Genetic variation in the *interleukin-28B* (*IL28B*) gene has been found to predict spontaneous clearance of hepatitis C virus (HCV) infection and response to treatment.

Research frontiers

The association between the *IL28B* genotype and response to treatment in individuals infected with HCV-G4 still needs further investigation, especially among the ethnic group living in the Middle East. The research focused on the sensitivity of this gene in the prediction of response and its importance in predicting liver histology progression.

Innovations and breakthroughs

In previous reports, a few G4 patients were studied which was mainly related to spontaneous clearance. This study provided information on the relation between *IL28B* and post-treatment viral clearance and its relation to hepatic fibrosis in G4.

Applications

The study results suggest that the *IL28B* single nucleotide polymorphism (rs12979860) is a sensitive predictor of viral clearance in G4 patients and may improve clinical prediction models for treatment and should be included as a test in pre-treatment investigations, providing an opportunity for clinicians to individualize treatment regimens for hepatitis C patients.

Peer review

The authors studied 201 G4 HCV infected patients. All patients were treated with Peginterferon/Ribavirin for 48 wk. They found in HCV-G4, *IL28B* single nucleotide polymorphism (rs12979860) is an independent predictor of viral clearance.

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Predictive factors of endoscopic submucosal dissection procedure time for gastric superficial neoplasia

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Abstract

AIM: To identify the determinants of endoscopic submucosal dissection (ESD) operation time.

METHODS: This investigation was conducted as a single-center, prospective study in which ESD was performed by the same endoscopist at the Chinese PLA General Hospital. A total of 173 patients underwent ESD operations performed by Dr. Lu from July 2007 to December 2011, and 183 lesions were enrolled. Patient gender, age, tumor location, gross type, tumor

size, pathological type and adhesions were recorded prospectively. The order of treatment represented the experience of the operator. Univariate analysis and multivariate analysis were performed to evaluate the relationships between these factors and ESD procedure time.

RESULTS: Univariate analysis showed the ESD time was closely related to the gender ($P = 0.0210$), tumor size ($P < 0.0001$), location ($P < 0.0001$), gross type ($P < 0.0001$) and adhesion ($P = 0.0010$). The surgical proficiency level was associated with ESD time in unit area ($P < 0.0001$). Multivariate analysis revealed that the ESD time was positively correlated with tumor size ($P < 0.0001$), adhesion ($P < 0.0001$) and location ($P < 0.0001$), but negatively correlated with surgical proficiency level ($P = 0.0046$).

CONCLUSION: Large tumor size, adjacency to the cardia, and adhesion are predictors of a long ESD time, whereas high surgical proficiency level predicts a short ESD time.

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Key words: Endoscopic submucosal dissection; Procedure time; Gastric superficial neoplasia; Predictive factors

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INTRODUCTION

Endoscopic submucosal dissection (ESD) has become a mature technique widely applied in the treatment of mucosal lesions. ESD has advantages compared with endoscopic mucosal resection. However, ESD is complex, and the associated high risk is another disadvantage of ESD that requires improvement^[1-7]. The time required to perform ESD is one of the best indicators of operation difficulty. Studies have demonstrated ESD time is closely related to bleeding, perforation and post-operative pneumonia^[8-12]. It is the goal of clinicians to overcome operation difficulty and reduce ESD time. Previous studies have reported on the determinants of ESD time, but these results reflected the operations conducted by several clinicians^[13,14]. In the present study, ESD was performed by the same endoscopist. A total of 183 lesions were collected, and the determinants of ESD time were analyzed.

MATERIALS AND METHODS

Patients

From July 2007 to December 2011, consecutive gastric superficial intraepithelial neoplasia (GIN) was treated by ESD that were performed by the same endoscopist at the Chinese PLA General Hospital. This study was approved by the medical ethical committee Chinese PLA General Hospital and the clinical study was registered with the Clinical Trial (NCT01378507). Partial resection, piecemeal resection and snare resection after circumferential cutting was excluded and complete *en bloc* resection was included in the study.

The morphology of the lesions of all patients was superficial intraepithelial neoplasia according to the Paris endoscopic classification^[15]. Adenoma, noninfiltrating carcinoma or intramucosal invasive carcinoma were confirmed by histologic evaluation of forceps biopsy specimens, which corresponding to criteria of no lymph node metastases^[16].

Patients were excluded from the study if they had a previous diagnosis of undifferentiated carcinoma, or Type 0-III or 0-I s adenocarcinoma. Systemic conditions were evaluated before each operation, and those with contraindications for anesthesia and endoscopy were excluded from the study. The existing strategies for the treatment of GIN and their advantages and disadvantages were explained to the patients and their family members before the operation. Written informed consent was obtained before ESD.

ESD procedure

The patients were sedated by intravenous injection of propofol (0.1-0.2 mg/kg per minute) (Xi'an Libang Pharmaceutical Co., Ltd. Xi'an, China). A gastroscope (GIF Q260J, Olympus Optical Co., Ltd, Tokyo, Japan) was used for the treatment, which was facilitated with a soft transparent front cap (Olympus Optical Co, Ltd.) and a high-

frequency surgical unit for cutting and coagulation (Erbotom VIO200; ERBE, Tübingen, Germany). A normal-saline solution of 10% glycerin and 5% fructose with epinephrine (0.025 mg/mL) was used for submucosal injections.

The first step was locating the lesion using gastroscopy with narrow-band imaging magnification to observe and distinguish the border between the lesion and normal mucosa. The border was marked with thermal coagulation markers at 0.5 cm intervals at a distance of 0.3 to 0.5 cm from the edge with a needle knife (KD-1L; Olympus Optical Co. Ltd., Tokyo, Japan). The glycerol fructose solution was then injected submucosally around the lesion to raise the mucosa thoroughly. Next, a pre-cut was made with needle knife to cut the mucosa through to the submucosa. Then insulation tipped (IT-2) knife (KD-610L; Olympus Optical Co, Ltd.) was inserted into the pre-cut incision to complete the incision around the lesion at 0.5 cm outside the markers (Endo cut Q 2). After the lesion was cut in a circle, submucosal injection was continued, and the mucosa was gradually dissected with an IT-2 knife (foced coag 40W) until the mucosa was completely excised. A needle knife or IT-2 knife was used for hemostasis of small blood vessels (foced coag 40W); an electric coagulation hemostat (FD-410LR; Olympus Optical Co., Ltd.) was used for hemostasis of large blood vessels (soft coag 80W). The ESD procedure is showed in Figure 1.

Definition

ESD time was defined as the time from circumferential marking around the lesion to the complete removal of GIN. The number of patients was used as the continuous numerical variable, which represented the proficiency level. The tumor size was calculated with two vertical maximum diameters.

Statistical analysis

The age, gender, number of patients, location, macroscopic appearance, tumor size, pathological type and adhesion were recorded as variables. Normally-distributed data were expressed as mean \pm SD, and data not conforming to a normal distribution were expressed as medians and inter-quartile ranges. The categorical variables were presented as constituent ratios. One way analysis of variance was employed to analyze the factors influencing procedure time. Simple correlation analysis was performed to investigate the association between numerical variables and procedure time. A rank sum test was used to investigate the association between categorical variables and procedure time. A value of $P < 0.05$ was considered statistically significant. Factors meeting the criteria for significance in the univariate analysis were included in the multivariate analysis. Multiple linear regressions were used to analyze the association between procedure time and factors influencing procedure time. The procedure time served as the dependent variable and those from univariate analysis as independent variables. Factors with a significant difference, as determined by multivariate analysis, were included

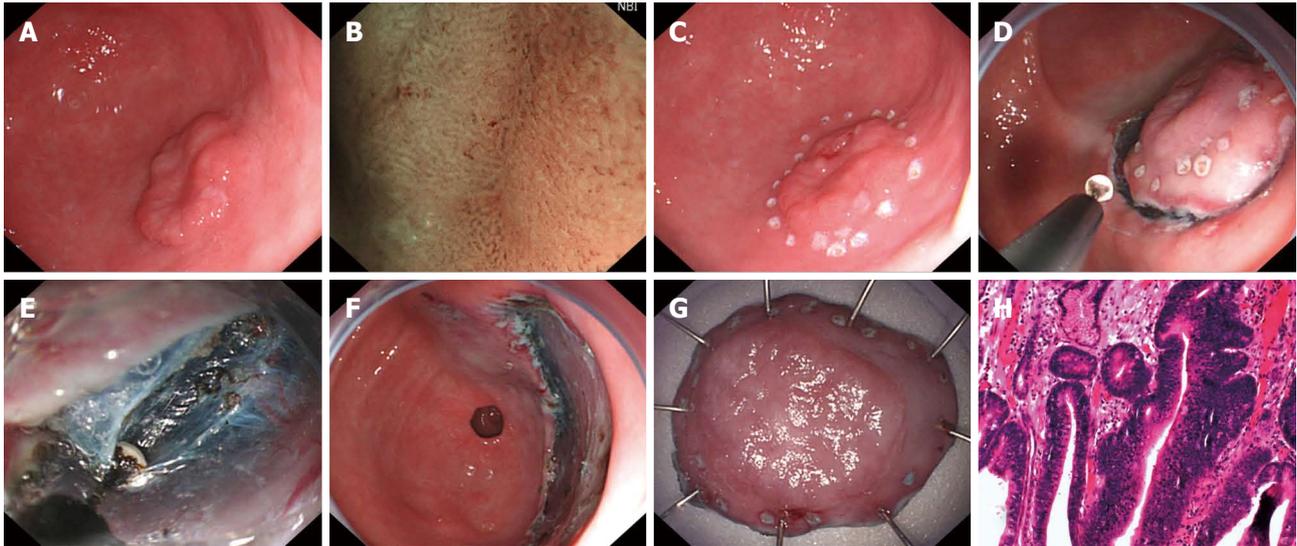


Figure 1 Procedure of endoscopic submucosal dissection: A male patient aged 56 years. A: Gastroscopy showed a type 0-IIa (2.5 cm × 1.8 cm) lesion in the greater curvature of the stomach and pathology revealed differentiated adenocarcinoma; B: Magnification was performed using narrow band imaging to determine the borderline; C: Point-like electric coagulation was done at 0.5 cm away from the borderline of lesion for marking; D: Following submucosal injection, a circumferential incision was made in the mucosa and submucosa at 0.5 cm away from the marks; E: The mucosae were dissected from submucosa using an insulation tipped-2 knife; F: Artificial ulcer following resection; G: The sample (4 cm × 3 cm) was unfolded and the marks were found in the sample; H: Pathological type was high-grade intraepithelial neoplasia (× 40).

in a step-forward linear regression model to compose a predictive formula of procedural time. In addition, 0.10 and 0.05 were applied for backward selection and forward selection, respectively. Statistical analysis was performed with SPSS version 13.0.

RESULTS

A total of 194 GIN lesions were treated by ESD, and 183 GIN lesions were completely removed (94.3%). In addition, 178 lesions met the criteria for curative resection (91.8%). In the present study, 173 cases were recruited, and a total of 183 lesions were collected. There were 126 males and 47 females, with a mean age of 62.0 years (29-82 years). The major diameter of the resected lesions was 3.0 cm (1.5-8.0 cm). The median area of resected mucosa was 7.0 cm² (1.5-33.25 cm²). The median procedure time was 41 min (8-221 min). Bleeding to different extents was observed during the operation but did not meet the criteria for bleeding as a complication^[17]. Delayed bleeding was observed in 7 cases, which resolved after endoscopic hemostasis ($n = 5$) or pharmacotherapy ($n = 2$).

Small muscularis propria resection was identified in 2 cases, but the serosa was intact and no pneumoperitoneum was observed. Because the defect was closed with the used of endoscopic clips after the *en bloc* dissection, the clip closure time was not including the ESD procedure time and was not analyzed as a risk factor.

Univariate analysis showed that the procedure time was related to tumor size, but not to age or the level of surgical proficiency. The procedure time was significantly different among groups in terms of gender, location, macroscopic appearance and adhesion. No marked dif-

ferences were noted among groups in terms of pathological types.

The irrelevance of some factors to ESD time may be attributed to small tumor size at early stage. For the same size lesion, the more experienced of the operator, the shorter of procedure time. Once ESD time per unit area was used as a factor, the influence of resection area was abolished. The ESD procedure time should be related to the proficiency of endoscopist because the resection size was increased as technical proficiency increased so the ESD procedure time was also increased although univariate analysis showed no correlation. If the ratio of procedure time to specimen area was transformed unit area, the ESD procedure time related to the proficiency of endoscopist and the number of procedure cases was conformed by univariate analysis because of elimination of interference of lesion area. So the number of procedure cases was independent predictors in multivariate analysis. (Tables 1 and 2).

Multiple linear regression analysis revealed that ESD time was positively correlated with tumor size, adhesion and location, but was negatively associated with surgical proficiency level. After comparison with the standardized regression coefficients, the correlation intensity was in the following order: tumor size, adhesion, lesions in upper stomach and proficiency level. Although multiple linear regression analysis showed the ESD procedure time was not correlation with the lesions in the middle of the stomach, the influence of lesions in the middle stomach should be used in prediction the time because ESD procedure time was related to the lesions in the upper stomach. Statistical analysis revealed tumor size and proximity to the cardia were positively correlated with longer ESD times. In addition, a submucosal adhesion could prolong

Table 1 Correlation analysis of numerical variables and endoscopic submucosal dissection time

Predictive factors	Data	ESD time (min) median (IQR)	Analysis method	P value
Age	mean ± SD 62.00 ± 11.69	41.00 (39.00)	Pearson correlation	0.3135
Tumor size (cm ²)	Median (IQR) 7.00 (8.82)	41.00 (39.00)	Pearson correlation	< 0.0001
Proficiency level	<i>n</i> 1-183	ESD time per unit area(min) 5.00 ± 3.87	Pearson correlation	< 0.0001

ESD: Endoscopic submucosal dissection; IQR: Interquartile-range.

Table 3 Multivariable analysis of factors related to endoscopic submucosal dissection time

Predictive factors	Beta	Standardized coefficients beta	95%CI for beta	P value
Resection area	3.500	0.581	2.829-4.180	< 0.0001
Adhesion	67.082	0.356	47.003-86.285	< 0.0001
Location (upper)	39.439	0.345	25.767-50.124	< 0.0001
Proficiency level	-0.755	-0.207	-0.545-(-0.124)	0.0046
Location (middle)	7.009	0.059	-2.890-17.778	0.1408

ESD: Endoscopic submucosal dissection.

ESD time. With the increase of proficiency level, ESD time was reduced. The results of multiple linear regressions are displayed in Table 3.

DISCUSSION

In the present study, ESD was performed by a single endoscopist. The complete resection rate, curative resection rate, and incidence of complications were similar to those previously reported^[18-25]. The procedures of ESD by the same operator were identical among different patients. Thus, the influence of procedural differences on ESD time was excluded. We tried our best to reduce pre-operative confounding factors, and then performed univariate and multivariate analysis. The analysis of information from operations performed by the same operator can help to identify the association between proficiency level and ESD time.

The pre-operative predictable factors served as variables for analysis, and the results can serve as a reference for predicting ESD time. Although an adhesion is only found during surgery, the scar and the cushion following submucosal injection can be identified to determine the adhesion. In the recruitment of patients, the indications were restricted and the post-operative pathology only confirmed two lesions with submucosal invasion. The remaining lesions were only found in the tunica mucosa. Lesions confined to the tunica mucosa may not affect the procedure time regardless of tumor depth. Thus, tumor depth was not applied as a viable.

Studies have shown that ESD time is related to the location and size of tumors. However, some studies are

Table 2 Categorical variables and endoscopic submucosal dissection time

Predictive factors	<i>n</i> (%)	ESD time (min) median (IQR)	Analysis method	P value
Location			Kruskal wallis	< 0.0001
Upper	44 (24.0)	52.00 (69.00)		
Middle	52 (28.4)	48.00 (33.00)		
Lower	87 (48.6)	26.00 (33.50)		
Macroscopic appearance			Kruskal wallis	< 0.0001
Protrusion	76 (41.5)	23.00 (31.00)		
Indentation	61 (33.3)	49.00 (31.00)		
Mixed	46 (25.2)	50.00 (43.50)		
Pathological type			Kruskal wallis	0.2410
Adenoma	36 (19.7)	25.00 (27.00)		
HIN	97 (53.0)	29.00 (36.00)		
Invasive carcinoma	50 (27.3)	51.00 (29.50)		
Adhesion			Mann-whitney	0.0010
Yes	14 (7.7)	81.50 (149.00)		
No	179 (92.3)	39.00 (35.00)		
Gender			Mann-whitney	0.0210
Male	134 (73.2)	47.00 (41.00)		
Female	49 (26.8)	29.00 (25.50)		

ESD: Endoscopic submucosal dissection; IQR: Interquartile-range.

derived from empirical analysis, and some from univariate analysis^[26]. Goto *et al*^[13] investigated 222 early gastric cancers, which were resected with a Flex knife by four operators. Their results showed that location in the upper-third of the stomach, the presence of ulcerative findings, and > 20 mm in size was independent factors affecting ESD time. In the study by Ahn *et al*^[14] complete ESDs were performed by four experts, primarily using an IT knife, for 916 early gastric cancers. The results revealed that proximal location, tumor size greater than 20 mm, submucosal fibrosis, and perforation during the procedure were independent predictors of a longer ESD time. In the present study, the multivariate analysis indicated that ESD time was positively correlated with tumor size, location and adhesion, but negatively associated with proficiency level. The correlation intensity was in the following order: resection area, adhesion, location in the upper-third of the stomach and the proficiency level.

The factors identified by the multivariate analysis can be applied to predict ESD time. However, the determinants are numerous, and some are unpredictable before an operation, including unstable anesthesia, equipment failure, and heavy bleeding. Therefore, ESD time cannot be accurately determined according to the factors identified in the present study. These factors may only be used to predict ESD time and the level of difficulty. These findings suggest that lesions with small size and without adhesion are suitable for the training of inexperienced endoscopists. The resection of lesions with larger size located in the upper to middle stomach should be performed by experienced operators or in the presence of experienced operators. Once it is determined that the predicted ESD time is relatively long, preventive mea-

tures should be prepared before operating, with the aim of preventing skin compression and deep vein thrombosis of the lower extremities. With respect to anesthesia, intubation followed by general anesthesia is preferred. Maintaining an open airway helps to assure satisfactory anesthesia status.

Although analysis of clinical information from operations performed by the same operator can exclude the influence of other confounding factors, such as different instruments and operation habits, we only analyzed the data from a single center and therefore the sample size was relatively small. Our experience revealed that the time consumed maintaining hemostasis can affect ESD time. However, this period of time is unpredictable and cannot be accurately recorded. Therefore, it was not used as a variable for analysis.

Taken together, our results revealed that large tumor size, adjacency to the cardia, and adhesion predict a long ESD time, but high a proficiency level predicts a short ESD time. Our results provide reference for the prediction of operation difficulty and ESD time.

COMMENTS

Background

Endoscopic submucosal dissection (ESD) is a therapeutic technique for the treatment of gastrointestinal neoplasms with high *en bloc* resection rate. However, owing to its technical difficulty, longer procedure time and increase risk of perforation, ESD is not as widely used in China. The procedure time of ESD is the most direct indicator of operation difficulty. An investigation of the determinants of the time required for ESD will offer guidance in predicting operation difficulty.

Research frontiers

Many investigators have shown that ESD time is related to the location and size of tumors. In the present study, the multivariate analysis indicated that ESD time was positively correlated with tumor size, location and adhesion, but negatively associated with proficiency level.

Innovations and breakthroughs

In this study, 183 ESD procedures were performed by a single endoscopist. Analysis of clinical information from operations performed by the same operator can exclude the influence of other confounding factors, such as different instruments and operation habits.

Applications

This study revealed that large tumor size, adjacency to the cardia, and adhesion predict a long ESD time, but high a proficiency level predicts a short ESD time. The results provide reference for the prediction of operation difficulty and ESD time.

Terminology

The ESD technique was first introduced in Japan. ESD is an innovative technique that improves the rate of successful *en bloc* resection of gastrointestinal neoplasms.

Peer review

This is a study done in one center by a single endoscopist, which in this case is a strength. The manuscript is well presented and easy to read.

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Patterns and prognosis of locally recurrent rectal cancer following multidisciplinary treatment

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Abstract

AIM: To investigate the patterns and decisive prognostic factors for local recurrence of rectal cancer treated with a multidisciplinary team (MDT) modality.

METHODS: Ninety patients with local recurrence were studied, out of 1079 consecutive rectal cancer patients who underwent curative surgery from 1999 to 2007. For each patient, the recurrence pattern was assessed by specialist radiologists from the MDT using imaging, and the treatment strategy was decided after discussion by the MDT. The associations between clinicopathological factors and long-term outcomes were evaluated using both univariate and multivariate analysis.

RESULTS: The recurrence pattern was classified as follows: Twenty-seven (30%) recurrent tumors were evaluated as axial type, 21 (23.3%) were anterior type,

8 (8.9%) were posterior type, and 13 (25.6%) were lateral type. Forty-one patients had tumors that were evaluated as resectable by the MDT and ultimately received surgery, and R0 resection was achieved in 36 (87.8%) of these patients. The recurrence pattern was closely associated with resectability and R0 resection rate ($P < 0.001$). The recurrence pattern, interval to recurrence, and R0 resection were significantly associated with 5-year survival rate in univariate analysis. Multivariate analysis showed that the R0 resection was the unique independent factor affecting long-term survival.

CONCLUSION: The MDT modality improves patient selection for surgery by enabling accurate classification of the recurrence pattern; R0 resection is the most significant factor affecting long-term survival.

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Key words: Rectal cancer; Local recurrence; Prognosis; Survival; Surgery

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INTRODUCTION

Local recurrence of rectal cancer (LRR) has dramatically fallen to 4%-10% after widespread application of total mesorectal excision (TME)^[1-5]. Nevertheless, LRR re-

mains a significant clinical problem and is associated with severe morbidity, low quality of life, and poor survival in the majority of patients^[6]. Although radical (R0) resection is the most effective way to improve prognosis, it is only achieved in 10%-50% of LRRC, and in general, the 5-year survival rate remains unfavorable, varying from 18% to 58% according to different reports^[7-10]. One decisive factor for R0 resection is the pattern of recurrence^[3,4,11,12], since an accurate assessment of the recurrence pattern before treatment is critical for the selection of indicated patients as candidates for surgery.

Currently, the treatment of rectal cancer has evolved toward a multidisciplinary team (MDT) modality^[13], and this modality has been successfully delivered in our center for ten years^[14]. The MDT approach can optimize treatment strategy by enabling accurate and integrative evaluation *via* discussion by the MDT before treatment^[15]. However, whether the MDT approach can improve the R0 resection rate and long-term survival in patients with LRRC is still unknown. The aim of this study was to investigate the long-term outcome and its influential factors in LRRC under the MDT modality.

MATERIALS AND METHODS

Patients

A total of 1079 consecutive patients who underwent curative surgery at the Beijing Cancer Hospital between January 1999 and December 2007 were reviewed. In this study, LRRC was defined as the occurrence of recurrent tumors after prior radical resection located within the pelvis, either alone or in conjunction with metastases^[16]. Ninety eligible patients were ultimately included in the study according to the following criteria: (1) the initial surgery was R0 resection, and transabdominal surgery must be delivered strictly according to TME principles^[17], without bowel resection margin or circumferential resection margin involvement; (2) no synchronous distant metastasis outside the pelvis; (3) no history of other malignant tumors; and (4) no severe surgical or medical complications that were likely to affect the long-term outcome after initial surgery.

Diagnosis and evaluation of local recurrence by the MDT

Confirmation of LRRC by biopsy was obtained in 68 patients, and 10 patients were diagnosed with positron emission tomography. The remaining 12 patients were clinically diagnosed by assessing clinical symptoms, serum carcinoembryonic antigen (CEA) levels, and imaging, including computed tomography (CT), magnetic resonance imaging (MRI), and sonography.

Data for each patient was evaluated and discussed by a special MDT. Recurrence patterns were evaluated by senior radiologists of the MDT based on CT or MRI, using the Memorial Sloan-Kettering classification^[6,12],

namely, axial: recurrence at anastomotic, residual mesorectum, or perirectal soft tissue within the center of the pelvis or perineum following an abdominoperineal resection; anterior: involving the genitourinary tract; posterior: involving the sacrum and presacral fascia; and lateral: involving the muscles or soft tissue of the pelvic sidewall, major iliac vessels, sacral nerve plexus, and lateral bony pelvis.

Multimodality treatment surgery

Surgery for indicated patients was performed after discussion by the MDT, which included a group of experienced colorectal surgeons. Surgery for recurrent tumors was defined as curative (R0) if the area where resection was performed was grossly and microscopically free of residual cancer. Resections were considered palliative if either gross (R2) or microscopic (R1) cancer remained at the end of the procedure. For each patient, a thorough abdominal exploration was carried out to rule out extrapelvic metastasis after division of adhesions. Recurrent tumors were resected, along with any adjacent structures involved. Frozen-section analysis was not routinely performed, except for the suspicious resection margin.

Chemoradiotherapy and palliative treatment

Decisions regarding whether and when patients needed to undergo chemoradiotherapy were arrived at *via* discussion by the MDT. In principle, preoperative external beam radiotherapy was applied in patients 70 years of age and under with the possibility of curative resection. A radiation dose of 50 Gy in 25 daily fractions of 2 Gy was planned if no neoadjuvant radiotherapy for the primary tumor had been delivered. Three fields (1 posterior and 2 lateral) were used. Chemotherapy with fluorouracil (350 mg/m² per day) and leucovorin (20 mg/m² per day) was administered concurrently in two 5 d courses. Surgery was performed 6-8 wk after the end of the preoperative treatment. None of the patients in this study underwent intraoperative or postoperative radiotherapy. Palliative chemotherapy for unresectable tumors was delivered by oncologists, and the regimens were based on fluorouracil, oxaliplatin, and irinotecan. If the patients could not receive radio- or chemotherapy due to poor status, supportive care, including nutrition support, analgesic therapy, and other measures were given to improve quality of life.

Follow-up

All included patients underwent regular follow-ups. Physical examination and laboratory testing (blood count, liver enzymes, and CEA) were performed at the time of each follow-up visit, every 3 mo for 2 years, and every 6 mo thereafter. Abdominal ultrasonography, a chest X-ray, and abdominal and pelvic CT were performed every 6 mo. Colonoscopy was performed every 12 mo. The follow-up was repeated for at least 5 years.

Table 1 Patient characteristics *n* (%)

Variables	Patient
Gender	
Male	54 (60)
Female	36 (40)
Age	
≤ 60 yr	50 (55.6)
> 60 yr	40 (44.4)
Primary surgery	
Anterior resection	54 (60)
Abdominoperineal resection	32 (35.6)
Hartmann	2 (2.2)
Local excision	2 (2.2)
Neoadjuvant chemoradiotherapy for primary tumor	
Yes	18 (20)
No	72 (80)
Primary tumor stage	
I	6 (6.6)
II A	8 (8.8)
II B	11 (12.2)
III A	10 (11.1)
III B	20 (22.2)
III C	26 (28.8)
Unknown	9 (10)
Serum CEA ¹	
≤ 5 ng/mL	27 (30)
> 5 ng/mL	48 (53.3)
Unknown	15 (16.7)
Preoperative chemoradiotherapy for recurrent tumor ²	
Yes	10 (24.4)
No	31 (75.6)
Surgery for recurrent tumor	41 (45.6)
Anterior resection	2 (4.9)
Abdominoperineal resection	30 (73.2)
Combined organ excision ³	5 (12.2)
Total pelvic exenteration	4 (9.8)
Conservative therapy	49 (54.4)
Resection status	
R0	36 (40)
R1-2	5 (5.5)

¹The serum CEA level after recurrence; ²For the patients who underwent surgery; ³Including excision of uterus, vagina, seminal vesicle, prostate, and sacrum. CEA: Carcinoembryonic antigen.

Statistical analysis

Statistical analyses were performed using the SPSS 16.0 Statistical Software (SPSS Inc., Chicago, IL). Categorical variables were compared using Pearson's χ^2 test. Survival rates were estimated using the Kaplan-Meier curve, and comparisons of survival between groups were made using the log-rank method. Multivariate analysis was performed using the Cox proportional-hazard method. All statistical tests were 2-tailed, and statistical significance was set at $P < 0.05$.

RESULTS

Baseline characteristics of patients

Of the 90 included patients, 54 were male and 36 were female. The mean age was 59.4 years (median 57.5; range 27-76). Median follow-up was 68.5 mo (range 6-96). Ten patients (11.1%) were lost to follow-up and their data

Table 2 The association between recurrence pattern and resectability

Recurrence pattern	Resection rate (%)	<i>P</i> value	R0 rate (%)	<i>P</i> value
Axial	88.9 (24/27)	< 0.001	85.2 (23/27)	< 0.001
Anterior	33.3 (7/21)		33.3 (7/21)	
Posterior	25 (2/8)		25 (2/8)	
Lateral	21.7 (5/23)		4.3 (1/23)	

The values shown are the resection rate expressed in %, with the number of patients with successful resection/total number of patients with locally recurrent rectal cancer in parentheses.

were included in the survival analysis until the date of loss. The interval to recurrence was a median of 18.5 mo (3-102). Forty-one patients had recurrent tumors that were deemed resectable or potentially resectable; of these patients, 10 received preoperative chemoradiotherapy and 8 had R0 resection. Of the 41 patients who underwent surgery, 36 (87.8%) had R0 resection. In total, 9 patients underwent extended resection: 4 patients underwent total pelvic exenteration, 3 were R0 resection and 1 was R1 resection; 5 patients underwent combined organ excision, 3 were R0 resection, 1 was R1 resection, and 1 was R2 resection. Within the combined organ excision, 2 combined with uterus and vagina resection, 1 combined with partial prostate resection, and 2 combined with sacrum resection. The remaining 49 patients with unresectable tumors received conservative therapy: 18 received chemoradiotherapy and consequent chemotherapy, 25 received palliative chemotherapy alone, and 6 underwent supportive care. The demographic and clinicopathological characteristics are summarized in Table 1.

Recurrence pattern and its relationship to resectability

The pattern of local recurrence was classified by the MDT as axial type in 27 (30%) patients, anterior type in 21 (23.3%), posterior type in 8 (8.9%), lateral type in 23 (25.6%), and unclassifiable in 11.

The recurrence pattern had a strong association with resectability of the recurrent tumor, with the highest resection rate (88.9%) in the axial type and the lowest resection rate (21.7%) in the lateral type ($P < 0.001$) (Table 2). A significant difference in R0 resection percentage was also observed among the different patterns of recurrence: the R0 resection rates in axial, anterior, posterior, and lateral type were 85.2%, 33.3%, 25%, and 4.3%, respectively ($P < 0.001$; Table 2).

Prognostic factors for long-term survival

The 5-year overall survival rate of all patients was 31.1%. Univariate analysis of patient survival with locally recurrent tumors was performed according to clinicopathological and surgical factors. Twenty-seven patients with a long recurrence interval (> 24 mo) had a significantly higher rate of survival than the 63 patients with a short recurrence interval (≤ 24 mo) (48.1% *vs* 23.8%, $P < 0.05$; Table 3). A significant difference in survival was seen in

Table 3 Univariate analysis of risk factors affecting long-term survival

Variables	n	5 yr OS rate (%)	P value
Gender			
Male	54	29.6	0.803
Female	36	33.3	
Age			
≤ 60 yr	50	26	0.187
> 60 yr	40	37.5	
Surgery of primary tumor			
Anterior resection	54	31.5	0.451
Abdominoperineal resection	32	31.2	
Pathologic stage of primary tumor			
I - II	25	33.3	0.473
III	56	30.8	
Serum CEA ¹			
≤ 5 ng/mL	26	38.5	0.35
> 5 ng/mL	49	28.6	
Interval to recurrence (mo)			
≤ 24	63	23.8	0.011
> 24	27	48.1	
Recurrence pattern			
Axial	27	63	< 0.001
Anterior	21	28.6	
Posterior	8	12.5	
Lateral	23	4.3	
Surgery for recurrent tumor			
R0 resection	36	55.6	< 0.001
R1-2 and non-surgery	54	14.8	

¹The serum carcinoembryonic antigen (CEA) level after recurrence. OS: Overall survival.

Table 4 Multivariate Cox regression (backward method) of prognostic factors in relation to 5 year overall survival

Variables	Hazard ratio (95%CI)	P value
R0 resection	2.734 (1.212-6.168)	0.015
Recurrence pattern	1.078 (0.716-1.625)	0.718
Interval to recurrence	0.978 (0.953-1.004)	0.102
Gender	1.776 (0.870-3.625)	0.114
Age	0.657 (0.326-1.322)	0.239
Serum CEA	1.088 (0.447-2.647)	0.853
Primary tumor stage	1.423 (0.575-3.517)	0.445

CEA: Carcinoembryonic antigen.

patients with different recurrence patterns: the 5-year survival rate was 63% in patients with the axial type, 28.6% in the anterior type, 12.5% in the posterior type, and 4.3% in the lateral type ($P < 0.001$). Univariate analysis also demonstrated that patients who underwent R0 resection had a significantly improved survival rate than those with palliative resection or conservative therapy (55.6% *vs* 14.8%, $P < 0.001$; Figure 1). Gender, age, primary surgery, pathological stage of primary tumor, and serum CEA were not associated with long-term survival (Table 3).

Multivariate analysis revealed that only the R0 resection independently influenced long-term survival of locally recurrent rectal cancer ($P < 0.05$). Although other factors, including the recurrence pattern, may associate

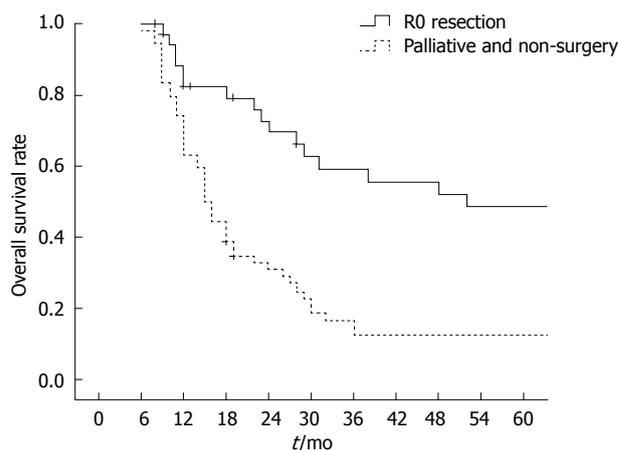


Figure 1 Survival after recurrence between the curative resection group and other groups. Patients who underwent curative resection ($n = 36$) had a significantly higher 5 year survival rate than those who underwent palliative resection or conservative treatment ($n = 54$) in both univariate and multivariate analysis (55.6% *vs* 14.8%, $P < 0.001$).

with overall survival, they were not as significant as R0 resection in prognosticating long-term survival (Table 4).

DISCUSSION

The aggressive use of multimodality therapy, including chemotherapy and perioperative or intraoperative radiotherapy, is being increasingly delivered in order to improve the outcomes of patients with LRRC^[8,18,19]. However, the prognosis of LRRC is far from satisfactory: the 5-year survival rate fluctuates between 18% and 58%, and only a minority of patients received curative surgery^[7-10]. To date, surgical resection with curative intent is still the most relevant prognostic factor for LRRC, and this was demonstrated in almost all reports published thus far^[7-10]. Unfortunately, the traditional pre-operative evaluation by surgeons does not allow for an accurate selection of patients as candidates for radical surgery, even though this selection is particularly important for prognosis. Nearly 30% to 65% of patients who underwent surgery did not achieve microscopically negative margins according to the majority of reports, which means that almost half of the patients would not benefit from surgery, whereas they still had to risk surgical morbidity^[8,9,20,21]. Therefore, the evaluation and treatment for LRRC needs more accurate and individualized strategies, and to this end, we propose the MDT approach.

MDT is a promising approach for identifying candidates who are most likely to benefit from surgical resection, since this modality concentrates the superiority of a series of colorectal cancer-associated disciplines, including imaging, radiology, surgery, and oncology^[13,22]. Our data revealed that an R0 resection rate of 87.8% could be achieved in patients who underwent surgery following MDT evaluation, which was significantly higher than that in previous reports^[8,9,20,21], and suggested that patient selection for surgery might be dramatically im-

proved *via* discussion by the MDT. Additionally, 8 out of 10 patients who were evaluated as marginally resectable gained R0 resection after preoperative chemoradiotherapy, based on the downsizing of the recurrent tumor; which suggested that the MDT approach may improve resectability by individual and multimodality treatment for LRRC. However, our findings need further studies to verify this, and our conclusion needs to be definitely proven by well-designed prospective cohort studies.

The basis of improved patient selection for surgery is the accurate evaluation of the recurrence pattern, which was also the main concern of the discussion by the MDT. Several studies have demonstrated that the recurrence pattern is a decisive factor for resectability^[3,4,11,12]. Moore *et al.*^[12] reported the axial and anterior type of recurrences had a R0 resection rate greater than 70%, whereas the lateral type had a R0 resection rate lower than 20%. Yamada *et al.*^[11] also reported that the involvement of the pelvic sidewall in LRRC is a dominant adverse factor for surgery and prognosis, with a 5-year survival rate lower than 5%. Our data revealed that the recurrence pattern was an optimal index to accurately select indicated patients for surgery, since the difference was dramatically significant in R0 resection and survival between each pattern, with a favorable outcome in the axial type and a poor prognosis in the lateral type. Therefore, based on the results of this study, we recommend including the recurrence pattern as an essential criterion in clinical decision making.

In terms of the independent prognostic factors of long-term survival, the conclusions from this study are in accordance with those of most current reports, in that R0 resection is the most critical factor compared to other clinicopathological variables^[3,4,10,11,20,21]. Although some studies have mentioned that elevated serum CEA, primary tumor stage, recurrence pattern, and other factors, may also have predictive value for long-term survival of LRRC^[5,9,10,23]; these factors were demonstrated to be not as strong as radical surgery in their influence on the 5-year survival rate^[10]. Therefore, the most pragmatic and effective way to improve long-term survival is to enhance radical resection for indicated patients *via* an MDT discussion.

In summary, this study reveals that the MDT approach optimizes the treatment strategy of LRRC; and among all the clinicopathological factors, R0 resection is the most significant factor affecting long-term survival.

COMMENTS

Background

Local recurrence of rectal cancer (LRRC) remains a significant clinical problem. Although radical resection is the most effective way to improve prognosis, it is only achieved in limited patients. Currently, the treatment of rectal cancer has evolved toward a multidisciplinary team (MDT) modality, and this modality is expected to improve the R0 resection rate and long-term survival of patients with LRRC. The aim of this study was to investigate the long-term outcome and its influential factors in LRRC under the MDT modality.

Research frontiers

This study addresses the role and effectiveness of MDT in the treatment of LRRC, which was rarely reported in other studies. The results of this study suggest that among all the clinicopathological factors, R0 resection is the most significant factor affecting long-term survival, and MDT modality could improve R0 resection by optimizing the treatment strategy.

Innovations and breakthroughs

This study demonstrated that the MDT approach could optimize the treatment strategy of LRRC; and R0 resection is still the most significant factor affecting long-term survival.

Applications

The MDT modality should be widely applied in the treatment of LRRC, since it could optimize treatment strategy and improve R0 resection.

Terminology

Local recurrence refers to the occurrence of recurrent tumors after prior radical resection located within the pelvis, either alone or in conjunction with metastases. A multidisciplinary team is a unit or working group composed of specialists from multiple disciplines which are associated with a certain disease.

Peer review

This is an excellent study in which the authors analyzed the associations between recurrence pattern and resectability, as well as the influential factors of long-term survival. All the pretreatment evaluation and therapy were made by a special MDT, which is a distinctive characteristic of this study. The results are interesting, and suggest that the recurrence pattern is a decisive factor of resectability, and that R0 resection is still the most significant factor affecting long-term survival. This study has important clinical significance for the identification of the indicated patients for surgery and those who had a better prognosis.

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Prealbumin is predictive for postoperative liver insufficiency in patients undergoing liver resection

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Abstract

AIM: To investigate the risk factors for postoperative liver insufficiency in patients with Child-Pugh class A liver function undergoing liver resection.

METHODS: A total of 427 consecutive patients undergoing partial hepatectomy from October 2007 to April 2011 at a single center (Department of Hepatic Surgery I, Eastern Hepatobiliary Surgery Hospital, Shanghai, China) were included in the study. All the patients had preoperative liver function of Child-Pugh class A and were diagnosed as having primary liver cancer by postoperative histopathology. Surgery was performed by the same team and hepatic resection was carried out by a clamp crushing method. A clamp/unclamp time of 15 min/5 min was adopted for hepatic inflow occlusion. Patients' records of demographic variables, intraoperative parameters, pathological findings and laboratory test results were reviewed. Postoperative

liver insufficiency and failure were defined as prolonged hyperbilirubinemia unrelated to biliary obstruction or leak, clinically apparent ascites, prolonged coagulopathy requiring frozen fresh plasma, and/or hepatic encephalopathy. The incidence of postoperative liver insufficiency or liver failure was observed and the attributing risk factors were analyzed. A multivariate analysis was conducted to determine the independent predictive factors.

RESULTS: Among the 427 patients, there were 362 males and 65 females, with a mean age of 51.1 ± 10.4 years. Most patients (86.4%) had a background of viral hepatitis and 234 (54.8%) patients had liver cirrhosis. Indications for partial hepatectomy included hepatocellular carcinoma (391 patients), intrahepatic cholangiocarcinoma (31 patients) and a combination of both (5 patients). Hepatic resections of ≤ 3 and ≥ 4 liver segments were performed in 358 (83.8%) and 69 (16.2%) patients, respectively. Seventeen (4.0%) patients developed liver insufficiency after hepatectomy, of whom 10 patients manifested as prolonged hyperbilirubinemia unrelated to biliary obstruction or leak, 6 patients had clinically apparent ascites and prolonged coagulopathy, 1 patient had hepatic encephalopathy and died on day 21 after surgery. On univariate analysis, age ≥ 60 years and prealbumin < 170 mg/dL were found to be significantly correlated with postoperative liver insufficiency ($P = 0.045$ and $P = 0.009$, respectively). There was no statistical difference in postoperative liver insufficiency between patients with or without hepatitis, liver cirrhosis and esophago-gastric varices. Intraoperative parameters (type of resection, inflow blood occlusion time, blood loss and blood transfusion) and laboratory test results were not associated with postoperative liver insufficiency either. Age ≥ 60 years and prealbumin < 170 mg/dL were selected on multivariate analysis, and only prealbumin < 170 mg/dL remained predictive (hazard ratio, 3.192; 95%CI: 1.185-8.601, $P = 0.022$).

CONCLUSION: Prealbumin serum level is a predictive factor for postoperative liver insufficiency in patients with liver function of Child-Pugh class A undergoing hepatectomy. Since prealbumin is a good marker of nutritional status, the improved nutritional status may decrease the incidence of liver insufficiency.

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Key words: Prealbumin; Hepatectomy; Liver insufficiency; Child-Pugh class A; Primary liver cancer

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INTRODUCTION

In spite of techniques such as local ablation, liver resection is still the accepted gold standard treatment for liver tumors. The aim of liver resection is to remove all macroscopic diseases (with negative resection margins), retain sufficient functioning liver^[1] and preserve vascular inflow and outflow. If too much healthy liver parenchyma is removed, patients may develop postoperative liver insufficiency or liver failure, which is a complication dreaded by surgeons.

The search for a method to categorically quantify the functional reserve of the liver and tailor surgical intervention has resulted in the development of a range of methods. These methods range from clinical scores such as the Child-Pugh classification, tests assessing complex hepatic metabolic pathways and radiological methods assessing functional reserve. Child-Pugh classification, a convenient and practical scoring system, has proven to be a useful tool in estimating the risks for both hepatic and nonhepatic surgery in patients with liver diseases^[2-4]. Liver resection is generally safe for patients with preoperative liver function of Child-Pugh class A, even for patients with cirrhosis. However, a minority of patients undergoing apparently safe resection still inexplicably develop postoperative liver insufficiency or liver failure despite seemingly sufficient liver remained preoperatively^[5].

The objective of this study is to determine the incidence of postoperative liver insufficiency in patients with preoperative liver function of Child-Pugh class A who underwent liver resection, and to clarify the risk factors for postoperative liver insufficiency in those patients.

MATERIALS AND METHODS

We reviewed the data of a single center database (De-

partment of Hepatic Surgery I, Eastern Hepatobiliary Surgery Hospital, Shanghai, China). This database comprises 427 patients undergoing partial hepatectomy with liver function of Child-Pugh class A observed during the period from October 2007 to April 2011. All the patients were diagnosed as having primary liver cancer by postoperative histopathology.

Before surgery, all patients had a chest X-ray, ultrasonography, esophagogastric endoscopy, and contrast computed tomography scan or magnetic resonance imaging of the abdomen. Laboratory blood tests included count of white blood cell and platelet, hepatitis B surface antigen, antibodies to hepatitis C, serum alpha-fetoprotein, carcinoembryonic antigen, carbohydrate antigen 19 to 9, serum albumin, serum prealbumin, serum total bilirubin, alanine aminotransferase (ALT) and prothrombin time (PT).

Surgery was performed through a right subcostal incision with a midline extension. As for hepatic inflow occlusion, normothermic intermittent interruption of the porta hepatis, with a clamp/unclamp time of 15 min/5 min, was adopted. Hepatic resection was carried out by a clamp crushing method, which has been reported previously^[6]. Blood loss was accurately recorded and blood transfusion was given when necessary. Serum albumin, serum prealbumin, serum total bilirubin, ALT, PT and ascites were monitored after surgery. Patients were discharged when the liver function was recovered (total bilirubin ≤ 34 $\mu\text{mol/L}$, ALT ≤ 40 IU/L, PT ≤ 15 s, no ascites on abdominal ultrasound and no appearance of hepatic encephalopathy).

We reviewed patients' records for demographic variables (age, gender, hepatitis background, liver cirrhosis and esophagogastric endoscopic findings), intraoperative parameters (type of resection, inflow blood occlusion time, blood loss and blood transfusion), pathological diagnosis and laboratory test results. Postoperative liver insufficiency and failure were defined as "prolonged hyperbilirubinemia unrelated to biliary obstruction or leak, clinically apparent ascites, prolonged coagulopathy requiring frozen fresh plasma, and/or hepatic encephalopathy"^[7], which was recommended by Mullin *et al.*^[5].

Statistical analysis

Data were collected and analyzed with the SPSS statistical software (SPSS version 16.0, Chicago, IL, United States). The variable data were expressed as means and SDs or median and range. Categorical variables were compared using χ^2 or Fisher's exact test when appropriate, and continuous variables were compared using the independent sample *t* test. A multiple logistic regression analysis was used to determine predictors of postoperative liver insufficiency.

Variables with a $P < 0.05$ in the univariable analysis were added to the multi-variable model. In the multivariate analysis, a stepwise method was used to select variables for the final model: the conditional probabilities for stepwise entry and stepwise removal of a factor were 0.05 and 0.20, respectively.

Table 1 Baseline characteristics of all 427 patients

Variables	<i>n</i> = 427
Sex (male/female)	362/65
Age (yr)	51.1 ± 10.4
Surgical indications	
HCC	391
ICC	31
HCC-ICC ¹	5
Tumor size (cm)	6.2 ± 4.0
WBC (× 10 ⁹ /L)	5.5 ± 1.8
PLT (× 10 ⁹ /L)	160.5 ± 66.4
PT (s)	12.2 ± 4.8
Total bilirubin (μmol/L)	14.6 ± 5.8
Albumin (g/L)	42.0 ± 4.0
Prealbumin (mg/dL)	217.6 ± 61.0
ALT (IU/L)	47.4 ± 43.7
Hepatitis virus background	
HBV	362
HCV	4
HBV-HCV ²	3
None	58
Liver cirrhosis	
Yes	234
No	193
Ascites	
Little	48
No	378
Esophageal varices	
Present	60
Absent	367
Types of liver resection	
Minor ≤ 3 liver segments	358
Major ≥ 4 liver segments	69
Inflow blood occlusion time (min)	15.8 ± 8.0
Blood loss (mL)	200 (50, 5500)
Blood transfusion (yes/no)	43/384

¹Concurrence of HCC and ICC; ²Concurrence of HBV and HCV. HCC: Hepatocellular carcinoma; ICC: Intrahepatic cholangiocarcinoma; WBC: White blood cell; PLT: Platelet; PT: Prothrombin time; ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

RESULTS

Baseline data

The clinical features of the 427 patients included in the study are reported in Table 1. There were 362 males and 65 females, with a mean age of 51.1 ± 10.4 years. Most patients (86.4%) had a background of viral hepatitis (hepatitis B and/or hepatitis C), 234 (54.8%) patients suffered from liver cirrhosis, and 48 (11.2%) patients had controlled ascites before surgery. Indications for partial hepatectomy included hepatocellular carcinoma (391 patients), intrahepatic cholangiocarcinoma (31 patients) and combination of hepatocellular carcinoma and intrahepatic cholangiocarcinoma (5 patients). Hepatic resection of ≤ 3 and ≥ 4 liver segments was performed in 358 (83.8%) and 69 (16.2%) patients, respectively.

Liver insufficiency and its risk factors

There were 17 (4.0%) patients who had liver insufficiency after hepatectomy, of whom 10 patients presented with prolonged hyperbilirubinemia unrelated to biliary

Table 2 Univariate analysis of factors related to postoperative liver insufficiency

Variables	Patients with liver insufficiency (<i>n</i> = 17)	Patients without liver insufficiency (<i>n</i> = 410)	<i>P</i> value
Sex (male/female)	17/0	345/65	0.075
Age (yr)	57.6 ± 7.1	50.8 ± 10.4	0.008
< 60	10	325	
≥ 60	7	85	
Tumor size (cm)	6.6 ± 5.0	6.2 ± 3.9	0.664
≥ 10	4	79	
< 10	13	331	
WBC (× 10 ⁹ /L)	5.2 ± 1.9	5.5 ± 1.8	0.581
PLT(× 10 ⁹ /L)	146.0 ± 88.2	161.1 ± 65.4	0.357
PT (s)	12.5 ± 1.3	12.2 ± 4.9	0.816
Total bilirubin (μmol/L)	16.3 ± 5.7	14.5 ± 5.8	0.205
Albumin (g/L)	40.2 ± 4.3	42.0 ± 4.3	0.074
Prealbumin (mg/dL)	191.4 ± 59.6	217.9 ± 61.1	0.049
< 170	8	84	
≥ 170	9	326	
ALT (IU/L)	52.2 ± 38.0	47.2 ± 43.9	0.647
≥ 100	3	29	
< 100	14	381	
Viral background			0.222
HBV and/or HCV	4	54	
None	13	356	
Liver cirrhosis			0.182
No	5	188	
Yes	12	222	
Ascites			0.415
Little	3	46	
No	14	364	
Esophageal varices			0.251
Present	4	56	
Absent	13	354	
Types of liver resection			0.130
Minor ≤ 3 liver segments	12	346	
Major ≥ 4 liver segments	5	64	
Inflow blood occlusion time (min)	17.2 ± 6.2	15.7 ± 8.0	0.438
> 20	4	102	
≤ 20	13	308	
Blood loss (mL)	200 (50-5500)	200 (50-2800)	0.362
≥ 800	1	36	
< 800	16	374	
Blood transfusion			0.558
Yes	1	42	
No	16	368	

WBC: White blood cell; PLT: Platelet; PT: Prothrombin time; ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

obstruction or leak, 6 patients had clinically apparent ascites and prolonged coagulopathy requiring frozen fresh plasma, and 1 patient had hepatic encephalopathy and died on day 21 after surgery.

To determine the risk factors for postoperative liver insufficiency, 427 patients were classified into two groups: patients with liver insufficiency (17 patients) and patients without liver insufficiency (410 patients). Eighteen variables as listed in Table 2 were analyzed. On univariate analysis, age ≥ 60 years and prealbumin serum level < 170 mg/dL were found to be significantly correlated with the postoperative liver insufficiency (*P* = 0.045 and *P* = 0.009, respectively). On multivariate analysis using

a logistic regression, only prealbumin serum level < 170 mg/dL remained predictive (hazard ratio, 3.192; 95%CI: 1.185-8.601, $P = 0.022$).

Other complications

Twenty-five patients had one or more complications, one (0.2%) them died in the hospital because of multiple organ failure including liver failure, the other patients recovered after a longer hospital stay. These complications included pleural effusion (15 patients), abdominal hemorrhage (3 patients), wound infection (2 patients), acute renal failure (2 patients) and bile leakage (1 patient).

DISCUSSION

Child score, first proposed in 1964^[8] and modified as Child-Pugh score thereafter, was originally developed to predict the risk of mortality in patients undergoing shunting procedures for portal hypertension. Its role has been expanded to predict risk for a range of procedures, including hepatectomy, and liver resection is generally safe in patients with class A liver function^[9]. However, in the present study, the minority of patients (4.0%) with good liver function (Child-Pugh class A) still had postoperative liver insufficiency, one of them even developed undesirable liver failure.

Because of several apparent limitations in the assessment of risk of liver failure following hepatectomy, to improve the Child-Pugh classification system, many quantitative techniques have been developed to assess postoperative risk of liver failure in patients undergoing hepatectomy. Indocyanine green (ICG) elimination is the most widely used assessment for liver function and a number of retrospective studies have found some efficacy in predicting liver dysfunction and mortality following hepatectomy^[10-13]. Unfortunately, ICG elimination is not a routine test in our hospital and we could not present such data in this study. However, with the finite data in our study, we found that patients with postoperative liver insufficiency were correlated with elder age and lower serum level of prealbumin, and prealbumin serum level < 170 mg/dL remained predictive for liver insufficiency after hepatectomy.

Prealbumin, also known as transthyretin, is synthesized in the liver and serves as a transport protein throughout the body. Serum prealbumin differs from albumin, which has a relatively short half-life of 48 h and does not accumulate in the body to undergo redistribution^[14-17]. Therefore, it might be a better indicator to assess nutritional status than the widely used albumin serum level, and any fluctuations in nutritional status can be detected rapidly^[18,19]. Prealbumin has been considered an effective indicator of malnutrition in cancer patients^[15,16]. Nutrition is an important part of the management of surgical patients, and poor nutritional status in patients undergoing surgery is well known to increase postoperative morbidity and mortality by deteriorating various organ functions and the immune system of the host^[14,20-23]. Thus, we could infer that it was the poor nutritional status that caused the postoperative liver insufficiency, and prealbumin serum

level < 170 mg/dL was an indicator of malnutrition.

With regard to the Child-Pugh score, we noticed that the initial version of Child or Child-Turcotte score^[8], included nutritional status which was classified as good, fair and poor. One of the limitations which is argued by studies reported thereafter was that some values (ascites, encephalopathy and nutritional status) were determined subjectively by clinicians^[24,25]. There was no quantitative index for clinicians to give the substantial score. Due to its good representation of nutritional status, we think that prealbumin could be a quantitative variable to evaluate the patient's nutritional status, and possibly as a modification to the Child-Pugh classification system, which will make the score system more objective to assess the liver function.

Almost all the serum prealbumin is synthesized in the liver, thus, its level can be influenced by the liver condition. It has been reported that the prealbumin level could be lowered in patients with acute or chronic liver diseases and alcoholism^[16]. We think that the decreased prealbumin level may reflect the damage of liver function, which also indicates the risk of liver insufficiency after hepatectomy. More importantly, oral and parenteral steroids can falsely elevate the prealbumin levels. This elevation can make patients on steroids appear to be at a lower risk for surgery than they really are^[26]. For those patients, more attention should be paid and other variables can be taken into account to justify the liver function.

In summary, the present study exhibited that the minority of patients (4.0%) with good liver function (Child-Pugh class A) still had postoperative liver insufficiency, one of them even developed undesirable liver failure. Patients' age ≥ 60 years and serum level of prealbumin serum level < 170 mg/dL were found to be significantly correlated with the postoperative liver insufficiency, and prealbumin serum level < 170 mg/dL remained predictive for liver insufficiency after liver resection. Considering prealbumin has served as a good marker of nutritional status in cancer patients, the improved nutritional status may decrease the incidence of liver insufficiency. However, further studies with a larger number of patients are needed to confirm this hypothesis.

COMMENTS

Background

In spite of techniques such as local ablation, liver resection is still the accepted gold standard treatment for liver tumors. The aim of liver resection is to remove all macroscopic diseases (with negative resection margins) and retain sufficient functioning liver with preservation of vascular inflow and outflow. If too much healthy liver parenchyma is removed, patients may develop postoperative liver insufficiency or liver failure, which is a complication dreaded by surgeons. Liver resection is generally safe in patients with preoperative liver function of Child-Pugh class A, even in patients with cirrhosis. However, a small number of patients undergoing apparently safe resection still inexplicably develop postoperative liver insufficiency or liver failure although seemingly sufficient liver remained preoperatively.

Research frontiers

The search for a method to categorically quantify the functional reserve of the liver and tailor surgical intervention has resulted in the development of a range of methods. These methods include clinical scores such as the Child-Pugh clas-

sification, tests assessing complex hepatic metabolic pathways and radiological methods assessing functional reserve. Child-Pugh classification, a convenient and practical scoring system, has proven to be a useful tool in estimating the risks for both hepatic and nonhepatic surgery for patients with liver diseases.

Innovations and breakthroughs

In this study, the authors exhibited that a small number of patients (4.0%) with good liver function (Child-Pugh class A) still had postoperative liver insufficiency, one of them even developed undesirable liver failure. Patients' age ≥ 60 years and serum level of prealbumin serum level < 170 mg/dL were found to be significantly correlated with the postoperative liver insufficiency, and prealbumin serum level < 170 mg/dL remained predictive for liver insufficiency after liver resection. Prealbumin has been considered an effective indicator of malnutrition in cancer patients. Nutrition is an important part of the management of surgical patients, and poor nutritional status in patients undergoing surgery is well known to increase postoperative morbidity and mortality by deteriorating various organ functions and the immune system of the host. Thus, the authors inferred that it was the poor nutritional status that caused the postoperative liver insufficiency, and prealbumin serum level < 170 mg/dL was an indicator of malnutrition.

Applications

One of the limitations which is argued by previous studies was that some values (ascites, encephalopathy and nutritional status) were determined subjectively by clinicians. There was no quantitative index for clinicians to give the substantial score. Due to its good representation of nutritional status, the authors think that prealbumin could be a quantitative variable to evaluate the patient's nutritional status, and possibly as a modification to the Child-Pugh classification system, which will make the score system more objective to assess the liver function.

Peer review

This manuscript demonstrates that preoperative serum level of prealbumin is a predictor of postoperative liver insufficiency in patients with Child-Pugh class A. This manuscript is well written and attractive.

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Contrast-enhanced ultrasonography assessment of gastric cancer response to neoadjuvant chemotherapy

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Abstract

AIM: To quantitatively assess the ability of double contrast-enhanced ultrasound (DCUS) to detect tumor early response to pre-operative chemotherapy.

METHODS: Forty-three patients with gastric cancer treated with neoadjuvant chemotherapy followed by curative resection between September 2011 and February

2012 were analyzed. Pre-operative chemotherapy regimens of fluorouracil + oxaliplatin or S-1 + oxaliplatin were administered in 2-4 cycles over 6-12 wk periods. All patients underwent contrast-enhanced computed tomography (CT) scan and DCUS before and after two courses of pre-operative chemotherapy. The therapeutic response was assessed by CT using the response evaluation criteria in solid tumors (RECIST 1.1) criteria. Tumor area was assessed by DCUS as enhanced appearance of gastric carcinoma due to tumor vascularity during the contrast phase as compared to the normal gastric wall. Histopathologic analysis was carried out according to the Mandard tumor regression grade criteria and used as the reference standard. Receiver operating characteristic (ROC) analysis was used to evaluate the efficacy of DCUS parameters in differentiating histopathological responders from non-responders.

RESULTS: The study population consisted of 32 men and 11 women, with mean age of 59.7 ± 11.4 years. Neither age, sex, histologic type, tumor site, T stage, nor N stage was associated with pathological response. The responders had significantly smaller mean tumor size than the non-responders (15.7 ± 7.4 cm vs 33.3 ± 14.1 cm, $P < 0.01$). According to Mandard's criteria, 27 patients were classified as responders, with 11 (40.7%) showing decreased tumor size by DCUS. In contrast, only three (18.8%) of the 16 non-responders showed decreased tumor size by DCUS ($P < 0.01$). The area under the ROC curve was 0.64, with a 95%CI of 0.46-0.81. The effects of several cut-off points on diagnostic parameters were calculated in the ROC curve analysis. By maximizing Youden's index (sensitivity + specificity - 1), the best cut-off point for distinguishing responders from non-responders was determined, which had optimal sensitivity of 62.9% and specificity of 56.3%. Using this cut-off point, the positive and negative predictive values of DCUS for distinguishing responders from non-responders were 70.8% and 47.4%, respectively. The overall accuracy of DCUS for therapeutic response assessment was 60.5%, slightly

higher than the 53.5% for CT response assessment with RECIST criteria ($P = 0.663$). Although the advantage was not statistically significant, likely due to the small number of cases assessed. DCUS was able to identify decreased perfusion in responders who showed no morphological change by CT imaging, which can be occluded by such treatment effects as fibrosis and edema.

CONCLUSION: DCUS may represent an innovative tool for more accurately predicting histopathological response to neoadjuvant chemotherapy before surgical resection in patients with locally-advanced gastric cancer.

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Key words: Gastric cancer; Chemotherapy; Ultrasonic imaging; Predictive value of tests; Disease management

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INTRODUCTION

Neoadjuvant chemotherapy (NAC) is a particularly promising component of the current multidisciplinary model for treating locally-advanced gastric cancer. Recent studies have shown that pre-operative NAC can increase the likelihood of curative resection, thereby improving long-term survival^[1]. However, in cases where patients prove unresponsive to the pre-operative NAC regimen, the result is higher risk of tumor progression and death since the surgical treatment is delayed. Thus, in contrast to a post-operative NAC regimen, pre-operative NAC requires an accurate, non-invasive technique to assess short-term therapeutic response. Such a technique should not only identify unresponsive cases in a timely manner to initiate individualized treatment options but also provide alternative endpoints for identifying non-responders and prognostic parameters for assessing individual prognosis^[2].

Currently, computed tomography (CT) is a key method for evaluating chemotherapy response in patients with advanced gastric cancer. The parameters of CT imaging to assess tumor response to treatment are well defined and established in the standard workup for gastric cancer staging^[3]. The response evaluation criteria in solid tumors (RECIST 1.1) were developed in 2009 as a robust and standardized guideline for clinical assessment of tumor

response to treatment^[4]. Unfortunately, the morphologic imaging techniques, including CT, magnetic resonance image (MRI) and endoscopic ultrasonography (EUS), have limited accuracy for detecting residual tumorous tissue within chemotherapy-treated areas due to occlusion by chemotherapy-induced fibrosis^[3,4].

Double contrast-enhanced ultrasound (DCUS) was recently developed as a complementary tool for the existing imaging modalities to help improve assessment of gastric cancer^[5,6]. In clinical application of this technology, use of an oral ultrasound contrast agent reveals the three-layered structure of the gastric wall, while use of intravenous contrast reveals the dynamic features of tumor vascularity. Thus, patient evaluation with double contrast provides qualitative and quantitative measures by which changes in gastric cancer pathophysiology may be evaluated and used to determine a patient's prognosis or treatment responsiveness^[5]. In addition, DCUS is superior to the traditional staging methods for gastric cancer since it can assess the depth of tumor penetration and the presence of lymph node metastases. However, no study to date has systematically evaluated the DCUS parameters for differentiating histopathological responders from non-responders after NAC and prior to surgical treatment.

The purpose of this study was to determine whether DCUS parameters are able to differentiate histopathological responders from non-responders early in the NAC course of treatment. In addition, the predictive value of DCUS was compared with the routine CT imaging technique.

MATERIALS AND METHODS

Patients

Forty-five consecutive patients who underwent pre-operative NAC followed by curative resection at the Second Affiliated Hospital at Zhejiang University College of Medicine between September 2011 and February 2012 were enrolled in the study. NAC was recommended for patients according to the following criteria: (1) a diagnosis of histologically proven gastric adenocarcinoma; (2) clinical stage of T4a or greater, and/or any T stage with lymph node metastasis; (3) Eastern Cooperative Oncology Group performance status of 2 or less; (4) adequate organ function; and (5) no active concomitant malignancy. All patients underwent pre-operative staging by both CT and DCUS. During the NAC treatment period, one of the study participants developed an irresectable tumor and a second developed metastatic disease; both of these patients were removed from the study and excluded from analysis.

The response to chemotherapy was evaluated for each patient after two courses of chemotherapy had been completed, according to the RECIST criteria. For cases showing partial response (PR), one or two more courses were administered or the patient underwent surgical resection. The study was carried out with pre-approval by the institute's Medical Ethics Committee. All subjects provided written informed consent prior to study participation.

Pre-operative chemotherapy regimens

Two NAC regimens were randomly used: either fluorouracil + oxaliplatin (FOLFOX) or S-1 + oxaliplatin (SOX) were administered for 2-4 cycles (as noted above) over a period of 6-12 wk. The fluorouracil was given on day 1 of each cycle at 400 mg/m² IVP, followed by 2400 mg/m² IVCI over 48 h. The S-1 was given at 80 mg/m² per day for 14 d, and then repeated three weeks later. For both regimens, the oxaliplatin was given on day 1 of each cycle at 130 mg/m² IV over 2 h.

Evaluation of tumor therapeutic response by CT and DCUS

CT scanning and DCUS were performed before and after two courses of the pre-operative NAC (either FOLFOX or SOX). For contrast-enhanced abdominal CT (Somatom Definition AS scanner; Siemens Medical Solutions, Forchheim, Germany), patients were fasted for six hours and given a 450 mL bolus of pure water immediately before the CT scanning to induce gastric distention. Contrast medium [OmnipaqueTM (iohexol); GE Healthcare, Cork, Ireland] was delivered intravenously at a rate of 3 mL/s by using an automatic injector to achieve a total injection volume of 2 mL/kg. For DCUS, the Sequoia 512 Acuson sonographic system (Siemens Medical Solutions) equipped with CadenceTM contrast pulse sequencing visualization technology and a 4V1 vector transducer to deliver low acoustic pressure frequencies (1.0-4.0 MHz) was used. Oral contrast agent and intravenous contrast medium were administered sequentially, as previously described^[5,6]. Briefly, the patients drank the Xinzhang oral contrast agent (Huqingyutang, Zhejiang, China; <http://dazzy007.cn.makepolo.com/product/8891276.html>) diluted in 500 mL of warm water to distend the stomach immediately before the procedure. Each dose of the microvesicle intravenous contrast medium (SonoVue, Bracco, Italy) was dissolved in 5 mL of saline and a 2.4 mL bolus was injected into the patient's superficial elbow vein.

CT and DCUS images were interpreted and reviewed by two independent diagnostic specialists, each with more than 10 years of experience in gastric imaging. All specialists were blinded to the histological findings and the same-sample findings from the other technique for evaluating the therapeutic response. The therapeutic response indicated by CT assessment was classified using the RECIST 1.1 criteria^[4] as: complete response, PR, stable disease, or progressive disease. Tumor area was assessed by DCUS as enhanced appearance of gastric carcinoma due to tumor vascularity during the contrast phase as compared to the normal gastric wall. Thus, the DCUS detected NAC response was evaluated according to: (1) the static change of ultrasonic echo; and (2) the dynamic (real-time) assessment of tumor vascularity and lymph nodes.

Surgery

Surgery was performed between weeks 3 and 5 after the completion of pre-operative NAC. All surgical procedures were carried out using an open laparotomy approach.

The resectability of the tumors, the extent of lymph node dissection, and the type of gastrectomy procedure were determined according to the perioperative observations. The gastrectomy procedure (total or subtotal) was selected based on the location and extent of the primary lesion. For successful resection, the resection lines had to be at least 5 cm from the edge of the macroscopic tumor. D2 (extended) or D3/D4 (super-extended) lymphadenectomies were performed according to the guidelines of the Japanese Research Society for Gastric Cancer (14th ed).

Assessment of pathologic response to NAC

Pathological findings served as the reference standard for all patients. The pathological response to NAC was evaluated according to the criteria of Mandard's tumor regression grade, which was based on the percentage of viable residual tumor cells in relation to fibrosis/necrosis^[7]. Patients with TRG1-2 were defined as responders, while patients with TRG3-5 were defined as non-responders.

Statistical analysis

All statistical analysis were carried out using SPSS software (version 16.0 for Windows; Chicago, IL, United States). The χ^2 or Fisher's exact tests were used to determine the significance of associations between pathologic findings and categorical variables. Receiver operating characteristic (ROC) curves were constructed to evaluate the ability of DCUS to identify a histopathological therapeutic response, with the area under the curves and the corresponding 95% CIs being calculated. The effects of several cut-off points on diagnostic parameters were determined by the ROC curve analysis. To compare the CT and DCUS procedures, the summary accuracy measure of Youden's index (sensitivity + specificity - 1) was used. By maximizing Youden's index, the best cut-off for distinguishing pathological responders from non-responders was identified. All tests were two-sided with *P* values of < 0.05 considered as indicating statistical significance.

RESULTS

The study population consisted of 32 men and 11 women, with a mean age of 59.7 ± 11.4 years (range: 34-79 years). The baseline patient and tumor characteristics are summarized in Table 1, with patients stratified according to the status as histopathological responders or non-responders.

Neither age, sex, histologic type, tumor site, T stage, nor N stage was significantly associated with the pathological response (χ^2 test, *P* > 0.05). However, the mean tumor size was significantly smaller in the responders than in the non-responders (15.7 ± 7.4 cm *vs* 33.3 ± 14.1 cm, *P* < 0.01).

The individual patient data of change in tumor size showed that 40.7% (11/27) of the responders experienced a decrease in tumor size that was detected by DCUS. However, significantly less, only 18.8% (3/16), of the non-responders showed a decrease in tumor size, as detected by DCUS (*P* < 0.01).

Table 1 Patient's clinical data and pathological features (n = 43)

Characteristic	Assessable patients with Mandard's TRG		P value
	Histopathologic responders	Histopathologic non-responders	
No.	27	16	
Age (yr), mean (range)	68.7 (34-75)	62.2 (39-79)	0.370
Gender			0.429
Male	19	13	
Female	8	3	
Histological type			0.934
Well differentiated	4	2	
Moderately differentiated	6	5	
Poorly differentiated	12	5	
Signet ring cell type	5	4	
Tumour size, cm (mean ± SD)	15.7 ± 7.4	33.3 ± 14.1	0.003
Tumour site			0.376
Fundus and cardia	3	4	
Body	7	5	
Antrum and pylorus	17	7	
Pathological T classification			0.475
T4a	22	13	
T4b	5	3	
Pathological N classification			0.744
N-	8	4	
N+	19	12	
Chemotherapy			0.965
FOLFOX	15	9	
SOX	12	7	
Chemotherapy cycle			0.432
2	12	4	
3	12	10	
4	3	2	

TRG: Tumor regression grade.

The ROC curve analysis for identifying histopathologic responders based on DCUS-detected changes in tumor size is shown in Figure 1. The area under the receiver-operating characteristic curve (AUC) was 0.64 (95%CI: 0.46-0.81). Using ROC curve analysis with Youden's index maximization, the best cut-off for distinguishing the responders from the non-responders was identified, which showed optimal sensitivity of 62.9% and specificity of 56.3%. For this cut-off point, the positive and negative predictive values of DCUS for distinguishing the responders from the non-responders were 70.8% and 47.4%, respectively. The overall accuracy of DCUS for therapeutic response assessment was 60.5%, compared with the slightly lower overall accuracy (53.5%) of CT assessment with RECIST criteria (P = 0.663, Table 2).

Interestingly, we found that DCUS was able to identify decreased perfusion in the tumors of responders who showed no morphological changes by the CT imaging technique. We believe the false negative findings of CT were likely due to occlusion by chemotherapy-induced effects, such as fibrosis and edema.

DISCUSSION

Given the generally poor long-term survival (< 20%-30%) achieved in advanced gastric cancer patients who undergo

Table 2 Comparison between computed tomography and double contrast-enhanced ultrasound for neoadjuvant chemotherapy response assessment n (%)

	Sensitivity	Specificity	PPV	NPV	Accuracy	χ^2	P value
CT	13 (48.1)	10 (62.5)	13 (66.7)	10 (41.7)	23 (53.5)	0.427	0.663
DCUS	17 (62.9)	9 (56.3)	17 (70.8)	9 (47.4)	26 (60.5)		

Double contrast-enhanced ultrasound (DCUS) had an overall accuracy of 60.5%, with a positive predictive value (PPV) of 70.8%. NPV: Negative predictive value; CT: Computed tomography.

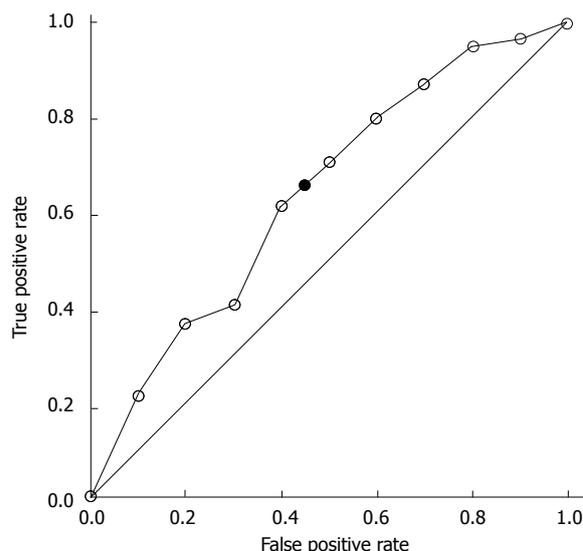


Figure 1 Receiver operator characteristics curve for the assessment of histopathologic response using double contrast-enhanced ultrasound. Area under the receiver-operating characteristic curve: 0.64 (95%CI: 0.46-0.81). The solid circle indicates the best cut-off point for distinguishing the responders from the non-responders.

surgery alone, clinicians and researchers have been actively pursuing methods to improve the survival rates of these patients. The approach of providing chemotherapy prior to the resection surgery (pre-operative/neoadjuvant) has proven beneficial for locally-advanced tumors^[8-10]. Patients receiving pre-operative NAC have shown increased likelihood of curative resection and improved overall survival. Although several studies have demonstrated the significant benefits of NAC, compared to surgery alone, for patients with resectable locally-advanced gastric cancer, the major clinical response rate has only reached 38%-69.7%^[11,12].

The current standard method for discriminating chemotherapeutic responders from non-responders is histopathologic analysis, which measures the extent of the residual tumor. This method, however, is applicable only in a post-operative setting and cannot be used for either the pre-operative design of personalized treatment or the planning of intra-operative strategies^[13]. Previous studies have revealed that the conventional imaging modalities (EUS, CT and MRI) to detect tumor volumetry lack reliability for predicting response to chemotherapy^[14,15]. Moreover, the response evaluation methods based on the

World Health Organization or RECIST criteria were reported to be highly inaccurate for gastric cancer^[16]. The recently developed technology of functional imaging, which detects or measures changes in metabolism, blood flow, regional chemical composition, and absorption, appears to be a promising alternative for monitoring chemotherapeutic effects in gastric tumors^[17]. In addition, the semi-quantitative approach of measuring glucose metabolism by means of positron emission tomography (PET) was shown to have clinical relevance in determining the response to chemotherapy for several tumor types, including gastric carcinomas^[18]. While both PET and PET/CT are well-established methods of molecular imaging, they are each limited by poor spatial resolution^[19]. In this regard, the CT and MRI technologies are preferable; however, these imaging technologies are restricted from widespread use due to their technical complexity and high cost which is often not amenable to smaller, underfunded clinics, especially those in rural areas.

Zhou *et al.*^[20] was the first to suggest that quantifying tumor perfusion with contrast-enhanced ultrasound may help detect changes in tumor perfusion after chemotherapy, based on their findings in an animal model. Our present study assessed the clinical value of a new functional imaging modality, DCUS, for non-invasive assessment of response to NAC in locally-advanced gastric cancer patients. The routine ultrasound techniques, US and EUS, rely purely on acoustic shadowing for visualization. As such, they are unable to differentiate conditions of inflammation and fibrosis in tumorous tissues, which leads to misinterpretation of the tumor depth^[3]. Chemotherapy-induced death of cancer cells results in reduced blood perfusion and decreased metabolic activity of the tumor. Since DCUS is capable of assessing the physiological blood flow within a tumor, it is also able to assess the depth of tumor penetration and lymph node metastasis^[21].

Quantitative assessment of tumor perfusion with contrast-enhanced ultrasound has already been successfully applied in both animal model^[22-24] and clinical^[25,26] studies. However, to the best of our knowledge, our study presented herein is the first to evaluate the efficiency of DCUS-detected tumor perfusion to monitor gastric cancer tumor response to chemotherapy. Although the indicated value of this technique in early assessment of tumor response to chemotherapy must be further tested in larger and more heterogeneous study populations, our preliminary results indicate that DCUS provides better diagnostic performance (AUC: 0.64) and accuracy (65.1%) for the assessment of a histopathological response than the standard CT imaging technique. Moreover, the results from the current study have clinical implications for customizing gastric cancer treatments to individual risk profiles, which should be explored in future studies.

In conclusion, in this study of patients with locally-advanced gastric cancer undergoing NAC, we found that DCUS may be as an innovative tool for predicting pathological response at an early stage of the NAC regimen

and prior to definitive resection. Because this technique is non-invasive and does not cause patient discomfort, it is particularly promising for repeated monitoring during the chemotherapy treatment period. Some limitations exist in the current study, however, that may impact the generalization of our findings. Despite the fact that the present study is the first to investigate the potential of DCUS response assessment during neoadjuvant chemotherapy in patients with gastric cancer, the total number of cases assessed was small ($n = 43$). We found that tumor size was affected by chemotherapy, and considered this a parameter of the assessment method, but we did not investigate any fluctuations in tumor size over time or in response to features of the chemotherapy regimen (doses, drug type, or cycle duration). Finally, our institute does not routinely use ultrasonography techniques as an evaluation modality for gastric cancer, so we were unable to draw conclusions about which analysis method is most useful (i.e., EUS *vs* DCUS). In order to determine whether DCUS can actually be used for accurate response prediction, a larger, multi-institute study is required.

COMMENTS

Background

Gastric cancer is currently the fourth most commonly diagnosed cancer worldwide, and it ranks second among cancer-related deaths. Currently, almost two-thirds of the gastric cancer cases occur in developing countries, with China alone accounting for 42%. Neoadjuvant chemoradiotherapy (NACRT) before surgery can improve survival in patients with locally-advanced gastric cancer, but not all patients respond to this treatment. An accurate method to assess short-term response to NACRT is critical for identifying responsive and non-responsive patients to design appropriate individualized treatment strategies.

Research frontiers

The criteria of computed tomography (CT) imaging for assessing tumor response to chemotherapy is well defined, and a well-established component of the standard workup for gastric cancer staging. However, CT imaging is usually not accurate for identifying the presence of residual tumorous tissues within areas with chemotherapy-induced effects, such as fibrosis. Their previous studies showed that double contrast-enhanced ultrasound (DCUS) is superior to the traditional CT-based gastric cancer staging methods to assess tumor penetration depth and lymph node metastases. In the current study, authors aimed to determine whether DCUS parameters are able to differentiate histopathological responders from non-responders in the early stage after neoadjuvant chemotherapy (NAC), and if so to compare the predictive efficacy of DCUS with that of CT imaging.

Innovations and breakthroughs

The results of the current study demonstrate that DCUS has optimal sensitivity and specificity for distinguishing histopathological responders from non-responders among NAC-treated gastric cancer patients. The overall accuracy of 60.5% was slightly higher than that of 53.5% for CT assessment with the response evaluation criteria in solid tumors criteria. Moreover, DCUS was able to identify decreased perfusion in cases of responders who showed no morphological changes by CT imaging, which had been likely occluded by treatment effects, such as fibrosis and edema.

Applications

DCUS is a feasible addition to the preoperative workup for NAC response assessment in gastric cancer patients. The results of DCUS may be beneficial and additive for formulating appropriate treatment plans for individual patients. Finally, ultrasound is a low-cost technique that is amenable to widespread application in small clinical setting with restricted funding and may prove a valuable technique for assessing the patients served by such clinics.

Terminology

Contrast-enhanced ultrasound is based on the traditional medical sonography

imaging technique but combined with contrast agents to enhance and differentiate various sections of an object under investigation. The substance composition of each of the various ultrasound contrast agents reflects sound waves in a distinctive manner. Neoadjuvant therapy is the administration of therapeutic agents prior to the main treatment regimen being initiated. The objective of neoadjuvant chemotherapy for gastric carcinoma is to reduce the size or extent of the cancer lesion prior to the curative resection surgery; smaller tumors are easier to resect and less extensive tumors have a higher chance of complete removal. Use of neoadjuvant chemotherapy is expected to reduce the potential risk and side effects of more radical surgical interventions required for larger and more extensive tumors.

Peer review

This is an interesting study in which authors evaluate the efficacy of double contrast-enhanced ultrasound for assessing tumor response to pre-operative chemotherapy in patients with gastric cancer. The results suggest that DCUS may represent an innovative tool for accurate predicting of histopathological response to neoadjuvant chemotherapy in patients with locally-advanced gastric cancer.

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Major influence of renal function on hyperlipidemia after living donor liver transplantation

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China Liver Transplant Registry retrospectively. PTHL was defined as serum triglycerides ≥ 150 mg/dL or serum cholesterol ≥ 200 mg/dL or the need for pharmacologic treatment at the sixth month after LDLT. Early renal dysfunction (ERD) was defined as serum creatinine ≥ 2 mg/dL and/or the need for renal replacement therapy in the first post-transplant week.

RESULTS: In 115 eligible patients, the incidence of PTHL was 24.3%. Recipients with PTHL showed a higher incidence of post-transplant cardiovascular events compared to those without PTHL (17.9% vs 4.6%, $P = 0.037$). Serum creatinine showed significant positive correlations with total serum triglycerides, both at post-transplant month 1 and 3 ($P < 0.01$). Patients with ERD had much higher pre-transplant serum creatinine levels ($P < 0.001$) and longer duration of pre-transplant renal insufficiency ($P < 0.001$) than those without ERD. Pre-transplant serum creatinine, graft-to-recipient weight ratio, graft volume/standard liver volume ratio, body mass index (BMI) and ERD were identified as risk factors for PTHL by univariate analysis. Furthermore, ERD [odds ratio (OR) = 9.593, $P < 0.001$] and BMI (OR = 6.358, $P = 0.002$) were identified as independent risk factors for PTHL by multivariate analysis.

CONCLUSION: Renal function is closely associated with the development of PTHL in LDLT. Post-transplant renal dysfunction, which mainly results from pre-transplant renal insufficiency, contributes to PTHL.

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Key words: Hyperlipidemia; Liver transplantation; Renal insufficiency; Graft function; Risk factors; Prognosis

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Abstract

AIM: To investigate the impact of renal and graft function on post-transplant hyperlipidemia (PTHL) in living donor liver transplantation (LDLT).

METHODS: A total of 115 adult patients undergoing LDLT from January 2007 to May 2009 at a single center were enrolled. Data were collected and analyzed by the

Ling Q, Wang K, Lu D, Guo HJ, Jiang WS, He XX, Xu X, Zheng SS. Major influence of renal function on hyperlipidemia after living donor liver transplantation. *World J Gastroenterol* 2012; 18(47): 7033-7039 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i47/7033.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i47.7033>

INTRODUCTION

Post-transplant hyperlipidemia (PHTL) is a common and serious complication in liver transplantation (LT). It is estimated that 45%-70% of LT recipients develop PHTL^[1-4]. Previous studies have shown that recipients with lipid metabolic disorders are approximately 3-4 times more likely to have cardiovascular events than recipients without lipid metabolic disorders^[5,6]. Therefore, PHTL is well recognized as one of the major risk factors for cardiovascular diseases, including myocardial infarction, ischemic stroke and peripheral arterial disease. Furthermore, cardiovascular complications after LT exert negative effects on the recipient's long-term survival and quality of life and are becoming the leading cause of non-graft related deaths in LT recipients^[7-9].

The pathogenesis of PHTL remains to be elucidated. Possible factors that may increase the risk of PHTL include overweight or obesity, advanced age, and the use of immunosuppressive agents^[2,10]. Immunosuppressive drugs were considered to have a major effect on the development of metabolic syndromes in previous years. Today, immunosuppressive drugs are known to have a reduced impact on glucose and lipid metabolism^[11]. Low toxicity immunosuppressive protocols (e.g., early withdrawal of steroids and low-dose calcineurin inhibitors) have decreased the impact of immunosuppressive drugs on PHTL. It is known that the liver plays an essential role in lipoprotein metabolism and is involved in almost every key step during lipid anabolism and catabolism. In living donor liver transplantation (LDLT), graft function can be influenced by reduced graft size, preservation and ischemia-reperfusion injuries. Early allograft dysfunction (EAD) may occur in more than 10% of LT recipients and result in an unfavorable prognosis^[12,13]. More importantly, renal insufficiency, which is common in liver transplant candidates, is closely associated with significant alterations in lipid metabolism. Early renal dysfunction (ERD), which is also common after LT, may indicate a prolonged recovery of kidney insufficiency and indicates a poor outcome^[14].

To date, it is still unknown whether impaired kidney or graft function can cause derangements in lipid metabolism after LDLT. To clarify these questions, we designed this retrospective study with the aim of evaluating the impact of renal and graft function on lipid metabolism after LDLT and to identify the possible risk factors for PHTL.

MATERIALS AND METHODS

Study population

All adult (age \geq 18 years old) patients undergoing LDLT

from January 2007 to May 2009 at the First Affiliated Hospital, Zhejiang University School of Medicine, China were included in this retrospective study. Patients who died within 3 mo of LDLT were excluded. A total of 115 (96 male and 19 female) patients with a mean age of 44.7 ± 10.11 years (median: 46 years, range: 18-64 years) at transplantation were finally included. The indications for LT included cirrhosis ($n = 63$, 54.8%), acute liver failure ($n = 28$, 24.3%) and hepatocellular carcinoma ($n = 24$, 20.9%). Patient characteristics are shown in Table 1. Written informed consent was acquired from all donors and recipients before transplantation. Each organ donation and transplantation at our center was strictly carried out under the guidelines of the Ethics Committee of the First Affiliated Hospital, Zhejiang University, the regulation of Organ Transplant Committee of Zhejiang Province and the Helsinki Declaration of 1975. No prisoners were included in this study.

All patients received a triple immunosuppressive regimen incorporating tacrolimus, prednisolone, and mycophenolate mofetil as described previously^[15]. An interleukin (IL)-2 receptor blocker was used in some patients. Prednisolone was withdrawn within the first post-transplant month. A reduced dose of tacrolimus was given to patients who developed post-transplant renal impairment. All patients were routinely followed up at the out-patient clinic and the mean follow-up time was 2.66 ± 1.02 years (median: 1.98 years, range: 0.50-4.63 years).

Data collection

The data were extracted from the China Liver Transplant Registry (CLTR) database: age (donor/recipient), gender (donor/recipient), underlying liver diseases, cold ischemic time, graft-to-recipient weight ratio (GR/WR), graft volume to standard liver volume ratio (GV/SLV), donor's hepatic steatosis, recipient's pre-transplant metabolic status [history of smoking, alcohol abuse, diabetes mellitus, hypertension, hyperlipidemia, body mass index (BMI)], recipient's post-transplant liver function (bilirubin, alanine transaminase, aspartate transaminase, cholinesterase and international normalized ratio), recipient's pre/post-transplant renal function (serum creatinine), donor's and recipient's pre/post-transplant serum lipid profile (total triglycerides, total cholesterol), immunosuppressive regimen (agents and plasma levels), and recipient's post-transplant complications.

Hyperlipidemia was defined as serum triglycerides \geq 150 mg/dL or serum cholesterol \geq 200 mg/dL or the need for pharmacologic treatment^[16]. Patients were divided into the PHTL group ($n = 28$) and non-PHTL group ($n = 87$) according to their serum lipid levels at the sixth month after LDLT. EAD was defined by the presence of at least one of the following features: total bilirubin $>$ 10 mg/dL, prothrombin time \geq 17 s, or hepatic encephalopathy from day 2 to 7 post-transplantation^[17]. ERD was defined as serum creatinine \geq 2 mg/dL and/or the need for renal replacement therapy in the first post-transplant week^[18]. Renal insufficiency was defined as pre-transplant serum creatinine $>$ 1.5 mg/dL^[18].

Table 1 Patients' characteristics in post-transplant hyperlipidemia group and non post-transplant hyperlipidemia group (means \pm SD)

	PTHL group (n = 28)	Non-PTHL group (n = 87)	P value
Donor			
Age (yr)	24.8 \pm 4.3	24.0 \pm 6.4	NS
Male/female (n)	27/1	80/7	NS
Cold ischemia time (h)	1.2 \pm 0.5	1.1 \pm 0.4	NS
GR/WR (%)	1.0 \pm 0.2	1.1 \pm 0.2	0.048
GV/SLV (%)	58.5 \pm 5.6	62.6 \pm 10.7	0.011
Hepatic steatosis n (%)	2 (7.1)	7 (8.0)	NS
Serum triglyceride (mg/dL)	90.9 \pm 42.4	96.2 \pm 45.0	NS
Serum cholesterol (mg/dL)	137.3 \pm 30.9	135.7 \pm 27.1	NS
Recipient			
Age (yr)	45.4 \pm 8.2	44.5 \pm 10.6	NS
Male/female (n)	27/1	69/18	NS
Primary liver diseases n (%)			
Cirrhosis	12 (42.9)	51 (58.6)	NS
Acute liver failure	9 (32.1)	19 (21.8)	NS
Hepatocellular carcinoma	7 (25)	17 (19.5)	NS
Pre-transplant metabolic status			
BMI (kg/m ²)	23.2 \pm 3.16	21.9 \pm 2.79	0.036
Smoking n (%)	7 (25)	19 (21.8)	NS
Alcohol abuse n (%)	3 (10.7)	13 (14.9)	NS
Diabetes mellitus n (%)	2 (7.1)	4 (4.6)	NS
Hypertension n (%)	3 (10.7)	5 (5.7)	NS
Hyperlipidemia n (%)	4 (14.3)	15 (17.2)	NS
Pre-transplant MELD score	23.8 \pm 12.4	20.1 \pm 9.6	NS
score			
Total bilirubin (mg/dL)	14.2 (1.0-36.5)	12.8 (0.8-33.6)	NS
International normalized ratio	1.6 \pm 1.1	1.4 \pm 0.6	NS
Serum creatinine (mg/dL)	0.9 (0.5-4.0)	0.7 (0.4-4.5)	0.004
Post-transplant tacrolimus level (ng/mL)			
Month 1	8.1 \pm 2.8	9.8 \pm 4.6	NS
Month 3	9.2 \pm 2.6	8.3 \pm 2.6	NS
Month 6	6.7 \pm 2.2	7.7 \pm 2.7	NS
Follow-up period (yr)	2.7 \pm 1.2	2.6 \pm 0.9	NS

PTHL: Post-transplant hyperlipidemia; GR/WR: Graft-to-recipient weight ratio; GV/SLV: Graft volume/standard liver volume; BMI: Body mass index; MELD: Model for end-stage liver disease; NS: Not significant.

Statistical analysis

Quantitative variables were presented as mean \pm SD or median with range. Categorical variables were expressed as numbers and percentages. The Student's *t* test or Mann-Whitney *U* test was used to compare quantitative variables and the χ^2 test was used to compare categorical variables. Pearson's correlation and scatter plot graph were used for correlation analysis. Logistic regression was used to identify the risk factors for PTHL. Variables that were statistically significant in univariate analysis were entered into multivariate analysis. The Kaplan-Meier method and log-rank test were used for survival analysis. SAS software version 9.2 (SAS institute, Cary, NC, United States) was used to complete all the analyses, and a *P* value of less than 0.05 was considered statistically significant. Data analysis was performed by the CLTR

Table 2 Comparison between patients with and without early renal dysfunction (means \pm SD)

	ERD (n = 16)	Non-ERD (n = 99)	P value
Pre-transplant			
MELD	30.1 \pm 11.7	19.5 \pm 9.4	< 0.001
Total bilirubin (mg/dL)	15.2 (1.8-29.8)	13.6 (0.8-36.5)	NS
International normalized ratio	1.6 \pm 0.9	1.4 \pm 0.7	NS
Post-transplant			
Serum creatinine (mg/dL)			
At month 1	1.4 (0.6-2.8)	0.7 (0.2-1.2)	< 0.001
At month 3	1.0 (0.6-3.0)	0.7 (0.3-1.3)	0.008
At month 6	1.1 (0.6-4.1)	0.7 (0.3-1.5)	< 0.001
Total bilirubin (mg/dL)			
At month 1	1.3 (0.4-4.3)	1.1 (0.3-5.8)	NS
At month 3	0.6 (0.2-2.5)	0.7 (0.3-2.5)	NS
At month 6	0.6 (0.3-2.0)	0.8 (0.4-2.8)	NS
Cholinesterase (U/L)			
At month 1	3681.3 \pm 1354.3	4403.9 \pm 1323.6	NS
At month 3	5996.7 \pm 1619.4	6830.7 \pm 1750.5	NS
At month 6	7255.4 \pm 1662.9	7171.5 \pm 1721.6	NS
Hypertriglyceridemia n (%)	9 (56.3)	12 (12.1)	< 0.001
Hypercholesterolemia n (%)	3 (18.8)	9 (9.1)	NS
PTHL n (%)	9 (56.3)	19 (19.2)	0.003

Hypertriglyceridemia was defined as serum triglycerides \geq 150 mg/dL; Hypercholesterolemia was defined as serum cholesterol \geq 200mg/dL. PTHL: Post-transplant hyperlipidemia; MELD: Model for end-stage of liver disease; ERD: Early renal dysfunction; NS: Not significant.

Statistical Department.

RESULTS

Prevalence and prognosis of hyperlipidemia

Of 115 eligible recipients, 19 (16.5%) had pre-transplant hyperlipidemia and 28 (24.3%) developed PTHL. Of the recipients with and without pre-transplant hyperlipidemia, the incidence of PTHL did not differ significantly (21.1% *vs* 25%, *P* = 0.714).

Of 115 donors, 13 (11.3%) were diagnosed with hyperlipidemia and 9 (7.8%) had hepatic steatosis. However, neither donor hyperlipidemia nor hepatic steatosis was found to be differently distributed between the PTHL group and non-PTHL group (both *P* > 0.05) (Table 1).

Nine recipients (7.8%) developed cardiovascular events, including four with nonfatal myocardial infarction, two with cardiac arrhythmia, one with cardiac arrest and two with stroke, with a mean onset time of 10.5 mo (median: 8 mo, range: 3-15 mo). Compared to the non-PTHL group, the PTHL group had a higher incidence of cardiovascular events after LDLT (*n* = 5, 17.9% *vs* *n* = 4, 4.6%, *P* = 0.037). The 1- and 3-year cumulative survival rates were 89.3% and 85.7% in the PTHL group, and 97.7% and 85.0% in the non-PTHL group, respectively.

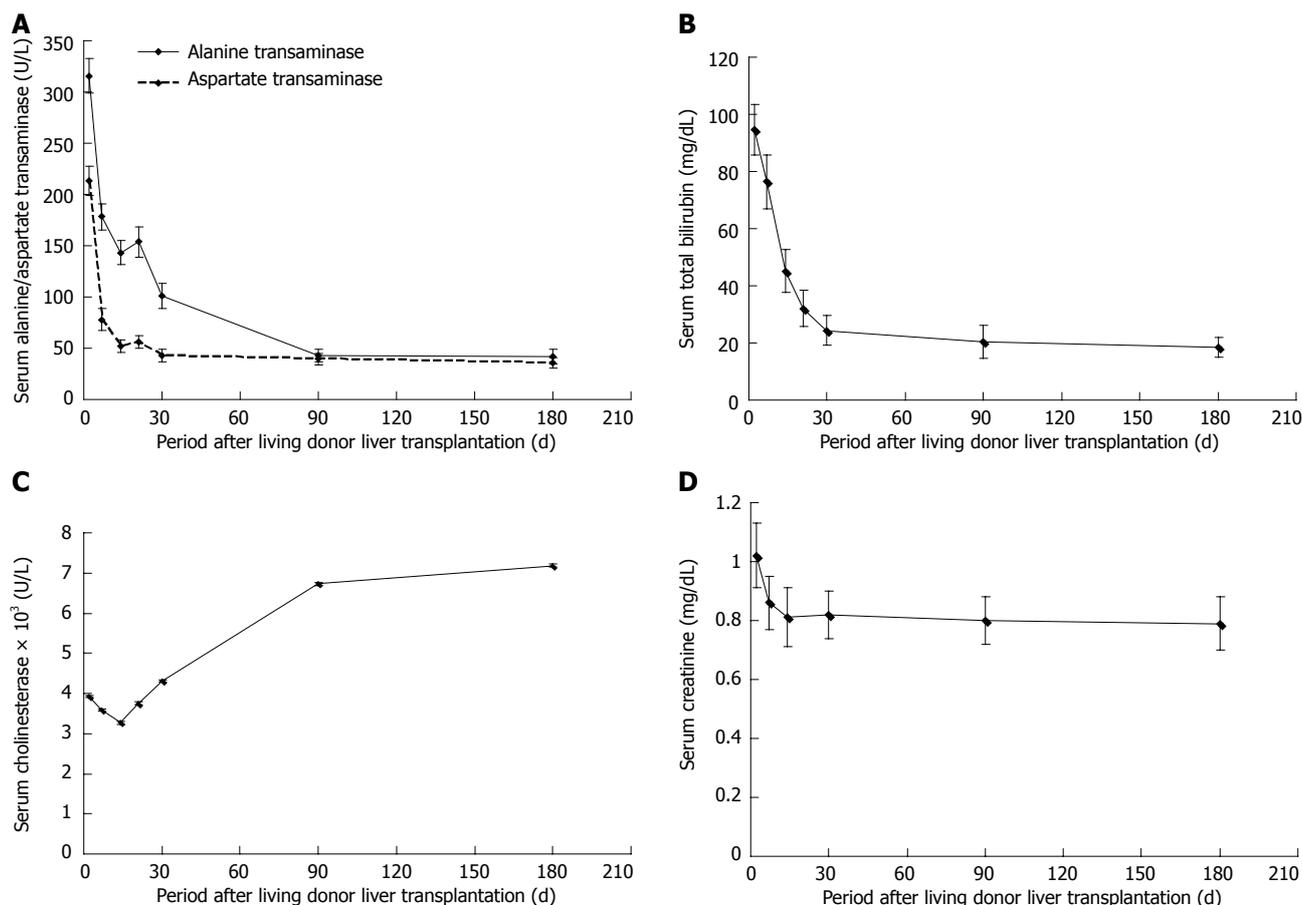


Figure 1 Variation tendency of graft and renal function by post-transplant month 6 in all recipients. A: Alanine/aspartate transaminase; B: Total bilirubin; C: Cholinesterase; D: Serum creatinine. Data were presented as means \pm SD.

There was no statistically significant difference between the two groups in cumulative survival ($P = 0.956$).

Graft function and post-transplant hyperlipidemia

As shown in Figure 1, alanine transaminase, aspartate transaminase and bilirubin, which indicate hepatic cellular injury, decreased sharply in the first post-transplant month and continued to decline to the normal range during the next two months. Cholinesterase, which represents hepatocellular synthetic function, remained at a low level during the first post-transplant month and gradually increased to an ideal level at post-transplant month 3. Of these parameters, cholinesterase had a significant positive correlation with serum cholesterol level at post-transplant month 1 ($r = 0.341$, $P = 0.001$) and month 3 ($r = 0.280$, $P = 0.003$), respectively (Figure 2A and B). However, cholinesterase was not associated with the development of PTHL following logistic regression analysis ($P > 0.05$).

EAD occurred in 18 (15.7%) of 115 recipients. At the third post-transplant month, EAD had resolved in 77.8% (14/18) of patients. Primary graft non-function was not observed. The incidence of PTHL did not differ significantly between patients with EAD and those without EAD (33.3% *vs* 20.6%, $P = 0.236$).

Renal function and post-transplant hyperlipidemia

Serum creatinine levels decreased during the first post-

transplant month (Figure 1) and were significantly related to serum triglyceride level at the first ($r = 0.440$, $P < 0.001$) and third ($r = 0.250$, $P = 0.007$) post-transplant month, respectively (Figure 2C and D). Serum creatinine levels at the first post-transplant month differed significantly between the PTHL and non-PTHL groups (1.10 ± 0.67 mg/dL *vs* 0.73 ± 0.25 mg/dL, $P = 0.001$).

ERD occurred in 16 (13.9%) of 115 post-transplant recipients. There was a statistically significant difference between ERD and non-ERD recipients in pre-transplant renal function (Table 2). Serum creatinine remained high during the first 6 post-transplant months in ERD recipients. Of the 16 recipients with ERD, 9 and 5 needed hemodialysis in the first and third post-transplant month, respectively. The prevalence of PTHL in ERD patients was significantly higher than that in non-ERD patients (56.3% *vs* 19.2%, $P = 0.003$), notably for hypertriglyceridemia (56.3% *vs* 12.1%, $P < 0.001$).

Risk factors for post-transplant hyperlipidemia

We postulated that donors' pre-transplant hyperlipidemia and hepatic steatosis, GR/WR (%) and GV/SLV (%); recipients' pre-transplant serum creatinine level, pre-transplant BMI and pre-transplant hyperlipidemia, post-transplant tacrolimus level, post-transplant diabetes within the first six months after LDLT^[15], post-transplant BMI at the sixth month after LDLT, EAD and ERD were poten-

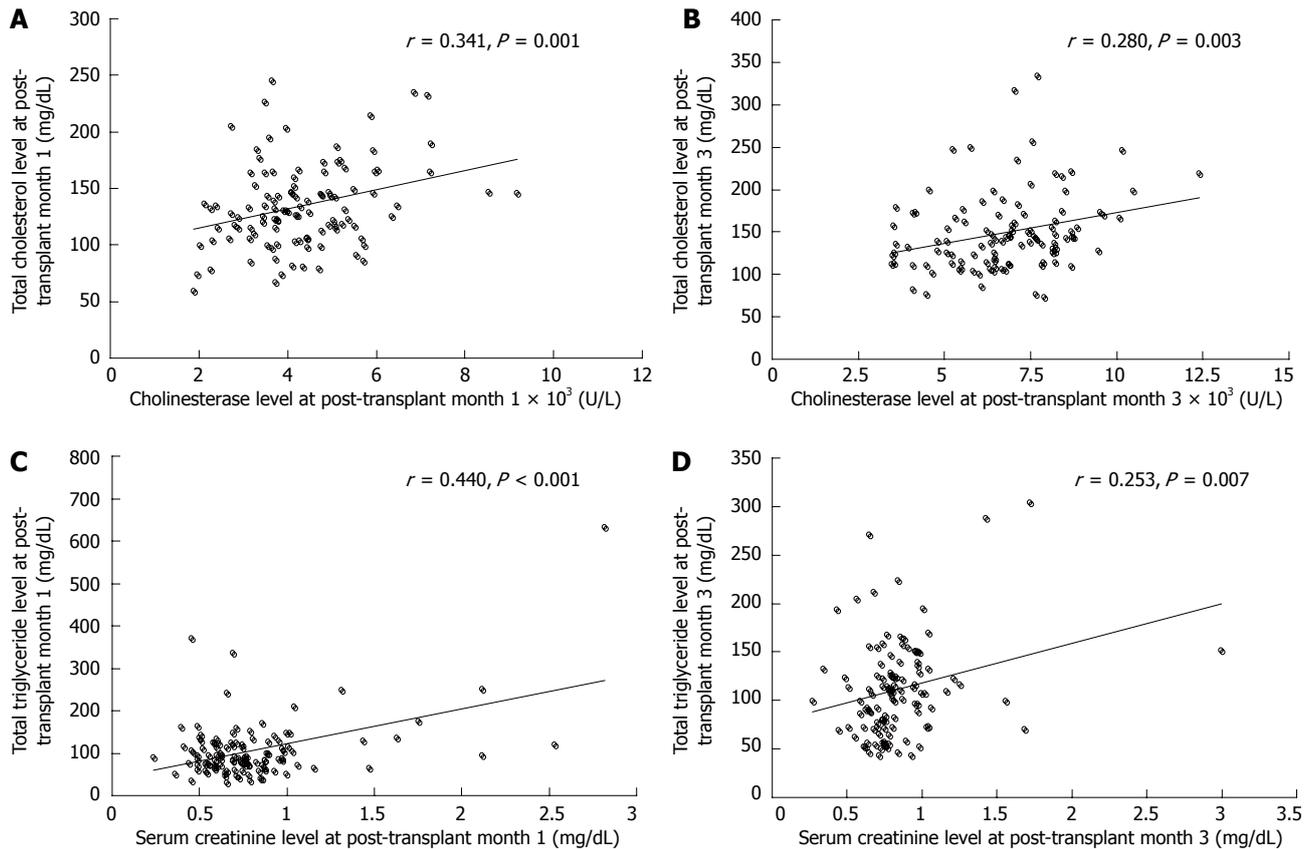


Figure 2 Correlation between serum cholesterol and triglyceride level and cholinesterase at post-transplant. A: Correlation between serum cholesterol level and cholinesterase at post-transplant month 1; B: Correlation between serum cholesterol level and cholinesterase at post-transplant month 3; C: Correlation between serum triglyceride level and serum creatinine at post-transplant month 1; D: Correlation between serum triglyceride level and serum creatinine at post-transplant month 3.

tial risk factors for PTHL. Following univariate analysis, five variables were identified as significant risk factors for PTHL and were entered into the multivariate analysis (Table 3). We subsequently found that ERD [odds ratio (OR) = 9.593, $P < 0.001$] and BMI (OR = 6.358, $P = 0.002$) were independent risk factors for PTHL.

DISCUSSION

The most important finding in this study was the correlation between renal function and PTHL, which may be of great help in better understanding the pathogenesis of PTHL, and as much as possible, preventing its development. It was demonstrated that post-transplant serum creatinine had a significant positive correlation with serum triglycerides both in the first and third post-transplant month. More importantly, using multivariate regression analysis, ERD was found to be an independent risk factor for PTHL. Patients with ERD usually had a relatively long-term and severe renal insufficiency before transplantation, and impaired renal function resolved very slowly during the first six months after transplantation. Here, we provide the first evidence that delayed recovery of renal function after transplantation, which was probably due to chronic kidney injury before transplantation, can result in PTHL, especially hypertriglyceridemia.

In fact, it is well known that chronic kidney disease is associated with hyperlipidemia. In the kidney, the apical surface of proximal tubules has a high capacity for receptor-mediated uptake of filtered lipid-binding plasma proteins^[19]. Therefore, hyperlipidemia induced by impaired renal function is characterized by abnormal metabolism of plasma lipoproteins^[20,21]. The reduced catabolic rate of triglyceride-rich lipoproteins and the growing hepatic production of triglyceride-high lipoproteins may explain why hypertriglyceridemia is one of the most common quantitative lipid abnormalities in patients with chronic kidney disease^[22,23]. Consistent with previous findings, the impaired renal function and recovered liver synthetic function in patients with ERD during the early post-transplant period may explain the development of hypertriglyceridemia in this study. Therefore, more emphasis should be placed on this issue, as more and more patients are undergoing liver transplantation with higher serum creatinine levels in the era of MELD.

Another finding in this study was that graft function was associated with cholesterol metabolism after LDLT. Our results showed the smooth recovery of decompensated liver function during the early post-transplant period. Furthermore, elevated serum cholinesterase level, which represents the recovery of hepatic cellular synthetic function, was significantly correlated with an increase

Table 3 Logistic regression analysis of variables relating to post-transplant hyperlipidemia

		Univariate analysis		Multivariate analysis	
		OR (95%CI)	P value	OR (95%CI)	P value
Pre-transplant serum creatinine	≥ 1.5 mg/dL	3.810 (1.203-12.063)	0.023		
	< 1.5 mg/dL				
GR/WR (%)	< 1.0	2.667 (1.116-6.372)	0.027		NS
	≥ 1.0				
GV/SLV (%)	< 60	2.806 (1.158-6.798)	0.022		NS
	≥ 60				
Pre-transplant BMI	≥ 25 kg/m ²	4.105 (1.435-11.752)	0.008	6.358 (2.026-19.958)	0.002
	< 25 kg/m ²				
ERD	With	5.143 (1.789-16.383)	0.003	9.593 (2.803-32.827)	< 0.001
	Without				

PTHL: Post-transplant hyperlipidemia; GR/WR: Graft-to-recipient weight ratio; GV/SLV: Graft volume/standard liver volume; BMI: Body mass index; ERD: Early renal deficiency; NS: Not significant.

in cholesterol level within the normal range. It is noteworthy that cholesterol plays an active role during liver regeneration. It is not only a structural component, but also a significant regulator in the control of the intermediate metabolism of different liver cell types^[24]. This implies that there may be mutual benefits between improved graft synthetic function and cholesterol homeostasis.

Since donor pre-transplant serum lipid level may play an essential role in the development of PTHL^[25], we also analyzed the association between pre-transplant donor serum lipid level and post-transplant recipient serum lipid level. No significant difference in the incidence of PTHL was found between the recipients receiving grafts from pre-transplant hyperlipidemic and non-hyperlipidemic donors. The pre-transplant serum triglyceride and cholesterol levels of donors in the PTHL group were not significantly higher than those in the non-PTHL group. Therefore, in this study, there was no obvious evidence to show that pre-transplant donors' serum lipid level or fatty liver exerted crucial effects on the development of PTHL.

High BMI was found to be another independent risk factor for PTHL in this study. As a main component of the metabolic syndrome, high BMI or obesity before LT has been reported to be associated with an increased prevalence of PTHL^[26]. It seems unlikely that dietary habits and unhealthy lifestyle can be changed and thus patients retain their obesity status, resulting in abnormalities in lipid metabolism. It was notable that some patients had pre-transplant hyperlipidemia, but did not have PTHL in this study. A possible reason for this is that pre-transplant hyperlipidemia, which resulted mainly from hepatorenal syndrome due to end-stage liver disease, could be improved or resolved by the recovery of liver and kidney function after liver transplantation.

A high blood concentration of tacrolimus has been reported to contribute to PTHL in LDLT^[27]. However, in the present study, we did not find an association between immunosuppressive drugs and PTHL. This may be due to the early steroid withdrawn and low tacrolimus concentration protocol. Furthermore, reduced-dose tacrolimus with or without an IL-2 receptor blocker was

given to patients who developed post-transplant renal impairment, which also minimizes the side-effects of immunosuppressive drugs on lipid metabolism.

There were some limitations in this study. Firstly, it was not a prospective study. The impact of graft and kidney function on the development of PTHL requires confirmation in prospective studies with larger samples. Secondly, a longer period of follow-up is necessary to identify the natural history of PTHL and to determine the influence of PTHL on a recipient's prognosis. Thirdly, a study at the molecular level should be performed as the donor's genotype may play a role in the development of metabolic diseases.

In conclusion, renal and graft function correlated with lipid metabolism after LDLT. Severe pre-transplant renal insufficiency may lead to long-term post-transplant renal dysfunction, and consequently cause PTHL, especially hypertriglyceridemia. Appropriate clinical treatment such as the prophylactic use of fibrates or statins may be considered to prevent PTHL in patients who develop ERD or have longstanding pre-transplant renal dysfunction. Well-designed and large-sample studies are needed to verify these results.

COMMENTS

Background

Post-transplant hyperlipidemia (PTHL) is a major and serious complication after liver transplantation. It is estimated that 45%-70% of recipients develop PTHL, which is well recognized as one of the major risk factors for cardiovascular diseases, including myocardial infarction, ischemic stroke and peripheral arterial disease, and exerts negative effects on the recipient's long-term survival and quality of life. It is well known that the liver plays an essential role in lipoprotein metabolism and is involved in almost every key step during lipid anabolism and catabolism. More importantly, renal insufficiency, which is common in liver transplant candidates, is closely associated with significant alterations in lipid metabolism. Therefore, it is necessary to determine the associations between both renal and hepatic function and PTHL.

Research frontiers

The pathogenesis of PTHL remains to be elucidated. Possible factors which may increase the risk of PTHL include overweight or obesity, advanced age, and the use of immunosuppressive agents. Immunosuppressive drugs were considered to have a major effect on the development of metabolic syndromes in previous years. Today, immunosuppressive drugs are known to have a reduced impact on glucose or lipid metabolism. Low toxicity immunosuppressive

protocols (e.g., early withdrawal of steroids and low-dose calcineurin inhibitors) have decreased the impact of immunosuppressive drugs on PTHL.

Innovations and breakthroughs

Over the past few years, most researchers have focused their attention on immunosuppressive protocols and obesity status. However, together with the application of lower toxicity immunosuppressive protocols, an increasing number of articles have shown that novel immunosuppressive therapy has less of an impact on glucose and lipid metabolism. Therefore, authors looked into the possibility that kidney and graft function may have a potential relationship with PTHL. From the results, renal and graft function were shown to correlate with lipid metabolism after living donor liver transplantation. Severe pre-transplant renal insufficiency may lead to long-term post-transplant renal dysfunction, and consequently result in PTHL, especially hypertriglyceridemia. Appropriate clinical treatment such as the prophylactic use of fibrates or statins may prevent PTHL in patients who develop early renal dysfunction or have longstanding pre-transplant renal dysfunction.

Applications

This study suggested that there was a correlation between renal function and PTHL, which may be of great help in better understanding the pathogenesis of PTHL, and as much as possible, preventing its development.

Peer review

This is a good retrospective study. There were some limitations in this study, but were reasonably explained by the authors. In my opinion, this study is original and it is acceptable for publication.

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Effect of salvianolate on intestinal epithelium tight junction protein zonula occludens protein 1 in cirrhotic rats

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Abstract

AIM: To study the effect of salvianolate on tight junctions (TJs) and zonula occludens protein 1 (ZO-1) in small intestinal mucosa of cirrhotic rats.

METHODS: Cirrhosis was induced using carbon tetrachloride. Rats were randomly divided into the untreated group, low-dose salvianolate (12 mg/kg) treatment

group, medium-dose salvianolate (24 mg/kg) treatment group, and high-dose salvianolate (48 mg/kg) treatment group, and were treated for 2 wk. Another 10 healthy rats served as the normal control group. Histological changes in liver tissue samples were observed under a light microscope. We evaluated morphologic indices of ileal mucosa including intestinal villi width and thickness of mucosa and intestinal wall using a pathological image analysis system. Ultrastructural changes in small intestinal mucosa were investigated in the five groups using transmission electron microscopy. The changes in ZO-1 expression, a tight junction protein, were analyzed by immunocytochemistry. The staining index was calculated as the product of the staining intensity score and the proportion of positive cells.

RESULTS: In the untreated group, hepatocytes showed a disordered arrangement, fatty degeneration was extensive, swelling was obvious, and disorganized lobules were divided by collagen fibers in hepatic tissue, which were partly improved in the salvianolate treated groups. In the untreated group, abundant lymphocytes infiltrated the fibrous tissue with proliferation of bile ducts, and collagen fibers gradually decreased and damaged hepatic lobules were partly repaired following salvianolate treatment. Compared with the untreated group, no differences in intestinal villi width between the five groups were observed. The villi height as well as mucosa and intestinal wall thickness gradually thickened with salvianolate treatment and were significantly shorter in the untreated group compared with those in the salvianolate treatment groups and normal group ($P < 0.01$). The number of microvilli decreased and showed irregular lengths and arrangements in the untreated group. The intercellular space between epithelial cells was wider. The TJs were discontinuous, which indicated disruption in TJ morphology in the untreated group. In the treated groups, the microvilli in the intestinal epithelium were regular and the TJs were gradually integrated and distinct. The expression of ZO-1 decreased in the small intestine of the un-

treated cirrhotic rats. The high expression rate of ZO-1 in ileal mucosa in the untreated group was significantly lower than that in the medium-dose salvianolate group (21.43% *vs* 64.29%, $\chi^2 = 5.25$, $P < 0.05$), high-dose salvianolate group (21.43% *vs* 76.92%, $\chi^2 = 8.315$, $P < 0.01$) and normal group (21.43% *vs* 90%, $\chi^2 = 10.98$, $P < 0.01$).

CONCLUSION: Salvianolate improves liver histopathological changes, repairs intestinal mucosa and TJ structure, and enhances ZO-1 expression in the small intestinal mucosa in cirrhotic rats.

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Key words: Salvianolate; Cirrhosis; Gut barrier; Tight junction; Zonula occludens protein 1

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INTRODUCTION

In liver cirrhosis, disruption of the intestinal mucosal barrier function and increased mucosal intestinal permeability lead to bacterial translocation and endotoxemia^[1], which increase susceptibility to infection, the most frequent and severe of which is spontaneous bacterial peritonitis^[2,3]. Endotoxemia may affect liver function, contribute to systemic hemodynamic derangement in liver cirrhosis^[4,5], and result in severe complications^[6-8]. Therefore, restoring the integrity of the intestinal barrier is an important goal in preventing deterioration of liver function in patients with cirrhosis^[3,9].

The intestinal mucosal barrier includes both secretory and physical preventive measures against the penetration of microbes. The secretory mechanism is realized through mucus secretion, local immunoglobulins and bile salts. The physical mechanism is represented by the intestinal epithelium^[10,11], specifically its lack of permeability and active antimicrobial peptide production. The tight junctions (TJs) of the intestinal epithelium allow only the passage of very tiny molecules^[12], preventing the transport of bacteria or macromolecules (lipopolysaccharides)^[13]. Disruption of the intestinal TJ barrier results in a “leaky” TJ barrier, allowing paracellular permeation of toxic luminal substances. Altered intestinal TJ expression in patients is a molecular mechanism of intestinal hyperpermeability. It was demonstrated that patients with decompensated and compensated cirrhosis had signifi-

cantly reduced expression of TJ proteins, higher Child-Pugh score, and decreased liver function^[14]. As an important contributor to the development of liver cirrhosis, the physical barrier of the small intestine represents an ideal therapeutic target^[15]. However, there are no reports on an effective means of preventing disruption of the intestinal physical barrier in liver cirrhosis.

Radix *Salvia miltiorrhiza*, a traditional Chinese medicinal herb known as “danshen” has been widely used for the treatment of various cardiovascular diseases^[16,17]. *Salvia miltiorrhiza* (*S. miltiorrhiza*) extracts contain lipid-soluble diterpene quinones (tanshinones) and water-soluble phenolic acid derivatives such as salvianolic acid A and B and lithospermic acid B^[18]. Recent pharmacological research has shown that *S. miltiorrhiza* eliminates oxygen free radicals, enhances antioxidant activity, decreases serum cytokine levels, and inhibits endotoxemia^[19]. Moreover, *S. miltiorrhiza* can block the lethal toxicity of lipopolysaccharides in mice *via* suppression of tumor necrosis factor- α (TNF- α) release^[20] and help to maintain the integrity of the endothelial junction structure^[21]. Salvianolate is a new water-soluble phenolic compound and is one of the most bioactive compounds in *S. miltiorrhiza* Bge. However, in carbon tetrachloride (CCl₄)-induced cirrhosis in rats, the effect of salvianolate on the physical barriers of the small intestine is less clear. A previous study demonstrated that salvianolate can reduce the endotoxin level, ameliorate injury to the intestinal mucosa, and inhibit the expression of TNF- α and interleukin-6 (IL-6) mRNA in the small intestine of cirrhotic rats^[22]. Therefore, we used CCl₄-induced cirrhotic rats to evaluate changes in the epithelial barrier of the ileal mucosa and the effect of different doses of salvianolate on TJs and zonula occludens protein 1 (ZO-1) in microvillus cells of the small intestine mucosa.

MATERIALS AND METHODS

A previous study demonstrated that salvianolate can reduce endotoxin levels in the portal vein, ameliorate injury to the intestinal mucosa, and inhibit cytokine gene expression in rats with CCl₄-induced liver cirrhosis^[22]. To further explore the mechanism of salvianolate in enhancement of the intestinal mechanical barrier, in the present study, we evaluated liver histopathological changes and morphologic indices of ileal mucosa using light microscopy, analyzed the ultrastructural changes using transmission electron microscopy and the expression of ZO-1, a TJ protein, using immunocytochemistry. The results of this study may provide a new strategy for the treatment of liver cirrhosis.

Animals

Ninety male Sprague-Dawley rats (weight: 180-220 g) were provided by the Department of Animal Care, Zhejiang Traditional University, Hangzhou, China. Experimental animals were housed in individual cages at 22 °C to 25 °C under a 12-h light/dark cycle and fed a standard

laboratory diet and tap water *ad libitum*.

Experimental protocol

The rats were randomly divided into two groups: the normal control group ($n = 10$, group A) and the model group. All model group rats received a subcutaneous injection of 40% CCl₄ in a 2:3 mixture with olive oil (0.3 mL/kg) once weekly for 12 wk. Liver cirrhosis was successfully induced in 55 rats at the end of 12 wk as shown by liver histological evaluation. The 55 model rats were further randomly divided into four subgroups: the untreated group ($n = 14$, group B), low-dose salvianolate-treated group (12 mg/kg) ($n = 14$, group C), medium-dose salvianolate-treated group (24 mg/kg) ($n = 14$, group D), and high-dose salvianolate-treated group (48 mg/kg) ($n = 13$, group E). Group A was injected intraperitoneally with 5% glucose solution once daily for 2 wk. Groups C to E were treated intraperitoneally with different doses of salvianolate dissolved in 5% glucose solution once daily for 2 wk. 40% CCl₄ administration was continued for the 14-wk experimental period. At the end of the experimental period, all rats were anesthetized with 3% chloral hydrate and dissected. Blood samples from the portal vein and intestinal tissue were obtained for further analysis.

Assessment of histological changes in the liver and morphologic indices of ileal mucosa

At the end of the 14-wk experimental period, the abdominal cavity in each rat was opened by a horizontal incision along the mid-section and the liver and intestines were excised. Tissue samples were taken immediately and washed three times with cold physiological saline and fixed in 10% formalin solution. After fixation, tissue specimens were dehydrated and embedded in paraffin. Sections from each sample were cut at a thickness of 4 μ m and stained with hematoxylin and eosin (Olympus BX50; Tokyo, Japan). We evaluated morphologic indices of the ileal mucosa including intestinal villi width, and thickness of the mucosa and intestinal wall using a pathological image analysis system (Leica Qwinv3, Jiangsu, China).

Ultrastructure with transmission electron microscopy

Ileum samples in the five groups were separated and fixed immediately with 2% glutaraldehyde, post-fixed with 1% osmium tetroxide, and embedded in resin (EM-bed 812 Embedding Kit, Electron Microscopy Sciences Company, United States). Ultrathin sections were cut and stained with uranyl acetate and lead citrate. Samples were examined using a transmission electron microscope (Philips Tecnai 12, Holland) and analyzed by an electron microscope image analyzer (Erlangshen ES500W, Holland).

Immunohistochemistry

Immunohistochemical analysis was performed to study ZO-1 expression in the intestinal mucous membranes. In brief, slides were baked at 55 °C for 2 h, deparaf-

finized with xylene, and rehydrated. The sections were submerged in EDTA antigenic retrieval buffer and microwaved for antigenic retrieval, after which they were treated with 3% hydrogen peroxide in methanol to quench endogenous peroxidase activity. They were then incubated with 1% bovine serum albumin to block nonspecific binding. Sections were incubated with mouse anti-ZO-1 antibodies (Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China) overnight at 4 °C. Normal goat serum was used as a negative control. After washing, tissue sections were treated with secondary antibody. Tissue sections were then counterstained with hematoxylin, dehydrated and mounted. The cytoplasm and stroma containing ZO-1 were stained as the buffy coat. The degree of immunostaining was reviewed and scored independently by two observers based on the proportion of positively stained epithelium and intensity of staining^[23-25]. The proportion of ZO-1 expression-positive areas was scored as follows: 0 ($\leq 5\%$ positive area expression), 1 (6%-25% positive area expression), 2 (26%-50% positive area expression), and 3 ($> 51\%$ positive area expression). Staining intensity was graded according to the following criteria: 0 (no staining), 1 (weak staining, light yellow), 2 (moderate staining, yellow-brown), and 3 (strong staining, brown). The staining index was calculated as the product of the staining intensity score and the proportion of positive cells. Using this method of assessment, we evaluated ZO-1 expression in the intestinal mucous membranes by determining the staining index with scores of 0, 1, 2, 3, 4, 6, or 9. The cutoff value for high and low expression levels was chosen based on a measure of heterogeneity using the log-rank test with respect to overall survival. An optimal cutoff value was identified as follows: a staining index score of ≥ 6 was used to define high ZO-1 expression and a staining index score of < 6 was used to indicate low ZO-1 expression.

Statistical analysis

Statistical analysis was performed using SPSS version 13.0 (Chicago, IL, United States). Results of the mucosal morphologic indices were assessed by analysis of variance. Data were expressed as the means \pm SD. The expression rate of ZO-1 protein was compared using the χ^2 test. A P value of < 0.05 was considered statistically significant.

RESULTS

Liver histological evaluation

In the untreated group, hepatocytes showed a disordered arrangement, fatty degeneration was extensive, and swelling was obvious. Spotty necrosis and eosinophilic bodies appeared in the hepatic tissue. Disorganized lobules were divided by collagen fibers. Central veins disappeared and pseudolobules proliferated in the hepatic tissue. Abundant lymphocytes infiltrated the fibrous tissue with proliferation of bile ducts (Figure 1A). Collagen fibers gradually decreased and damaged hepatic lobules were partly repaired by salvianolate treatment. Fatty degeneration of

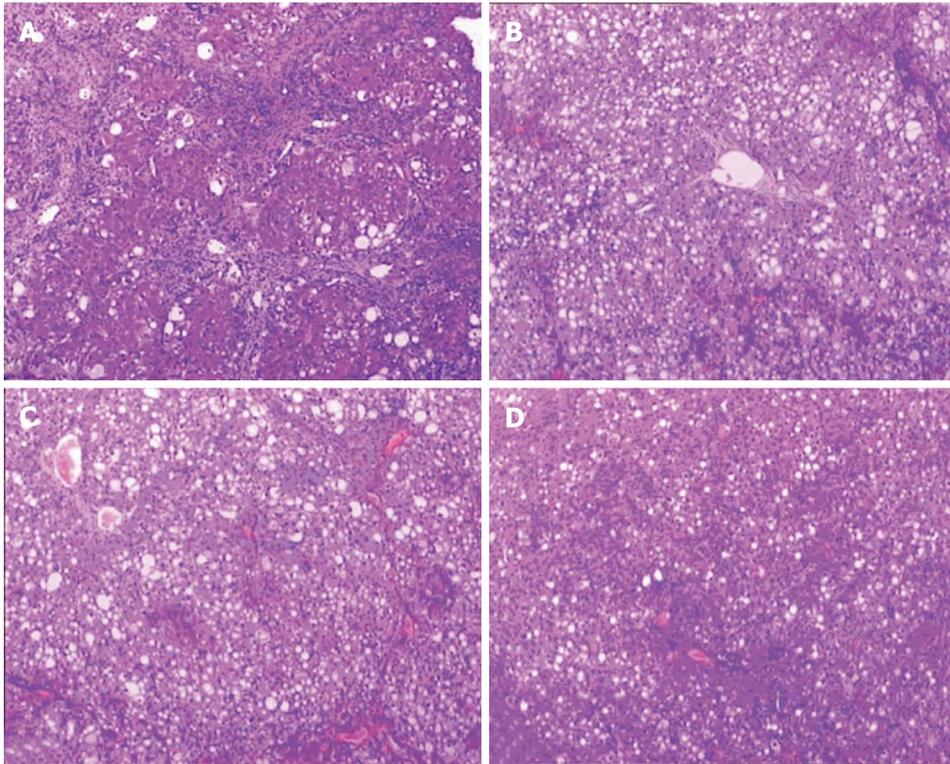


Figure 1 Liver histopathology in cirrhotic rats. A: Untreated group; B: Low-dose salvianolate-treated group; C: Medium-dose salvianolate-treated group; D: High-dose salvianolate-treated group (hematoxylin and eosin staining, × 200).

Table 1 Microscopic evaluation scores of mucosa in the five groups (mean ± SD, μm)

Group	n	Villi height	Villi width	Mucosa thickness	Intestinal wall thickness
A	10	221.82 ± 37.97 ^b	42.08 ± 8.74	260.55 ± 16.57 ^b	337.49 ± 36.92 ^b
B	14	99.14 ± 14.57	38.84 ± 11.08	131.43 ± 18.83	165.93 ± 23.02
C	14	126.43 ± 22.12 ^b	40.28 ± 8.98	179.02 ± 26.99 ^b	226.37 ± 40.66 ^b
D	14	128.71 ± 20.33 ^b	40.59 ± 11.70	181.10 ± 30.71 ^b	229.95 ± 42.08 ^b
E	13	147.10 ± 19.48 ^b	41.32 ± 6.17	230.28 ± 20.67 ^b	278.94 ± 39.94 ^b

^bP < 0.01 vs group B.

hepatocytes was reduced and inflammatory cell infiltration decreased in the fibrous tissue, especially in the high-dose salvianolate group (Figure 1B-D).

Morphologic indices of ileal mucosa

Histological changes observed in ileal tissue under light microscopy were previously published^[22]. In that study, we found that salvianolate ameliorated injury to the intestinal mucosa. In the present study, we measured intestinal villi width and thickness of the mucosa and intestinal wall using a pathological image analysis system. There were no differences in intestinal villi width among the five groups (P > 0.05). Villi height was significantly shorter in group B than in groups C, D, E and the normal group (99.14 ± 14.57 μm vs 126.43 ± 22.12 μm, 128.71 ± 20.33 μm, 147.10 ± 19.48 μm and 221.82 ± 37.97 μm, respectively; P < 0.01). Mucosal thickness was significantly less in the untreated group than in groups C, D and E (131.43 ±

18.83 μm vs 179.02 ± 26.99 μm, 181.10 ± 30.71 μm and 230.28 ± 20.67 μm, respectively; P < 0.01). The intestinal walls were significantly thicker in groups C, D and E compared with group B (226.37 ± 40.66 μm, 229.95 ± 42.08 μm and 278.94 ± 39.94 μm vs 165.93 ± 23.02 μm, respectively; P < 0.01) (Table 1). These results show that the intestinal mucosa was gradually thickened and repaired by salvianolate treatment.

Ultrastructural morphology of intestinal mucosa

In the normal group, regularly aligned microvilli and intact TJs were observed in the intestinal epithelium (Figure 2A). In the untreated group, the number of microvilli decreased and showed irregular lengths and arrangements. The intercellular space between epithelial cells widened. TJs were discontinuous, which indicated disruption in TJ morphology in the untreated group (Figure 2B). In the treated groups, the microvilli in the intestinal epithelium were regular and the TJs were gradually integrated and distinct (Figure 2C-E).

Expression of ZO-1 in ileal epithelial mucosa shown by immunohistochemistry:

Ileal epithelial mucosa from the five groups was assayed for ZO-1 expression using immunohistochemistry. Normal mice showed expression of ZO-1 at the apical pole of epithelial cells^[26] and within the cytoplasm (Figure 3A). Nine (90%) rats in group A showed high expression of ZO-1. Staining of ZO-1 was less in group B; there was almost no expression at the apical pole of epithelial cells and slight expres-

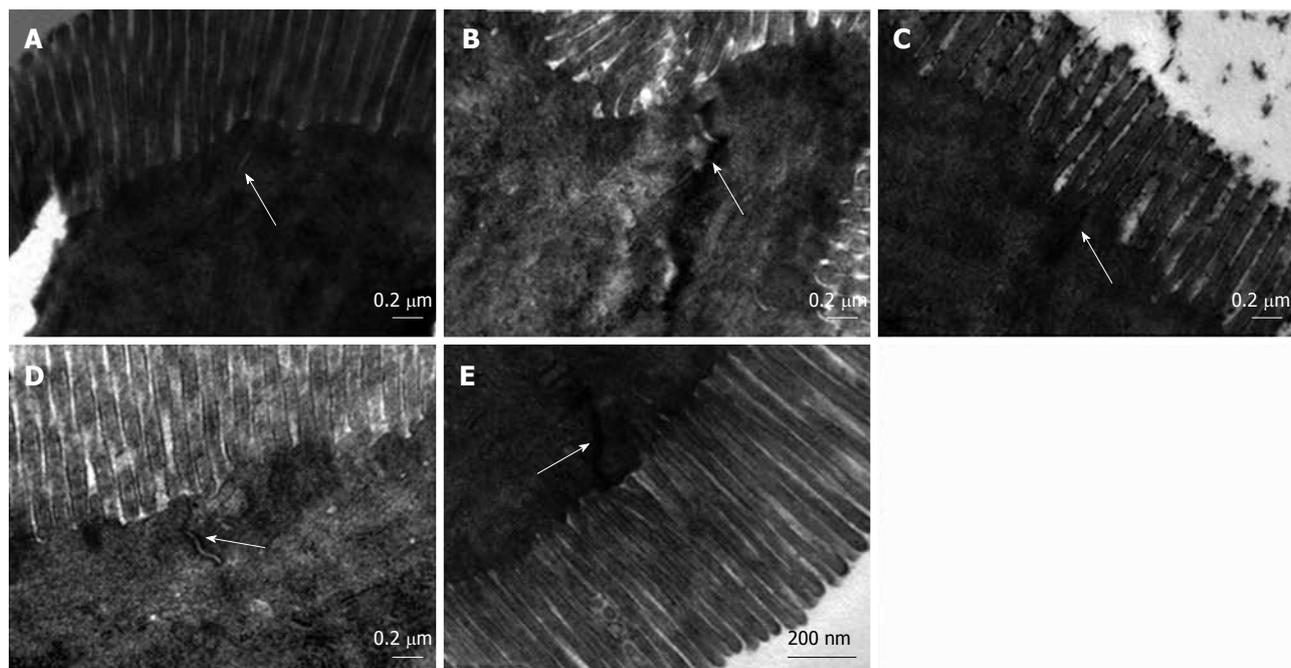


Figure 2 Tight junction structural morphology in ileal mucosa. A: Normal control group; B: Untreated group; C: Low-dose salvianolate-treated group; D: Medium-dose salvianolate-treated group; E: High-dose salvianolate-treated group (transmission electron microscopy, $\times 26\,500$).

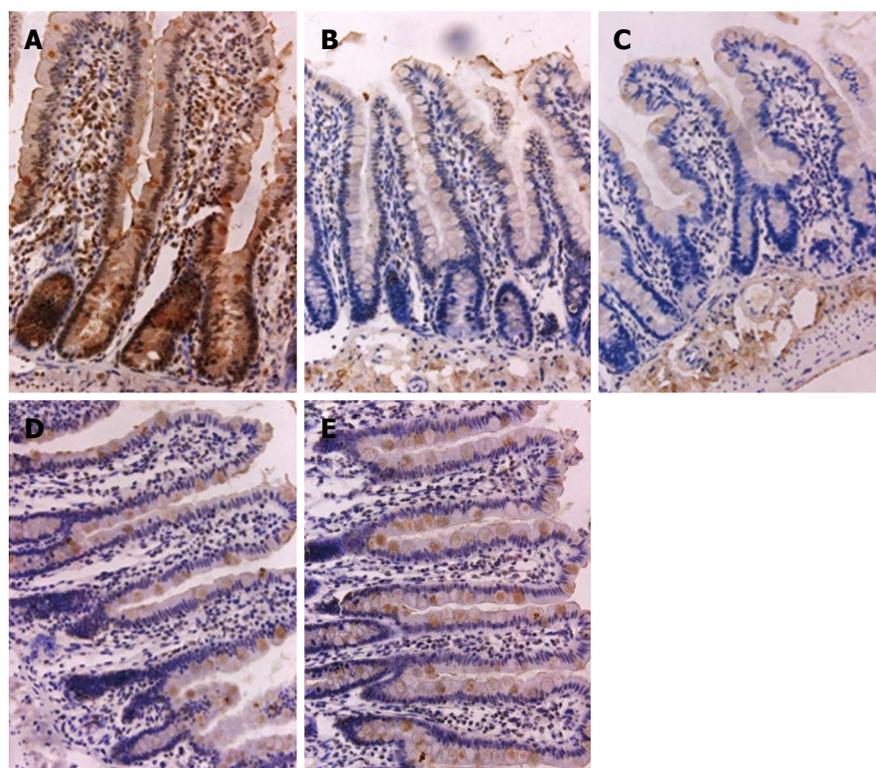


Figure 3 Immunohistochemical staining for zonula occludens protein 1 in ileal mucosa. A: Normal control group; B: Untreated group; C: Low-dose salvianolate-treated group; D: Medium-dose salvianolate-treated group; E: High-dose salvianolate-treated group ($\times 40$).

sion within the cytoplasm (Figure 3B). The expression of ZO-1 gradually increased in epithelial cells following salvianolate treatment. Group B showed a high expression rate of 21.43%, which was significantly lower than that in group A (21.43% *vs* 90%, $\chi^2 = 10.98$, $P < 0.01$) (Figure 4).

In the treated groups, brown grains again appeared at the apical pole of epithelial cells and within the cytoplasm of epithelial cells (Figure 3C-E). Groups C, D and E showed high ZO-1 expression rates of 57.14%, 64.29% and 76.92%, respectively. The expression rate in group B

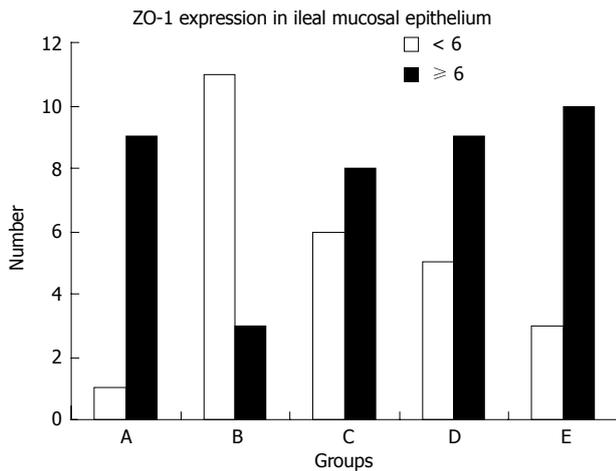


Figure 4 Expression of zonula occludens protein 1 in ileal mucosa. A: Normal control group; B: Untreated group; C: Low-dose salvianolate-treated group; D: Medium-dose salvianolate-treated group; E: High-dose salvianolate-treated group. The staining index was calculated as the product of the staining intensity score and the proportion of positive cells. We evaluated zonula occludens protein 1 (ZO-1) expression in the five groups by determining the staining index with scores of 0, 1, 2, 3, 4, 6, or 9. An optimal cutoff value was identified as follows: a staining index score of ≥ 6 was used to define high ZO-1 expression and a staining index score of < 3 was used to indicate low ZO-1 expression.

was significantly lower than that in groups D (21.43% *vs* 64.29%, $\chi^2 = 5.25$, $P < 0.05$) and E (21.43% *vs* 76.92%, $\chi^2 = 8.315$, $P < 0.01$) (Figure 4).

DISCUSSION

Protection of the intestinal barrier function and decreased bacterial translocation are important in patients with liver cirrhosis^[27]. In our previous study^[22], a significant increase in plasma endotoxins and high mortality were observed in untreated cirrhotic rats. Endotoxemia was significantly reduced and intestinal histopathological changes were improved by administration of salvianolate in cirrhotic rats. We showed that salvianolate has a direct anti-inflammatory effect on the intestine by inhibiting the expression of TNF- α and IL-6 mRNA. These favorable effects of salvianolate on the intestine can be partially explained by direct protection of the integrity of the mucosal barrier.

In this study, we observed improvement in liver histopathological changes (Figure 1) as well as thickening and gradual repair of the intestinal mucosa with salvianolate treatment (Table 1). To further elucidate the mechanisms of salvianolate in protecting the mucosal barrier in cirrhotic rats, we investigated the effect of salvianolate therapy on ultrastructural analysis using transmission electron microscopy and ZO-1 expression changes using immunocytochemistry.

The physical intestinal barrier comprises an intact layer of epithelial cells, which are tightly connected by a surrounding system of TJ strands that seal the lateral intercellular space^[28]. The intercellular space between

epithelial and endothelial cells is bridged by a set of specialized structures: TJs, or ZO; zonula adherens (ZA); desmosomes; and gap junctions. The TJ is an essential component of this barrier. The junctional complexes of the plasma membrane are not simply epithelial barriers in paracellular transport or barriers preventing diffusion across the plasma membrane, but also contain proteins involved in signal transduction and maintenance of the physiological epithelial cell state^[29,30]. Increased permeability is caused by disruption of intercellular TJs in the intestine and plays an important role in the pathogenesis of chronic liver disease^[31]. Previous studies have shown that impaired intestinal permeability may increase the risk of endotoxemia and spontaneous bacterial peritonitis in liver cirrhosis^[14].

The intracellular complex of TJ-associated proteins includes ZO-1, ZO-2, ZO-3, cingulin, 7H6, symplekin and ZA-1^[32]. This complex appears to link TJs to a perijunctional actomyosin ring, which supports and regulates TJ permeability in epithelial cells. ZO-1, one of the plaque proteins, was the first TJ protein to be characterized. It is a 225-kDa membrane-bound protein that localizes to the TJ. It binds the occluding and claudin transmembrane proteins, linking them to cytoskeletal actin^[33]. ZO-1 may be the direct link between actin and the transmembrane proteins; it has its own specific function^[34] and is a particularly important molecule in terms of the formation of TJs^[35]. ZO-1 alterations may contribute to disturbances of the TJ barrier, which lead to enhanced intestinal permeability^[36]. Disruption of this structure could lead to an imbalance in the normal interaction between aggressive factors and mucosal defense, resulting in increased intestinal permeability and bacterial translocation.

Further investigations revealed that mucosal damage was accompanied by ultrastructural changes in TJs. TJ injury is associated not only with increased transcellular permeability, but also with increased paracellular permeability because of the rearrangement of TJ proteins. In this study, we found that the expression of ZO-1 was decreased in the small intestine of untreated cirrhotic rats. Administration of salvianolate may recover the TJ structure and enhance the expression of ZO-1 in the small intestine of cirrhotic rats. This may explain the effect of salvianolate on the epithelial barriers of the small intestine in cirrhotic rats.

In summary, the epithelial barriers of the small intestine were destroyed in cirrhotic rats. These changes may promote bacterial translocation and intestinal endotoxemia. Salvianolate administration caused reduced mucosal damage and maintained the epithelial mucosal barrier function in the small intestine of cirrhotic rats. Furthermore, salvianolate may be a potent traditional Chinese medicinal herb for reducing the incidence of spontaneous bacterial peritonitis and influencing the natural history of liver cirrhosis. These results indicate that salvianolate may be a new therapy for liver cirrhosis.

COMMENTS

Background

In liver cirrhosis, disruption of the intestinal barrier function increases intestinal permeability and bacterial translocation, which contribute to endotoxemia and derangement in liver cirrhosis. Abnormalities of the epithelial mucosal barrier represented by tight junction structure damage play an important role in the pathogenesis of altered intestinal permeability. Improving the epithelial mucosal tight junctions (TJs) and epithelium tight junction proteins is a key goal in preventing intestinal endotoxemia in patients with cirrhosis.

Research frontiers

Recent studies have shown that the physical barrier of the intestinal mucosa plays an important role in intestinal defense mechanisms in hepatic cirrhosis. Previous experiments confirmed that salvianolate can reduce the endotoxin level, ameliorate injury to the intestinal mucosa and inhibit the expression of tumor necrosis factor- α and interleukin-6 mRNA in the small intestine of cirrhotic rats.

Innovations and breakthroughs

Disturbance of the TJs and zonula occludens protein 1 (ZO-1) alterations lead to increased intestinal permeability and bacterial translocation in hepatic cirrhosis. This study showed, for the first time, that salvianolate can restore ultra-structural changes in the intestinal mucosa and significantly increase TJ protein expression in cirrhotic rats. The authors demonstrated that the pharmacological activities of salvianolate represent an important cellular mechanism for preventing intestinal epithelial barrier function damage in liver cirrhosis.

Applications

By demonstrating the effects of salvianolate in maintaining the mucosal physical barrier function in the small intestine of cirrhotic rats, this study provides a new strategy for the treatment of liver cirrhosis. Salvianolate can be applied in clinical practice due to its potential pharmacological activities.

Terminology

Radix *Salvia miltiorrhiza* is a traditional Chinese medicinal herb known as "dan-shen". Salvianolate is a water-soluble phenolic compound isolated from Radix *Salvia miltiorrhiza*.

Peer review

The authors have illustrated that the administration of salvianolate may recover the TJ structure and enhance the expression of ZO-1 in the small intestine of cirrhotic rats. This topic is of significant clinical importance and the results provide a new strategy for the treatment of spontaneous bacterial peritonitis.

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Hepatic focal nodular hyperplasia in children: Imaging features on multi-slice computed tomography

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Abstract

AIM: To retrospectively analyze the imaging features of hepatic focal nodular hyperplasia (FNH) in children on dynamic contrast-enhanced multi-slice computed tomography (MSCT) and computed tomography angiography (CTA) images.

METHODS: From September 1999 to April 2012, a total of 218 cases of hepatic FNH were confirmed by either surgical resection or biopsy in the Sun Yat-sen Memorial Hospital of Sun Yat-sen University and the Cancer center of Sun Yat-sen University, including 12 cases (5.5%) of FNH in children (age \leq 18 years old). All the 12 pediatric patients underwent MSCT. We retrospectively analyzed the imaging features of FNH le-

sions, including the number, location, size, margin, density of FNH demonstrated on pre-contrast and contrast-enhanced computed tomography (CT) scanning, central scar, fibrous septa, pseudocapsule, the morphology of the feeding arteries and the presence of draining vessels (portal vein or hepatic vein).

RESULTS: All the 12 pediatric cases of FNH had solitary lesion. The maximum diameter of the lesions was 4.0-12.9 cm, with an average diameter of 5.5 ± 2.5 cm. The majority of the FNH lesions (10/12, 83.3%) had well-defined margins. Central scar (10/12, 83.3%) and fibrous septa (11/12, 91.7%) were commonly found in children with FNH. Central scar was either isodense ($n = 7$) or hypodense ($n = 3$) on pre-contrast CT images and showed progressive enhancement in 8 cases in the equilibrium phase. Fibrous septa were linear hypodense areas in the arterial phase and isodense in the portal and equilibrium phases. Pseudocapsule was very rare (1/12, 8.3%) in pediatric FNH. With the exception of central scars and fibrous septa within the lesions, all 12 cases of pediatric FNH were homogeneously enhanced on the contrast-enhanced CT images, significantly hyperdense in the arterial phase (12/12, 100.0%), and isodense in the portal venous phase (7/12, 58.3%) and equilibrium phase (11/12, 91.7%). Central feeding arteries inside the tumors were observed on CTA images for all 12 cases of FNH, whereas no neovascularization of malignant tumors was noted. In 9 cases (75.0%), there was a spoke-wheel shaped centrifugal blood supply inside the tumors. The draining hepatic vein was detected in 8 cases of pediatric FNH. However, the draining vessels in the other 4 cases could not be detected. No associated hepatic adenoma or hemangioma was observed in the livers of the 12 pediatric cases.

CONCLUSION: The characteristic imaging appearances of MSCT and CTA may reflect the pathological and hemodynamic features of pediatric FNH. Dynamic multi-phase MSCT and CTA imaging is an effective method for diagnosing FNH in children.

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Key words: Focal nodular hyperplasia; Liver; Children; Benign hepatic lesions; X-ray; Computed tomography**Peer reviewer:** Dr. Koike Naoto, Department of Surgery, Seirei Sakura Citizen Hospital, 2-36-2 Ebaradai, Chiba 2858765, Japan

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INTRODUCTION

Hepatic focal nodular hyperplasia (FNH) is the second most common benign liver tumor after hemangioma, occurring in approximately 3%-5% of the general population^[1]. The mechanism for the pathogenesis of FNH is unclear, although it may be caused by a proliferative response to local vascular abnormalities^[2]. FNH is most common in adult females, with a female to male ratio of 2-4:1^[1,3], whereas it is rare in children. If a focal hyper-vascular liver lesion is accidentally detected in children during imaging, it is important to differentiate FNH from other malignant lesions to avoid unnecessary surgical resection. Since FNH is a slow growing tumor without malignant transformation and is rarely accompanied by the complications of hemorrhage or rupture, conservative "wait and see" strategy is recommended for asymptomatic children^[4]. Surgical treatment would be considered only for symptomatic pediatric patients, patients with increasing size of FNH, or patients in whom malignancy cannot be ruled out confidently^[5-7].

Multi-slice spiral computed tomography (CT) technology has rapidly developed since the clinical application of 16-slice spiral CT was introduced in 2001. Multi-slice computed tomography (MSCT) (16 slices, 64 slices or 128 slices) features a fast scanning speed (the time required for a single tube rotation is sub-second) and is ideal for assessing pediatric patients. With MSCT, dynamic multi-phase scanning is allowed in a very short period of time, and increased detection and characterization of focal liver lesion can be achieved^[2]. In addition, the ability of MSCT to acquire data with "isotropic voxel" makes high-quality two- or three-dimensional vascular reconstruction images possible^[3]. To the best of our knowledge, there are few reports on the imaging features of FNH in children^[4,8,9]. The previous imaging procedure most often utilized for the assessment of the hemodynamic characteristics of FNH was angiography, which is invasive and labor-intensive. Studies that analyze the imaging characteristics of FNH in children on MSCT images and the angioarchitecture of pediatric FNH on computed tomography angiography (CTA) have not reported in the literature. This paper retrospectively reviewed the imaging features

of MSCT in 12 cases of FNH in children.

MATERIALS AND METHODS

Patients

The database of the Sun Yat-sen Memorial Hospital of Sun Yat-sen University and the Cancer Center of Sun Yat-sen University was searched from September 1999 to April 2012, and 218 cases of hepatic FNH confirmed by surgical resection or biopsy were identified. Out of the 218 cases, there were 12 cases (5.5%) of FNH in children (aged ≤ 18 years).

Out of the 12 pediatric cases, 8 were males and 4 were females. The age of the patients ranged from 2 years old to 18 years old, with an average of 12.1 ± 6.4 years. Four pediatric cases presented with abdominal pain, 2 cases presented with palpable abdominal masses, 1 case presented with upper abdominal discomfort, and the other 5 asymptomatic cases were discovered accidentally during imaging check-up for related reasons. In 1 patient, hemangiomas were found in the right shoulder and nasal alae when the diagnosis of hepatic FNH was established. All 12 cases were negative for hepatitis B surface antigen markers and for tumor markers, such as alpha-fetoprotein (AFP), carcinoembryonic antigen 125, and carbohydrate antigen 19-9. All the 12 pediatric patients did not have a history of malignancy or stem cell transplantation.

Imaging protocols

MSCT examinations were performed on either a 64-slice spiral CT scanner (Sensation 64, Siemens Medical Solutions, Erlangen, Germany) or a 16-slice spiral CT scanner (BrillianceTM 16, Philips Medical Systems, Best, The Netherlands) in all 12 pediatric patients. The CT scan parameters were as follows: a tube voltage of 120 kV, an effective tube current of 85-200 mAs, and collimation of $64 \text{ mm} \times 0.6 \text{ mm}$ or $16 \text{ mm} \times 0.75 \text{ mm}$. After pre-contrast scanning images were obtained, multi-phase contrast-enhanced CT scan was performed after intravenous administration of nonionic contrast material of Iopamidol (370 mgI/mL, Bracco, Milan, Italy) or Iopromide (370 mgI/mL, Schering, Erlangen, Germany) by using a power injector at a rate of 1.5-2.5 mL/s, and the dose of the contrast agents was 1.5 mL/kg. Images of the hepatic arterial phase, portal vein phase and equilibrium phase were acquired at 20, 60 and 120 s, respectively. Vascular reconstruction processing was performed using a commercially available workstation and software with interactive maximum-intensity-projection (MIP) and volume-rendering techniques. The slice thickness of reconstructed image for retrospective review was 3.0 mm.

Image analysis

Two experienced abdominal radiologists retrospectively reviewed the imaging features of FNH by consensus, who were unaware of the patient's clinical data and pathological results. The following imaging features were assessed: the number of FNH lesion, location, maximum

Table 1 The multi-slice computed tomography findings of the 12 pediatric focal nodular hyperplasia cases

Case	Sex/age (yr)	Location/size (cm × cm)	Margin	Central scar	Fibrous septa	Pseudocapsule	Precontrast/HAP/PVP/EP	Spoke-wheel pattern	Draining vein
1	M/18	S4/5.2 × 3.7	Well-circumscribed	+	+	-	Hypo/hyper/iso/iso	+	Hepatic vein
2	M/4	S5/4.3 × 3.2	Well-circumscribed	+	+	-	Hypo/hyper/hyper/iso	-	Hepatic vein
3	M/2	S5/5.5 × 5.2	Well-circumscribed	+	+	+	Hypo/hyper/hyper/slight hyper	+	Hepatic vein
4	F/14	S1/4.3 × 3.0	Well-circumscribed	-	+	-	Hypo/hyper/iso/iso	+	Not identified
5	M/16	S5,S6/12.9 × 10.1	Well-circumscribed	+	+	-	Hypo/hyper/hyper/iso	+	Hepatic vein
6	F/4	S4/4.5 × 3.7	Well-circumscribed	+	+	-	Slight hypo/hyper/hyper/iso	+	Hepatic vein
7	M/16	S6,S7/4.1 × 3.2	Well-circumscribed	-	-	-	Slight hypo/hyper/iso/iso	-	Not identified
8	F/16	S4/5.5 × 3.8	Well-circumscribed	+	+	-	Slight hypo/hyper/iso/iso	+	Hepatic vein
9	M/16	S6/6.5 × 5.2	Ill-defined margin	+	+	-	Hypo/hyper/iso/iso	+	Hepatic vein
10	F/17	S6/4.2 × 3.6	Ill-defined margin	+	+	-	Hypo/hyper/iso/iso	+	Not identified
11	M/18	S7/4.0 × 4.0	Well-circumscribed	+	+	-	Hypo/hyper/hyper/iso	+	Not identified
12	M/4	S8/4.8 × 4.5	Well-circumscribed	+	+	-	Slight hypo/hyper/iso/iso	-	Hepatic vein

HAP: Hepatic arterial phase; PVP: Portal venous phase; EP: Equilibrium phase; M: Male; F: Female.

axial diameter, margin, density of the lesions on the pre-contrast images and contrast-enhanced CT images, central scar, fibrous septa, pseudocapsule, the morphology of the feeding artery, and the presence of draining vessels (portal vein or hepatic vein). Other associated lesions in the liver were also recorded, such as hepatic adenomas and hemangiomas. Compared to the surrounding liver parenchyma, the density of FNH was classified as hypo-, iso-, or hyperdense on CT images. Homogeneous enhancement was considered if consistent enhancement was observed in all parts of the lesions, with the exception of the central scars and fibrous septa. The central scar was defined as the area in or near the center of the lesion that showed a significantly different density on pre-contrast or contrast enhanced CT images when compared to the surrounding component of the lesion. Fibrous septa were defined as the linear structures that radiated from the center to the periphery of the lesions.

RESULTS

The 12 cases of FNH were solitary lesions with a maximum lesion diameter of 4.0-12.9 cm and an average maximum diameter of 5.5 ± 2.5 cm. Out of the 12 cases of FNH, 2 cases (cases 3 and 5) had an exophytic growth. No hemorrhage, necrosis or calcification was observed in the FNH lesions. The detailed MSCT findings of the 12 FNH cases are listed in Table 1. Most FNH (10/12, 83.3%) had a well-defined margin. Central scar (10/12, 83.3%) and fibrous septa (11/12, 91.7%) were commonly detected in the pediatric cases of FNH (Figures 1-3). The central scar was found to be isodense ($n = 7$) or hypodense ($n = 3$) on pre-contrast CT images and was

hypodense in the arterial phase. The central scar showed progressive enhancement, and became isodense in the portal vein phase ($n = 2$) and in the equilibrium phase ($n = 8$). However, the central scar was still hypodense in other two cases in the equilibrium phase. Fibrous septa were isodense on pre-contrast CT images, hypodense in the arterial phase images, and isodense in the portal vein phase and equilibrium phase images. Pseudocapsule was very rare in the cases of pediatric FNH (1/12, 8.3%) and was confirmed pathologically as the surrounding displaced veins, showing a hyperdense rim enhancement in the equilibrium phase.

All 12 cases of FNH were hypodense on pre-contrast scans, and were homogeneously enhanced in all phases of the enhanced scans with the exception of the central scars and fibrous septa. FNH was significantly hyperdense in the arterial phase (12/12, 100%), and most of them were isodense in the portal vein phase (7/12, 58.3%) and equilibrium phase (11/12, 91.7%) (Figures 1A-D, 2A-D and 3A-D). The central feeding arteries in the FNH lesions were observed on CTA images in the arterial phase in all 12 cases of pediatric FNH (Figures 1B, 2B and 3C). However, no neovascularization of malignant tumors was observed. In 9 cases (75.0%), spoke-wheel shaped angioarchitecture of FNH was observed, with the feeding vessels radiating peripherally from the center and tapering gradually, suggesting a centrifugal blood supply of the tumors (Figures 1E, 2E and 3F). In 8 cases of FNH, the draining vessels of FNH entered the hepatic vein instead of the portal vein (Figures 1F and 2F). The draining vessels of the other 4 cases of FNH were not observed on CTA images in the portal vein phase or equilibrium phase.

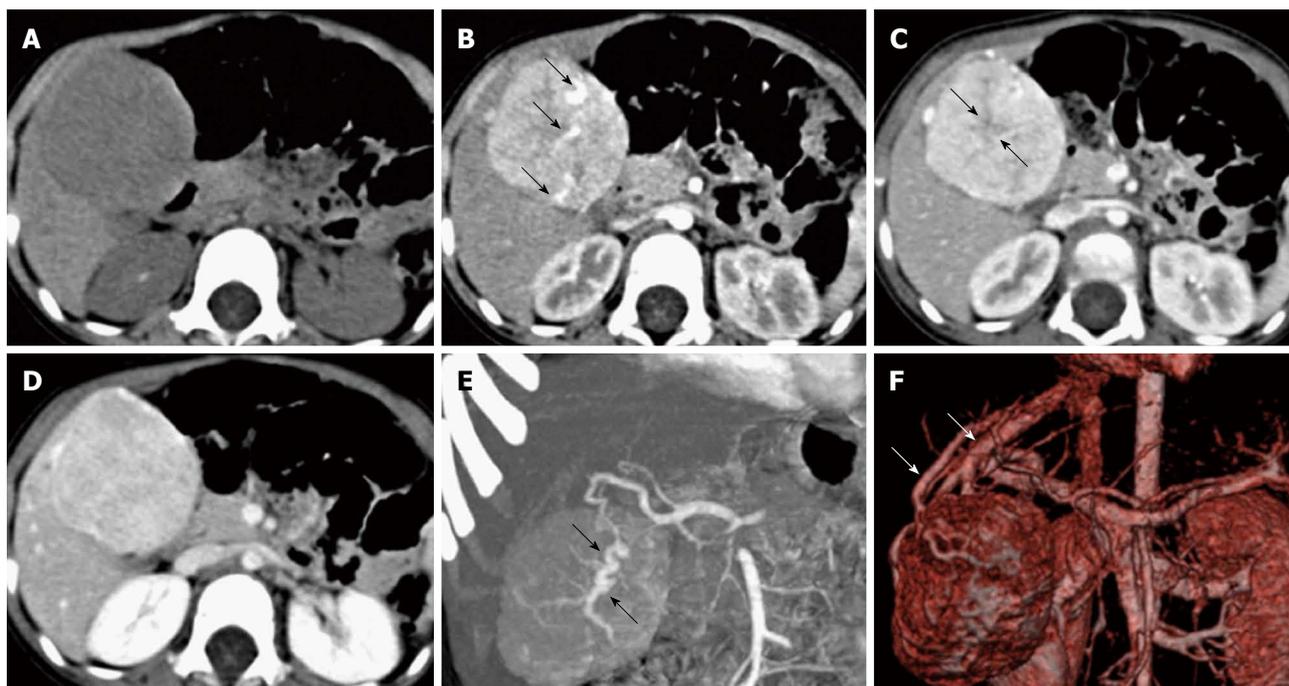


Figure 1 Focal nodular hyperplasia in a 2-year-old boy. A: The lesion was hypodense on pre-contrast computed tomography scan; B: The lesion was significantly enhanced in the arterial phase with enlarged feeding arteries (arrows); C: The lesion was hyperdense in the portal vein phase with hypodense central scar (arrows) and fibrous septa; D: The lesion was isodense in the equilibrium phase, and the scar shows delayed enhanced; E: Computed tomography angiography (CTA) in the arterial phase showed that the enlarged feeding artery was distorted with a spoke-wheel shaped blood supply (arrows); F: CTA in the portal vein phase showed the draining vessels directly into the hepatic vein (arrows).

No associated hepatic adenoma or hemangioma was observed in the liver of the 12 pediatric cases of FNH. Only 1 case (case 3) was associated with hemangioma in other parts of the body.

DISCUSSION

Pediatric FNH is a rare entity, accounting for approximately 2% of hepatic tumors and 0.02% of all pediatric tumors^[10]. The exact pathogenesis of FNH is unclear, although it might be a proliferative response to intrahepatic vascular malformations^[2]. Many studies have shown that the incidence of FNH increases after chemotherapy or radiotherapy for the treatment of solid tumors in children, suggesting that anti-tumor therapy-induced intrahepatic vascular injury may be risk factor for the development of FNH^[4,11-13]. Current molecular pathological data revealed an activation of the β -catenin pathway, a marked upregulation of angiopoietin-1 and downregulation of angiopoietin-2 in FNH^[14].

Most of the studies show that FNH in children is more common in females^[4,6]. In a large series of 172 cases of pediatric FNH, Lautz *et al*^[6] found that 66% (113/172) of the cases occurred in females. However, the majority of our cases were males, with a male to female ratio of 2:1. The high male to female ratio may be due to the bias inherent in the choice of cases enrolled in our study. All cases enrolled in this study were instances of FNH that had been confirmed by surgical resection or biopsy, and cases that were diagnosed by imaging modali-

ties and close follow-up were not included. There are no specific clinical manifestations for FNH in children. The majority of the cases of FNH are usually discovered accidentally for unrelated reasons, with only 36% of cases showing symptoms^[6]. The common symptoms of pediatric FNH include palpable abdominal mass and abdominal pain. Tumor rupture and hemorrhage are rare^[4]. Laboratory test results often do not show clinical significance, and tumor markers such as AFP are usually in normal range^[4].

After review the clinical features of 172 cases of pediatric FNH, Lautz *et al*^[6] found an interesting result: pediatric FNH patients with a history of malignancy were significantly less likely to be symptomatic (12% *vs* 45%, $P < 0.0001$), were much smaller in size (2.8 ± 2.2 cm *vs* 8.0 ± 3.3 cm, $P < 0.0001$), and fewer patients required resection of the lesions (10% *vs* 78%, $P < 0.0001$) as compared with patients without a malignancy history. The pediatric FNH patients with a history of malignancy were more likely to have multiple FNH nodules and less likely to have central scars. In contrast, pediatric patients without a history of malignant lesions generally had larger FNH nodules and were more likely to have central scars^[15,16]. The 12 patients in this group had no history of malignancy and had the features mentioned above: solitary FNH nodule, relatively large size (an average of 5.5 ± 2.5 cm), and high proportion of central scars (10/12, 83.3%).

Adult FNH patients may have associated hepatic adenomas (3.6%-6%) and hemangiomas (17%-23%)^[2,3,17].

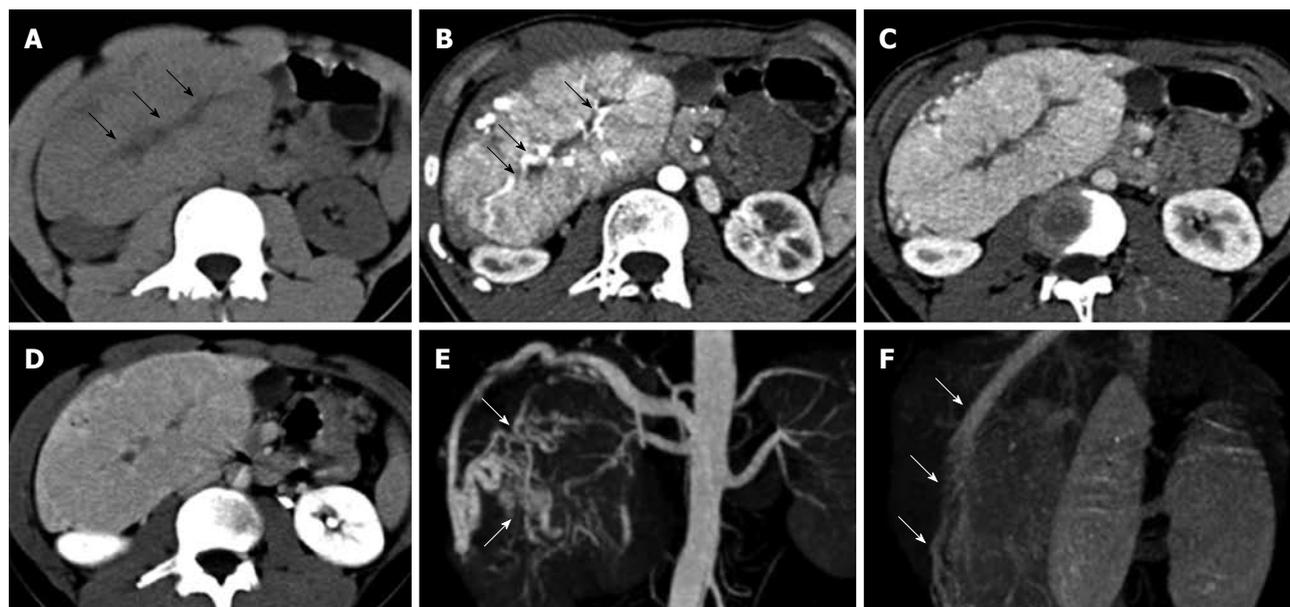


Figure 2 Focal nodular hyperplasia in a 16-year-old boy. A: The lesion was hypodense on pre-contrast computed tomography scan, and central scar with much lower density were identified (arrows); B: The lesion was significantly enhanced in the arterial phase with enlarged feeding arteries penetrating into the central scar (arrows); C: The lesion was slightly hyperdense in the portal vein phase; D: The lesion was isodense in the equilibrium phase with delayed enhancement of the central scar; E: Computed tomography angiography (CTA) in the arterial phase showed that the enlarged feeding arteries were distorted, and exhibited a spoke-wheel shaped blood supply (arrows); F: CTA in the portal vein phase showed the draining hepatic vein (arrows).

However, in our group of pediatric patients, there were no instances of associated hepatic adenomas or hemangiomas. More studies are needed to see whether FNH in adults and children differs in this respect.

The imaging appearances of FNH in pediatric patients on MSCT images reflect its pathological features. A typical case of FNH has the following pathological characteristics: well-differentiated hepatocytes forming nodules subdivided by fibrous septa, proliferation of ductules and malformed blood vessels in the central scars and fibrous septa, and no fibrous capsule^[4]. The fibrous septa are the linear structures that radiate from the central scar to the periphery of the lesions and can sometimes be detected in the absence of a central scar. The hypodense fibrous septa were clearly depicted by CT in the arterial phase, and become isodense with the surrounding component of nodules in the portal vein and equilibrium phases. The sign of fibrous septa is a characteristic finding of FNH, and has not been reported in other tumors of the liver. However, after analyzing 78 cases of FNH using an obsolete spiral CT scanner with thick slice thickness (slice thickness of 5-7 mm), Brancatelli *et al*^[17] found that only 8% of the patients showed this feature. Our results showed that, by using thin-slice MSCT imaging (slice thickness of 3.0 mm), this feature can be commonly observed (11/12, 91.7%). Kamel *et al*^[1] suggest that this finding is best seen on volume-rendered images of the portal venous phase, and frequently detected with fine manipulation of the degree of tissue opacity of the volume-rendered images. However, the subtle changes of fibrous septa on volume-rendered images are difficult to detect in our experience.

The vascular central stellate scar is another patho-

logical characteristic of FNH. The CT findings of the central scar in pediatric FNH are consistent with those of the central scar in adult FNH: compared to the surrounding nodules, the central scar was either hypodense or isodense on pre-contrast CT images, hypodense in the arterial phase (90%), and hypodense (68%) or isodense (22%) in the portal vein phase^[17]. Due to the retention of contrast material within the myxoid matrix of the central scar, the scars show delayed contrast enhancement in the imaging scans at 5-20 min^[17]. The central scar can be seen in 42.1%-50.0% of FNH nodules^[17-19]. However, it was more common in the 12 pediatric cases of FNH in our group (10/12, 83.3%). This discrepancy could be explained by the larger diameter of the lesions (average 5.5 ± 2.5 cm) observed in our group of FNH, since detection of a central scar is clearly related to the size of the FNH (central scars are identified in only 35% of FNH ≤ 3.0 cm but in 65% of FNH > 3.0 cm)^[17]. In addition to FNH, the central scar has also been described in fibrolamellar hepatocellular carcinoma (HCC) and large hemangiomas. A central scar width greater than 2 cm is helpful in differentiating between these three entities, since it is often detected in fibrolamellar HCC and sometimes in large hemangiomas, however never seen in FNH^[20]. The presence of delayed contrast enhancement of the central scar and radiating fibrous septa were not statistically significant for the identification of these three entities^[20].

With the development of CT software and hardware technology, the spatial and temporal resolutions of the images have significantly improved, which enables high-resolution CTA images possible. The post-processed high-resolution CTA images provide unique insights into the angioarchitecture of FNH. Although the hemodynamic

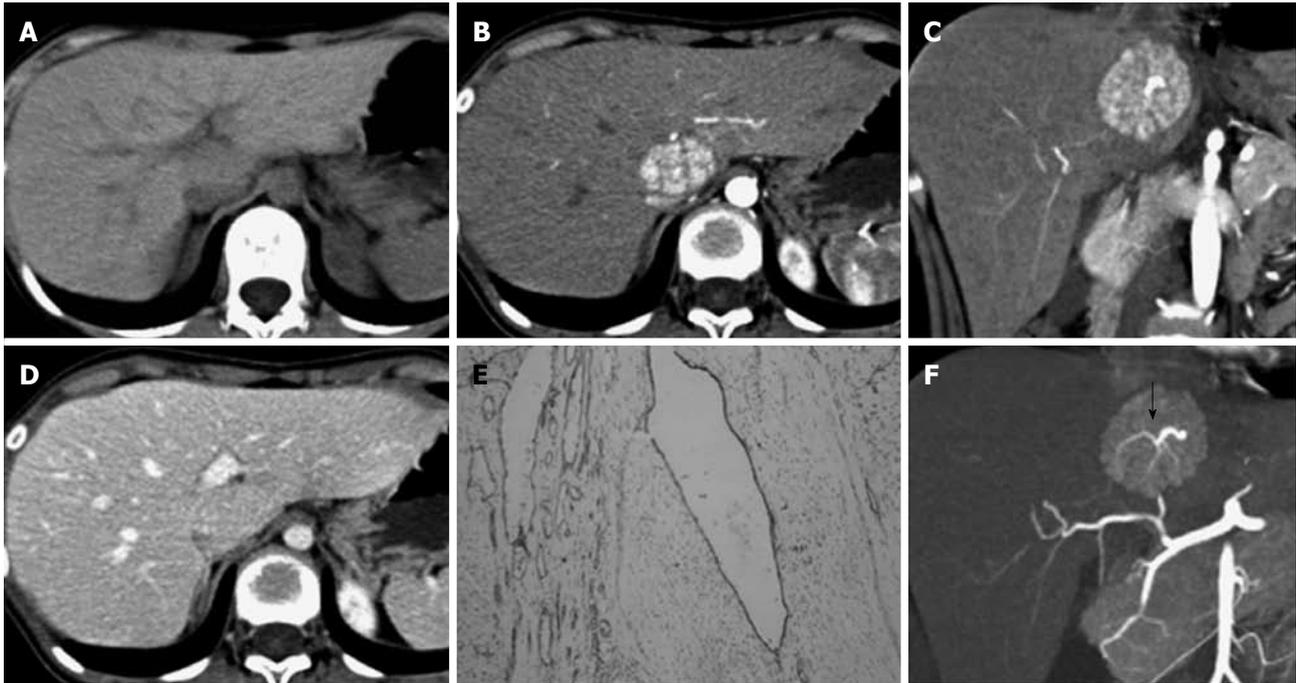


Figure 3 Focal nodular hyperplasia in a 14-year-old girl. A: The lesion was hypodense on pre-contrast computed tomography image; B, C: The lesion was significantly enhanced in the arterial phase with hypodense fibrous septa; D: The lesion was isodense in the portal vein phase; E: Immunohistochemical staining of CD34 revealed the abnormal blood vessels within fibrous septa ($\times 100$); F: Computed tomography angiography in the arterial phase showed that a spoke-wheel shaped blood supply of the focal nodular hyperplasia (arrow).

characteristics of FNH (such as one or more enlarged feeding arteries entering into the center of the tumor and radiating to the periphery, a spoke-wheel shaped centrifugal blood supply) could be observed through the use of the invasive and time-consuming process of transcatheter angiography, it can also be non-invasively depicted on three-dimensional CTA images. Liu *et al.*^[18] compared CTA in 28 cases of FNH and 75 cases of other hypervascular hepatic tumors (hepatocellular carcinoma, hemangioma, and hepatic adenoma), using 16-MSCT with the MIP imaging and volume rendering technology. They found that the centrifugal arterial supply pattern was only seen in FNH, and this finding will assist in differentiating FNH from other hypervascular hepatic tumors. The three-dimensional display of the feeding artery provided by CTA may be valuable in planning for embolization or ligation of vessels in symptomatic pediatric FNH patients.

CTA can clearly show the draining vessels of pediatric FNH. FNH lesion exclusively drains into the hepatic vein branches either directly or *via* perinodular sinusoids^[21,22]. HCC is drained almost always into the portal vein, only 1.8% of HCC draining into the hepatic vein. The identification of the draining blood vessels is therefore a reliable criterion for the differentiation between HCC and FNH^[22].

FNH has no fibrous capsule and shows a well-defined margin on contrast-enhanced CT images in arterial phase. However, pseudocapsules can be detected in 8%-36% of FNH on CT images, which appearing as hypodense rims in the arterial phase and hyperdense rims in the portal vein phase and the delayed phase. This sign is especially

obvious in large lesions, and may be a result of dilated surrounding vessels or sinusoids or compressed liver parenchyma^[1,2,17]. The sign of pseudocapsule is also visible on magnetic resonance imaging (MRI) (9% of FNH in pre-contrast scanning and 18% in contrast enhanced scanning)^[2]. Tumor capsule is a specific sign of HCC and is present in 60%-80% of cases. The capsule of HCC is mainly composed of fibrotic tissue and appears hypointense on both T1W and T2W images, whereas the pseudocapsule of FNH is hyperintense on T2W^[23]. This discrepancy might help to differentiate between these two entities.

The enhancement pattern of pediatric FNH on multi-phase CT images typically shows as early homogeneous enhancement (with the exception of the central scar and fibrous septa) in the arterial phase, hyperdense or isodense in the portal vein phase, and isodense in the equilibrium phase. These findings are consistent with the enhancement pattern of adult FNH reported in the literature^[17].

If all of the imaging characteristics of FNH are identified on CT images, FNH can be diagnosed with confidence. However, approximately 50% of FNH lesions lacking typical imaging manifestations, especially the small lesions, are reported in the literature, such as the absence of central scar, rapid washout of contrast agents in the portal vein phase, absence of delayed enhancement in the central scar, and presence of rim enhancement of the pseudocapsule^[3,4]. In these circumstances, it is necessary to distinguish FNH from other pediatric solid hypervascular tumor, and percutaneous fine needle biopsy

may be suggested when necessary. The malignant hepatic tumors, such as hepatoblastoma and hepatocellular carcinoma, manifest with elevated AFP, and heterogeneous density on CT images due to hemorrhage, necrosis and calcification, which are helpful for distinguishing these malignant tumors from FNH^[4]. MRI examinations with hepatobiliary-specific gadolinium-based contrast agents (specifically gadobenate dimeglumine, gadoxetate disodium or gadoxetic acid) may be valuable for the diagnosis of FNH, particularly on the delayed hepatobiliary phase of imaging where FNHs are usually iso- or hyperintense relative to the liver parenchyma but rarely hypointense, presumably because of the presence of functioning hepatocytes and focal abnormal biliary excretion^[24]. Hepatic adenoma in children is usually accompanied by glycogen storage disease and is prone to having multiple lesions and hemorrhage. Hepatic adenomas usually have no central scar and show heterogeneous density due to intratumoral hemorrhage. In the arterial phase, the CT attenuation values and relative enhancement of the lesion were significantly higher in FNH than in hepatic adenoma ($P < 0.05$). A threshold value of 1.6 for relative enhancement of the lesion in the arterial phase seems to be valuable to distinguish FNH from hepatic adenoma with an accuracy of 96%^[25]. FNH has a centrifugal and spoke-wheel shaped blood supply, whereas hepatic adenoma has centripetal and subcapsular feeding blood supply^[25]. Infantile hemangioendothelioma, or infantile hepatic hemangioma, is a benign vascular tumor that is unique to pediatric patients. Punctate calcification is seen in 50% of these cases^[4]. The enhancement features are similar to those of adult hepatic hemangioma: intense peripheral nodular or corrugated enhancement in the arterial phase, and progressive centripetal fill-in of contrast material in the portal venous and delayed phase, which are different from those of FNH^[4].

In summary, FNH in children manifests as solitary large nodule. The thin-slice MSCT images (slice thickness of 3.0 mm) facilitate in revealing the fibrous septa and central scar within FNH nodules. Multi-phase enhanced scanning has the capacity to demonstrate the enhancement characteristics of pediatric FNH: except for fibrous septa and central scars, FNH is homogeneously enhanced in the arterial phase, and it appears isodense or hyperdense in the portal vein phase and isodense in the equilibrium phase. CTA provides unique insight into the hemodynamic of FNH: the enlarged feeding arteries in the center of the nodules, the spoke-wheel shaped and centrifugal blood supply, and the drainage into the hepatic vein. For asymptomatic pediatric FNH patients with typical MSCT imaging findings, unnecessary surgical resection can be avoided and close follow-up may be suggested. For pediatric FNH patients who cannot definitely be diagnosed by MSCT, further invasive and expensive tests, such as biopsy or surgical resection, are required.

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COMMENTS

Background

Hepatic focal nodular hyperplasia (FNH) is the second most common benign liver tumor after hemangioma in adult, whereas it is rare in children. It is important to differentiate FNH from other malignant lesions to avoid unnecessary surgical resection in pediatric patients. Since FNH is a slow growing tumor without malignant transformation and is rarely accompanied by the complications of hemorrhage or rupture, conservative "wait and see" strategy is recommended for asymptomatic children.

Research frontiers

Multi-slice computed tomography (MSCT) features a fast scanning speed and is ideal for assessing hepatic lesions in pediatric patients. With MSCT, dynamic multi-phase scanning is allowed in a very short period of time, and increased detection and characterization of focal liver lesion can be achieved. In addition, MSCT makes high-quality two- or three-dimensional vascular reconstruction images possible. There are few reports on the imaging features of FNH in children, especially the angioarchitecture of pediatric FNH on computed tomography angiography.

Innovations and breakthroughs

Twelve pediatric patients with FNH were reported in this study, and their MSCT features were reviewed. This study showed that central scar and fibrous septa were commonly found in children with FNH. Central scar was either isodense or hypodense on pre-contrast computed tomography (CT) images and showed progressive enhancement in the equilibrium phase. Fibrous septa were linear hypodense areas in the arterial phase and isodense in the portal and equilibrium phases. With the exception of central scars and fibrous septa within the lesions, pediatric FNH were homogeneously enhanced on the contrast-enhanced CT images, and usually isodense in the portal venous phase and equilibrium phase. Central feeding arteries inside the tumors and a spoke-wheel shaped centrifugal blood supply were usually observed on computed tomography angiography (CTA) images. The characteristic imaging appearances on MSCT and CTA could reflect the pathological and hemodynamic features of pediatric FNH.

Applications

The study results suggest that dynamic multi-phase MSCT and CTA imaging is an effective method for diagnosing FNH in children.

Terminology

MSCT features a fast scanning speed (the time required for a single tube rotation is sub-second), and allows dynamic multi-phase scanning in a very short period of time. The ability of MSCT to acquire data with "isotropic voxel" makes high-quality two- or three-dimensional vascular reconstruction images possible.

Peer review

This manuscript is well written, and the authors retrospectively analyze the imaging features of FNH in children by MSCT and CTA, and suggested that it is an effective method for diagnosing FNH in children.

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Radical treatment of stage IV pancreatic cancer by the combination of cryosurgery and iodine-125 seed implantation

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Abstract

AIM: To investigate the therapeutic effect of radical treatment and palliative treatment in stage IV pancreatic cancer patients.

METHODS: 81 patients were enrolled in the study. Radical treatment was performed on 51 patients, while 30 patients were put under palliative treatment. The procedural safety and interval survival for stage IV pan-

creatic cancer (IS-IV) was assessed by almost 2.5 years of follow-ups. The IS-IV of patients under the two kinds of treatment, and the effects of treatment timing and frequency on IS-IV, were compared.

RESULTS: The IS-IV of patients who received radical treatment was significantly longer than those who received palliative treatment ($P < 0.001$). The IS-IV of patients who received delayed radical or palliative treatment was longer than those who received accordingly timely treatment ($P = 0.0034$ and 0.0415 , respectively). Multiple treatments can play an important role in improving the IS-IV of patients who received radical treatment ($P = 0.0389$), but not for those who received palliative treatment ($P = 0.99$).

CONCLUSION: The effect of radical treatment was significantly more obvious than that of palliative treatment, and multiple radical treatments may contribute more to patients than a single radical treatment.

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Key words: Cryosurgery; Stage IV pancreatic cancer; Iodine-125 seed

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INTRODUCTION

Pancreatic cancer is the fourth-leading cause of cancer-related death in Western societies^[1]. The incidence almost equals the mortality rate, since pancreatic cancer has one of the worst prognoses of all human malignancies. Less than 20% of pancreatic cancers are curable on diagnosis^[2]. Without effective treatment, the overall survival for patients with advanced pancreatic cancer is usually less than 1 year, and for those with metastatic stage IV pancreatic cancer the median survival is only 3–4 mo^[3,4]. Chemotherapy is currently the standard treatment for stage IV pancreatic cancer^[5,6], but it cannot radically eliminate larger tumors. Radiofrequency ablation has been widely used in radical treatment of metastatic pancreatic cancer: in 2007, Spiliotis *et al*^[7] reported the effect of radiofrequency ablation combined with palliative surgery on patients with stage IV pancreatic cancer; in 2010, Zou *et al*^[8] reported the effect of intraoperative radiofrequency ablation combined with iodine-125 seed implantation on patients with stage IV pancreatic cancer. The median overall survival of patients in both reports was 10 mo, but laparotomy is necessary for this technique. Cryosurgery, which is widely accepted as an invasive technique for curing solid tumors, has emerged as a new therapy for pancreatic cancer. The combination of cryosurgery and the implantation of iodine-125 seeds (CandS), which eliminate residual tumors in the treatment of advanced pancreatic cancer, was first reported by us in 2008^[9,10]. In our reports, the median survival was 16.2 mo, with 26 patients (53.1%) surviving for 12 mo or more. Overall 6-, 12-, 24-, and 36-mo survival rates were 94.9%, 63.1%, 22.8%, and 9.5%, respectively.

Here we aimed to compare the effects of radical treatment (CandS for intrapancreatic and extrapancreatic tumors) and palliative treatment (seed implantation for intrapancreatic tumors, CandS for extrapancreatic tumors) on stage IV pancreatic cancer, and assess the influence of treatment timing and frequency on the survival time of patients. To concentrate on the survival time of patients with stage IV cancer, the interval survival of stage IV pancreatic cancer (IS-IV) was used as the main evaluation index.

MATERIALS AND METHODS

Ethics

This study protocol received ethical approval from the Regional Ethics Committee of Guangzhou Fuda Cancer Hospital. Written informed consent was obtained from each participant in accordance with the Declaration of Helsinki.

Patient selection

This was a retrospective study of patients treated for metastatic pancreatic tumors in our tumor hospital from November 2008 through to August 2010. Before hospitalization, 145 patients received a comprehensive

evaluation, with their tumors being considered unresectable. These evaluations were multidisciplinary decisions incorporating a radiologist, a gastrointestinal pancreatic surgeon, and an oncologist in our hospital. Their diagnoses were principally based on computed tomography (CT) imaging and CT-guided needle biopsy to obtain a definitive diagnosis as pancreatic adenocarcinoma histologically. An 18-gauge Tru-Cut (Baxter, Deerfield, IL, United States) biopsy needle was used percutaneously to obtain one to two cores of tissue from the solid pancreatic tumor. Patients received their final treatments in our hospital and almost 2.5 years of follow-ups were reviewed.

A patient was deemed unsuitable for surgery and chemotherapy due to any of the following reasons: (1) multifocal disease; (2) an unresectable primary tumor; (3) the patient refusing surgery and chemotherapy, or seeking further treatment after previous chemotherapy; (4) severe complications (e.g., hypertension, hydrothorax, and ascites); or (5) advanced age. Written informed consent was obtained from all patients. The inclusion criteria were as follows: (1) the Karnofsky performance status (KPS) score was ≥ 70 ; (2) the platelet count was $\geq 80 \times 10^9/L$, white blood cell count was $\geq 3 \times 10^9/L$, neutrophil granulocyte count was $\geq 2 \times 10^9/L$, or hemoglobin ≥ 90 g/L; (3) the prothrombin time international normalized ratio was ≥ 1.5 ; (4) the largest primary or metastatic tumor diameter was < 6 cm, as measured by preoperative CT; (5) the pancreatic tumor did not obviously invade the main pancreatic duct, postcava, duodenum or colon; (6) absence of level 3 hypertension, severe coronary disease, myelosuppression, respiratory system disease, acute and chronic infection; (7) the basic normal liver function and puncture release ascites $< 1L$; and (8) the patient was deemed incapable of cooperating during the procedure.

When the patient had primary or metastatic tumors with a diameter ≥ 6 cm, or obviously invading the main pancreatic duct, postcava, duodenum or colon, we preferentially performed the treatment in other ways^[11,12], and so these patients were not enrolled in this study.

Percutaneous cryosurgery and iodine-125 seed implantation

According to the patients' own selection, 51 patients were under radical treatment and 31 patients received palliative treatment. Their treatments had been reported by us^[9,10]. In the radical treatment group, the CandS treatment of the pancreatic tumor was performed under double row helical CT (Somatom Emotion Duo; Siemens, Germany) and color ultrasound device (ALOKA SSD-5500SA; Aloka, Japan) guidance. Before the cryosurgery, the patients were administered general or local anesthesia, and positioned for an upper abdominal incision. All cryosurgery were performed by Dr. Niu LZ and assistants (Zhou L and Zhang C). Based on the location of the pancreatic tumor, the cryoprobes were inserted percutaneously *via* the retroperitoneal, transhepatic, or transgastric approach. For tumors greater than 3 cm in length, more than two 1.7 mm cryoprobes (CRYO-42; Endocare, Irvine, CA,

United State) were used, in an attempt to avoid puncturing the main pancreatic duct and duodenum. A 1-3 cycle freeze/thaw procedure was used with an argon gas-based cryosurgical unit (Endocare, United State)^[9,10]. The iodine-125 seed (SynCor Pharmaceuticals, Shanghai, China) implantation was performed by PTC needle, either at the time of cryosurgery or after cryosurgery through the percutaneous approach under a 3D treatment planning system. The seeds (activity of a single seed 0.7 mCi, half-life period 1-6 mCi) were implanted at the tumor borderline. The number of seeds deployed depended on the tumor size (matching dose around 120Gy, usually ≤ 20 particles), with the seeds implanted at intervals of 0.5 cm. In the palliative treatment group, the iodine-125 seeds were implanted in all parts of the pancreatic tumor under ultrasound or CT guidance, and the planting density and quantity were both more than the radical treatment group. For extrapancreatic metastases in the two groups, the CandS treatment were performed percutaneously *via* the retroperitoneal or transabdominal approach at the same time, or after the treatment of the intrapancreatic tumor.

Postoperative treatment

Once cryoablation was completed, 1 mL of both fibrinogen and thrombin for each probe were injected into the sheath simultaneously. The patients were then observed in the intensive care unit for at least 6 h, and fasted for at least 24 h. Therapies of anti-infection and inhibition of pancreatic juice secretion were given for a few days. Patients under radical treatment received some special treatment: for patients under the transgastric approach of cryosurgery, antacid and stomach mucosa-protecting drugs were delivered for a few days; for patients under the transhepatic approach of cryosurgery, oppressing hemostasis, bellyband, and liver-protecting drugs were all administered for a few days.

Statistical analysis

Complications were recorded and classified in accordance with the Common Terminology Criteria of Adverse Events v4.0. Local tumor control and IS-IV were also evaluated. Radiographic local tumor control was assessed by image-guided tumor ablation criteria^[13]. A post-operative plain abdominal CT was performed immediately after the removal of the cryoprobes for verification as to whether any major complications, such as pancreatic fistula, bile leakage, or intestinal fistula, had occurred. Abdominal ultrasound was performed at both 1 d and 1 wk after the cryoablation procedure. Follow-up dynamic CT abdominal scans of patients were carried out at 1 mo, and then at 3 to 4 mo intervals. The revised RECIST criteria (version 1.1) were used to assess the basic response of the intrapancreatic and extrapancreatic cancer^[14]. Three diagnostic radiologists (Piao XH, Zhou Q, and Tang J) with 17, 20, and 13 years of clinical experience, respectively, determined whether progression or recurrence had occurred; reviewing CT scans in every

case. Diagnoses were made independently, and the radiologists discussed with each other when the results were different. The IS-IV was calculated from the date when a patient was first diagnosed as suffering stage IV pancreatic cancer, and compared using the Kaplan-Meier test with long-rank analysis. A significant difference was indicated by $P < 0.05$. All analyses were performed using GraphPad Software (San Diego, CA, United States).

RESULTS

Clinical data

Percutaneous cryoablation was performed on 81 patients (42-84 years of age, median age: 65 years; 43 male patients, 38 female patients). The patients of each treatment half were from both China (38 patients) and abroad (43 patients). Two-thirds of patients (54 patients) were treated in our hospital when diagnosed as having stage IV pancreatic cancer; one-third of patients were diagnosed and treated with chemotherapy (27 patients, 95 sessions) and/or radiation (9 patients, 24 sessions) in other hospitals first, and came to our hospital 2-14 mo later for further treatment. Liver metastases (75 lesions) were found in 47 patients, peritoneum and liver metastases (76 lesions) were found in 27 patients, and all other metastases (26 lesions) were found in seven patients. Diabetes (16 patients), hydrothorax/ascites (15 patients), and hypertension/coronary disease (8 patients) were common complications in these patients. Radical treatment was performed on 50 patients (79 sessions), and 31 patients (48 sessions) were put under palliative treatment. The therapeutic option of cryoablation was chosen according to the previously-stated criteria.

The mean intrapancreatic tumor diameter in the radical treatment group was 4.3 ± 0.9 cm (range: 3.2-5.7 cm). For the first treatment of the 50 lesions, 13 were treated by using 2 cryoprobes; 16 by 3 cryoprobes; and 21 by four cryoprobes. The mean intrapancreatic tumor diameter in the palliative treatment group was 4.5 ± 0.8 cm (range: 3.5-5.8 cm). For the first treatment of the 31 lesions, 6 were implanted by using about 20 iodine-125 seeds; 9 by about 30 seeds; and 16 by about 40 seeds.

The mean extrapancreatic tumor diameter in the radical treatment group was 3.5 ± 0.8 cm (range: 2.1-4.5 cm). For the first treatment of the 110 lesions, 46 were treated by using 2 cryoprobes; 43 by 3 cryoprobes; and 21 by four cryoprobes. The mean extrapancreatic tumor diameter in the palliative treatment group was 3.7 ± 0.9 cm (range, 2.3-5.0 cm). For the first treatment of the 67 lesions, 31 were treated by using 2 cryoprobes; 27 by 3 cryoprobes; and 9 by 4 cryoprobes.

Perioperative outcomes

All percutaneous cryoablations of primary and metastatic pancreatic tumors by ultrasound and CT monitoring were performed successfully. No severe complications, such as pancreatic fistula, bile leakage, and intestinal fistula were discovered post-cryoablation. In the radical treat-

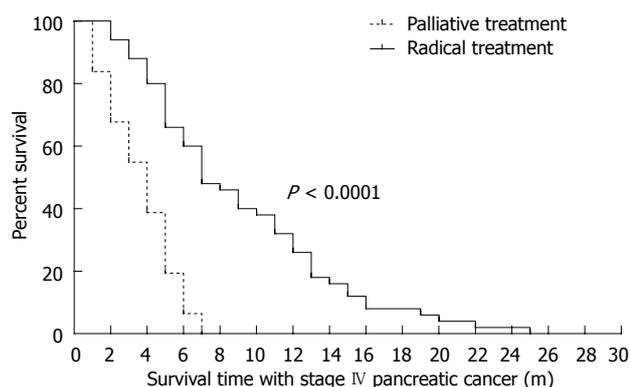


Figure 1 IS-IV of patients under radical or palliative treatment. All 81 patients had suffered from stage IV pancreatic cancer and died before October of 2011. There were 51 patients in the radical treatment group, and 30 patients in the palliative treatment group. The median follow-up period was 8 mo. The IS-IV of patients was accumulated from the early diagnosis of stage IV pancreatic cancer in our own or other hospitals.

ment group, some common adverse effects were found and are shown below. Serum amylase, an important index of acute pancreatitis, increased in 25 sessions (31%) for 16 patients (32%) on the first day after the procedure, unaccompanied by ascites or leukocytosis, but returned to normal levels in the following 5 d after symptomatic treatment. Seven patients (14%) with diabetes after 12 sessions (15%) experienced a rise in fasting blood glucose levels up to 20-25 mmol/L on the first day post-cryoablation, which were well-controlled with insulin injections. A mild decrease in the platelet count occurred after 14 sessions (18%) for 10 patients (20%), which returned to normal within 8-13 d without any treatment. Abdominal distension and nausea occurred after 23 sessions (29%) for 14 patients (28%) on the first day post-cryoablation, and disappeared automatically the following day. Eight patients (16%) in 11 sessions (14%) complained of poor appetite after the cryosurgery and were found to have ascites by ultrasound, which improved the following 3-5 d without any treatment. Eleven patients (22%) in 17 sessions (22%) were found to have abdominal bleeding, 8 patients (16%) of whom developed fever and a mild increase in white blood cells, and neutrophil granulocytes; they improved in the following 7 d after symptomatic treatment. Similar adverse effects were also found in the palliative treatment group, and disappeared in 2 wk after symptomatic treatment. There were no treatment-related deaths or any conversions to chemotherapy.

Within 1 wk after the first CandS treatment, 64 patients (79%) experienced a $\geq 50\%$ reduction in pain score, 57 patients (70%) experienced a 50% decrease in analgesic consumption, and 43 patients (53%) experienced a ≥ 20 increase in KPS score.

Influence of therapies, treatment timing, and treatment frequency on IS-IV of patients

The median follow-up period was 8 mo. Up to the date of the last follow-up of each patient, the median IS-IV of all patients was 7.12 mo (95%CI: 3.79-13.37). The me-

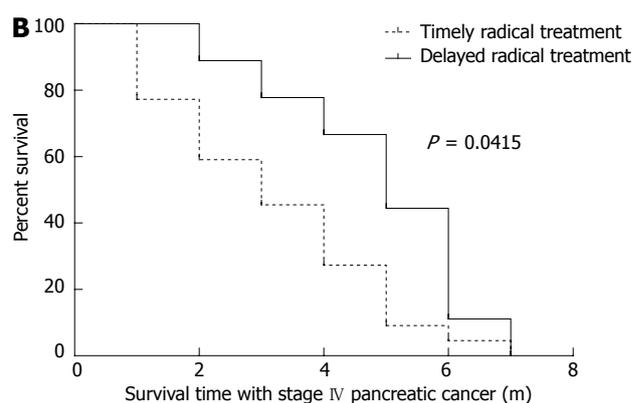
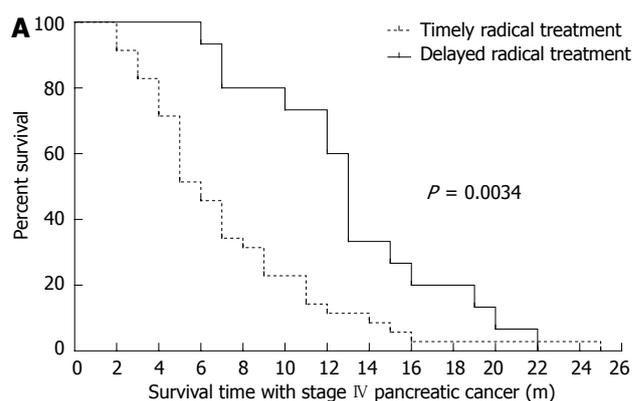


Figure 2 IS-IV of patients under timely or delayed treatment. A: Comparison between patients in the radical treatment group: 32 patients under timely treatment and 18 patients under delayed treatment; B: Comparison between patients in the palliative treatment group: 22 patients under timely treatment and 9 patients under delayed treatment.

dian IS-IV of patients with radical treatment was 8 mo, and those with palliative treatment was 4 mo. The IS-IV of the two groups differed significantly by long-rank test ($P < 0.001$, Figure 1).

Two-third of patients received CandS treatment 2 mo after the diagnosis of stage IV pancreatic cancer, and one-third of patients received CandS treatment 2-14 mo after diagnosis. The influences of treatment timing on IS-IV of patients were detected: for the radical treatment group, the median IS-IV of patients under timely treatment was 6 mo, and 13 mo for those under delayed treatment ($P = 0.0034$, Figure 2A); for the palliative treatment group, the median IS-IV of patients under timely treatment was 3 mo, and 5 mo for those under delayed treatment ($P = 0.0415$, Figure 2B). In the two groups, the patients who received CandS treatment ≥ 2 mo after diagnosis were associated with a longer IS-IV, which was more obvious in the radical treatment group.

According to disease progression, tumor recurrence and individual intents of patients, 31 patients (39%) received repeated CandS treatment when re-examined. For the radical treatment group, the median IS-IV of patients under single treatment was 7 mo, and 11 mo for those under multiple treatments ($P = 0.0389$, Figure 3A); for the palliative treatment group, the median IS-IV of patients under single treatment was 3 mo, and 4 mo for

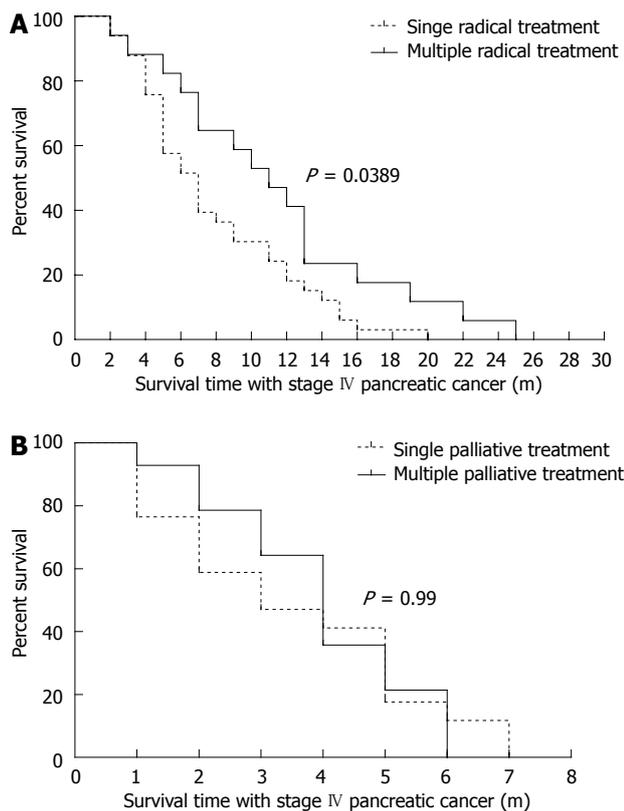


Figure 3 IS-IV of patients under different treatment frequency. A: Comparison among patients in radical treatment group, 17 patients under multiple radical treatment (2 times, by 10 patients; 3 times, by 4 patients; 4, 5 and 9 times, by 1 patient each) and 33 patients were under single radical treatment; B: Comparison among patients in palliative treatment group, 14 patients under multiple palliative treatment (2 times, by 11 patients; 3 times, by 3 patients) and 17 patients under single palliative treatment.

those under multiple treatments ($P = 0.99$, Figure 3B). Only in the radical treatment group did multiple treatments show a significant superiority in prolonging the IS-IV of patients.

DISCUSSION

Cryoablation has been performed for hepatic, renal, breast, and prostate cancer, and it has shown acceptable results. During cryoablation, the formation of an oversized ice ball increases the complication rate and can endanger a patient's life; an undersized ice ball can lead to the recurrence of the tumor at the edge of the frozen area^[15]. As the volumes of primary or metastatic tumors are sometimes large, adhesions to other organs or tissues and invasive growth are often present. Percutaneous cryoablation cannot guarantee complete ablation, and combination with brachytherapy might be a better choice^[16-20]. This combination of minimal invasive therapies (namely CandS treatment) can minimize the damage of radiotherapy and cryoablation, and increase the treatment response^[21,22].

In $\geq 80\%$ of patients with pancreatic cancer, the tumor is unresectable at time of diagnosis. Chemotherapy, palliative surgery, and radiofrequency ablation may be

the best options for patients with metastases. Along with advances in the cryosurgical system and imageology, percutaneous cryosurgery has been increasingly successful in the treatment of pancreatic cancer^[9,10]. This method avoids the risks of laparotomy, decreases the likelihood of complications^[23-25], and improves the quality of life, but the data is still lacking as to whether it can extend the IS-IV of patients. Cryoablation and chemotherapy have different effects on patients with stage IV pancreatic cancer. In 2010, Stathis *et al*^[26] reported that the median survival for patients treated with single-agent 5-FU or gemcitabine were 4.41 and 5.65 mo, respectively. Combined applications of other agents with gemcitabine had been frequently attempted in order to get a better IS-IV, but the statistical indication for such a benefit failed to materialize until now^[4,27-30]. In our study, the IS-IV of patients under radical treatment was significantly longer than for those under palliative treatment ($P < 0.0001$), with a 4 mo extension of median IS-IV (8 mo *vs* 4 mo, respectively). The 1-year survival rate of patients in the radical treatment group was 32%, showing the greater superiority of radical treatment, as this was better than palliative treatment and chemotherapy. Interestingly, patients who delayed CandS treatment were associated with better IS-IV, regardless of radical or palliative treatment. For this reason we re-analyzed the data, and found that all the patients under delayed treatment had received chemotherapy before admission to our hospital, and so chemotherapy may be an important reason for the extension of IS-IV. It seems that if chemotherapy is delivered early in stage IV pancreatic cancer, it will change the systemic disease into local disease, and improve the benefit to survival time of patients. As for the frequency of CandS treatment, multiple treatments only showed a significant advantage in the radical treatment group (median survival: 11 mo *vs* 7 mo, $P = 0.0389$). Therefore, in order to get the best therapeutic effect, early chemotherapy, radical, and multiple treatments are all very important.

With regard to the complications associated with radical treatment, pancreatic fistula, bile leakage, and intestinal fistula were seldom observed in our study, maybe due to the evasion of patients with a high surgical risk when enrolling. Other minor complications associated with radical or palliative treatment were common after cryoablation of the pancreas and liver, including an increase of serum amylase and blood glucose, a decrease in the number of platelets, abdominal distension, ascites, fever, infection, and abdominal bleeding. All complications can be decreased to normal within 2 wk after symptomatic treatment. In effectively reducing tumors and removing obstructions, the physical strength and energy of patients in the two groups improved obviously and pains were reduced significantly. These achievements are inseparable with effective tumor reduction and close postoperative monitoring.

The present study represents an early experience, with a small number of patients. Hence, extrapolation of the results to clinical practice should be performed with cau-

tion. This was not a definitive study for assessing the effects of chemotherapy on IS-IV of patients, the effectiveness of CandS in the treatment of newly diagnosed stage IV pancreatic cancer, or for determining whether CandS treatment was as effective as surgery for pancreatic tumors.

In conclusion, percutaneous CandS treatment may have a useful role in the management of stage IV pancreatic cancer evading vital organs and those < 6 cm in diameter when surgery and chemotherapy are not options. To further increase the IS-IV of patients, close postoperative monitoring and multiple treatments will be needed for recurrent tumors.

COMMENTS

Background

Pancreatic cancer is the fourth leading cause of cancer-related death clinically. Less than 20% of pancreatic cancers are curable on diagnosis. Without effective treatment, the overall survival for patients with advanced pancreatic cancer is usually less than 1 year, and for those with metastatic stage IV pancreatic cancer, the median survival is only 3-4 mo. Treatment effects of radiation and chemotherapy are poor, and radiofrequency ablation can not be applied for patients percutaneously.

Research frontiers

This research concerns the field of ablation for pancreatic cancer. Currently, cryosurgery is the only useful method for *in situ* ablation of pancreatic cancer.

Innovations and breakthroughs

Three methods were used to treat different parts of the pancreatic cancer by percutaneous ablation, which increased the safety and efficiency of cryosurgery for patients. The relationship between treatment timing, treatment frequency, and survival time of patients with metastatic pancreatic cancer were analyzed.

Applications

Depending on the retrospective analysis of radical and palliative treatment on patients with stage IV pancreatic cancer, clinical evidence was provided concerning survival time, which will benefit the future application of this technology.

Terminology

Percutaneous cryosurgery: Under the guidance of B ultrasound or computed tomography (CT), cryoprobes are directly stuck into the tumor through skin, and cryoablation is performed *in situ* by the ultra-low temperature of the probe tip; Seed implantation: under the guidance of B ultrasound or CT, radioactive seeds are implanted into the tumor to act as brachytherapy; IS-IV: since the patients enrolled in this study were all suffered from metastatic tumors (stage IV pancreatic cancer), and many treatments had been carried out in other countries and hospitals before, the ideal evaluating method for survival time for our treatment is interval survival for stage IV pancreatic cancer.

Peer review

This study is the first retrospective analysis for the radical and palliative treatment of patients with stage IV pancreatic cancer, which clarified the benefits of percutaneous cryosurgery for this disease, along with the relationship between treatment timing, treatment frequency, and survival time of patients. These findings can provide important evidence for the clinical treatment of stage IV pancreatic cancer.

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Protective role of metalloproteinase inhibitor (AE-941) on ulcerative colitis in rats

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Abstract

AIM: To evaluate the protective role of AE-941, a matrix metalloproteinase (MMP) inhibitor, on ulcerative colitis (UC) in rats.

METHODS: Sprague Dawley (SD) rats were randomly divided into three groups: a control group, an AE-941 treatment group, and an UC model group. Rats were sacrificed on days 7, 21, or 56 following administration of treatment by enema and the disease activity index (DAI), colonic mucosa damage index (CMDI) and colonic expression of MMP-2 and MMP-9 were assessed.

RESULTS: DAI and CMDI scores in the UC model group increased significantly compared to the control group at all timepoints ($P < 0.001$), and also increased significantly at the 21- and 56-d timepoints compared to the AE-941-treated group (DAI: 21- and 56-d = 2.09

± 0.25 , 1.52 ± 0.30 vs 1.55 ± 0.28 , 0.59 ± 0.19 , respectively, $P = 0.040$ and 0.007 , CMDI: 21- and 56-d = 3.03 ± 0.42 , 1.60 ± 0.35 vs 2.08 ± 0.46 , 0.86 ± 0.37 , respectively, $P = 0.040$ and 0.005). Furthermore, the colonic expression of MMP-2 and MMP-9 in the UC model group increased significantly compared to the control group ($P < 0.001$), and also increased compared to the AE-941-treated group on the 21- and 56-d timepoints (MMP-2: 21- and 56-d = 0.6048 ± 0.0522 , 0.4163 ± 0.0330 vs 0.3983 ± 0.0218 , 0.1093 ± 0.0072 , respectively, $P = 0.010$; MMP-9: 21- and 56-d = 0.6873 ± 0.0472 , 0.4328 ± 0.0257 vs 0.5179 ± 0.0305 , 0.2673 ± 0.0210 , respectively, $P = 0.010$ and 0.040).

CONCLUSION: Expression of MMP-2 and MMP-9 increased significantly in rats with UC. AE-941 can reduce colonic mucosal damage by downregulating the expression of MMP-2 and MMP-9.

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Key words: AE-941; Extracellular matrix; Matrix metalloproteinase-2; Matrix metalloproteinase-9; Ulcerative colitis

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INTRODUCTION

Ulcerative colitis (UC) is a type of nonspecific inflammatory bowel disease for which the etiology and disease mechanism are not completely clear. It has recently

become apparent that the synthetic and degradative imbalance of the colonic extracellular matrix (ECM) may be associated with the progression of UC^[1,2]. The aberrant expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) can cause a synthetic and degradative imbalance of the ECM, which then leads to a series of pathological reactions such as colonic mucosa inflammation, erosion, and ulceration^[3,4]. TIMPs can inhibit the activity of MMPs, which are able to correct MMP-TIMP imbalances. Our previous research indicated that MMPs may be involved in the occurrence and development of UC.

Expression of MMP-2 and MMP-9 can be blocked by AE-941, a natural inhibitor of MMPs, which is a new and effective treatment of malignant tumors^[5]. In this study, we used a rat model of UC and administered AE-941 by gastric lavage. We then observed the therapeutic effect of AE-941. We will discuss the mechanism of AE-941 alleviation of UC to provide a theoretical basis for a novel treatment for UC.

MATERIALS AND METHODS

Materials

Healthy male Sprague Dawley (SD) rats weighing 180-220 g and aged 4-8 wk were supplied by the SPF laboratory animal center of Dalian Medical University. 2,4,6-trinitrobenzenesulfonic acid solution (TNBS) was purchased from Sigma. AE-941, MMP-2 and MMP-9 polyclonal antibodies were supplied by Bioworld Technology. The MaxPoly Plus Anti-Mouse/Rabbit horseradish peroxidase IHC Kit was supplied by the Fujian Maixin Biological Technology Co. Primers, DNA markers (DL 2000), Takara RNA polymerase chain reaction (PCR) kit 3.0 (AMV) kit were from Dalian, Takara Co. Ltd.

Animal treatment

A total of 54 SD rats were randomly divided into three groups: a control group, an AE-941 group, and an UC model group; each group had 18 rats. The SD rat model of UC was established by administering a mixture of TNBS (100 mg/kg) and 50% ethanol (0.25 mL) by enema. The control group was subject to enema and gastric lavage with normal saline. For the AE-941 group, TNBS was administered by enema and AE-941 was administered by gastric lavage (10 mg/kg once a day). The UC model group was subjected to TNBS enema and the gastric lavage was composed of a saline control. Rats from all groups were sacrificed on days 7, 21 and 56. Six rats per group were sacrificed each day, after the enema and colonic tissue 2.0-10.0 cm from the anus was collected for reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemical analysis.

Disease activity index and colonic mucosa damage index scoring

The rats were weighed and checked for behavior, stool

Table 1 Oligonucleotide of primers of target genes

mRNA species		mRNA	PCR product (bp)
MMP-2	Sense	5'-ACCATCGCCCCATCATCAAGT-3'	348
	Antisense	5'-CGAGCAAAAGCATCATCCAC-3'	
MMP-9	Sense	5'-CCCTGCGTATTCCATTCAT-3'	600
	Antisense	5'-ACCCCACTTCTTGTGACGGTC-3'	
β-actin	Sense	5'-AAGCCTAAGGCCAACCCGTGAAA AG-3'	241
	Antisense	5'-TCAATGAGGTAGTCTGTGACGGT-3'	

MMP: Metalloproteinase; PCR: Polymerase chain reaction.

consistency and the presence of gross blood in the stools every day. The scores were assigned as follows: percentage of body weight reduction (0, no change; 1, 1%-5%; 2, 6%-10%; 3, 11%-15%; 4, > 15%); stool consistency (0, normal; 2, loose; 4, diarrhea); and the presence of fecal blood (0, normal; 2, positive occult blood test; 4, visible bleeding)^[6]. The disease activity index (DAI) was calculated as the sum of these scores.

Rats were sacrificed at the timepoints indicated and the entire colon was excised from the cecum to the anus and opened longitudinally. Macroscopic damage was evaluated using a validated [colonic mucosa damage index (CMDI)] scoring system with slight modifications^[7]. The numerical rating score was as follows: 0, no inflammation; 1, local hyperemia without ulcers, and/or stool consistency; 2, ulceration without hyperemia; 3, ulceration and adhesions at one site; 4, two or more sites of inflammation and ulceration extending > 1 cm; 5, ulceration extending more than 2 cm.

MMP-2 and MMP-9 mRNA expression

mRNA was extracted from colonic tissue samples using Trizol according to the manufacturer's protocols (Invitrogen) and RT-PCR was performed according to the instructions of the Takara RNA PCR kit 3.0 (AMV). An equal amount of cDNA from each sample was amplified using primers specific to each gene (Table 1). DNA amplification was done using a thermocycler under the following conditions: for MMP-2, 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 45 s, and extension at 72 °C for 90 s; for MMP-9, 35 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 30s, and extension at 72 °C for 90 s; for β-actin, 35 cycles of denaturation at 94 °C for 30s, annealing at 62 °C for 30s, and extension at 72 °C for 90s. RT-PCR products were measured by photodensitometry using a gel image analysis system after agarose gel electrophoresis and ethidium bromide staining.

Measurement of MMP-2 and MMP-9 protein expression

Immunohistochemistry was performed according to the Max Vision kit protocol. Image analysis software (Image-pro plus 6.0) was used to measure the light density

Table 2 Disease activity index and colonic mucosa damage index of three groups on different time (mean \pm SD)

Time	Control group		Model group		AE-941 group	
	DAI	CMDI	DAI	CMDI	DAI	CMDI
7th day	0.06 \pm 0.14	0.00 \pm 0.00	2.89 \pm 0.00 ^a	4.83 \pm 0.55 ^a	2.76 \pm 0.32	4.74 \pm 0.39
21st day	0.00 \pm 0.00	0.00 \pm 0.00	2.09 \pm 0.25 ^a	3.03 \pm 0.42 ^a	1.55 \pm 0.28 ^c	2.08 \pm 0.46 ^c
56th day	0.00 \pm 0.00	0.00 \pm 0.00	1.52 \pm 0.30 ^a	1.60 \pm 0.35 ^a	0.59 \pm 0.19 ^c	0.86 \pm 0.37 ^c

^a*P* < 0.05 vs control group; ^c*P* < 0.05 vs model group. DAI: Disease activity index; CMDI: Colonic mucosa damage index.

Table 3 Colonic mucosal mRNA expression of matrix metalloproteinase-2 and matrix metalloproteinase-9 (mean \pm SD)

Time	Control group		Model group		AE-941 group	
	MMP-2	MMP-9	MMP-2	MMP-9	MMP-2	MMP-9
7th day	0.0062 \pm 0.0017	0.0068 \pm 0.0096	0.8563 \pm 0.1132 ^a	0.9936 \pm 0.1187 ^a	0.7509 \pm 0.0693	0.8375 \pm 0.1054
21st day	0.0056 \pm 0.0012	0.0071 \pm 0.0134	0.6048 \pm 0.0522 ^{a,c}	0.6873 \pm 0.0472 ^{a,c}	0.3983 \pm 0.0218 ^c	0.5179 \pm 0.0305 ^c
56th day	0.0058 \pm 0.0015	0.0069 \pm 0.0011	0.4163 \pm 0.0330 ^{a,b}	0.4328 \pm 0.0257 ^{a,b}	0.1093 \pm 0.0072 ^c	0.2673 \pm 0.0210 ^c

^a*P* < 0.05 vs control group; ^c*P* < 0.05 vs model group; ^b*P* < 0.05 vs day 7 timepoint; ^b*P* < 0.05 vs day 21 timepoint. MMP: Matrix metalloproteinase.

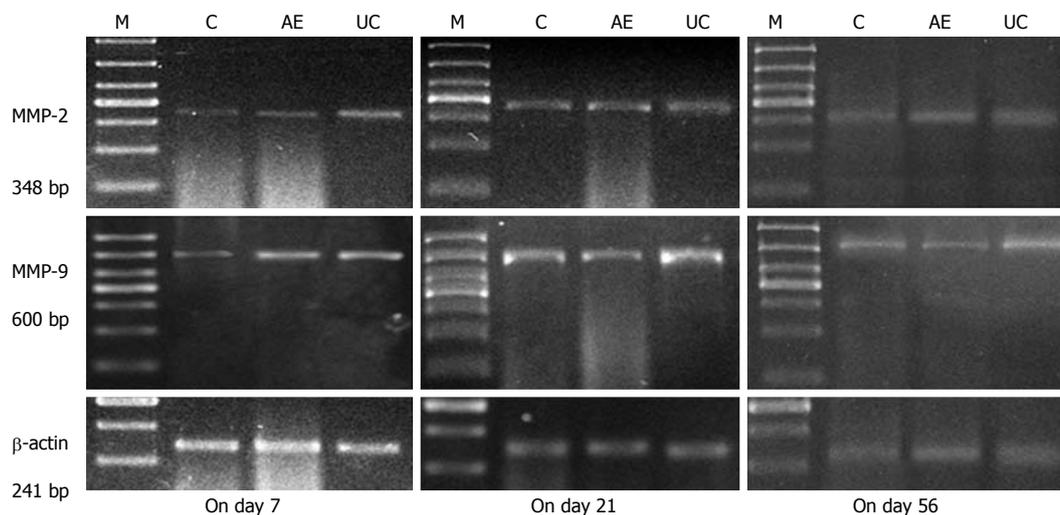


Figure 1 The colonic mucosal mRNA expression of matrix metalloproteinase-2 and matrix metalloproteinase-9 in control, AE-941 and ulcerative colitis model groups at different timepoints. Expression of matrix metalloproteinases (MMP)-2 and MMP-9 in the colonic mucosa was significantly higher in the ulcerative colitis model group compared to the control group (*P* < 0.05). On days 21 and 56, the two index values in the AE-941 treatment group were significantly less than that of the model group (*P* < 0.05). M: Marker; C: Control group; AE: AE-941 group; UC: UC model group.

of positive control cells in which the cytoplasm was tan-yellow or brown after 3,3'-diaminobenzidine staining. For each section, the positive integrated optical density (IOD) and total area of five representative visual fields without overlap were observed under high-power microscope (\times 400). The ratio of IOD and total area represents the mean value of optical density, with a higher ratio indicating a higher level of protein expression.

Statistical analysis

All data were analyzed using the SPSS statistical package version 11.5. Data showed a normal distribution and are expressed as means \pm SD. The responses of different experimental groups were analyzed using one-way analysis of variance. A *P*-value of < 0.05 indicates a statisti-

cally significant difference between different groups.

RESULTS

Results of disease activity index and colonic mucosa damage index

The DAI and CMDI of the UC model group was significantly higher than that of the control group (*P* < 0.05) at all timepoints. Compared with the UC model group, the DAI and CMDI values of the AE-941 treatment group were reduced on day 7, although not significantly (*P* > 0.05). On days 21 and 56 after treatment, the DAI and CMDI scores of the AE-941 treatment group were significantly lower than that of the UC model group (*P* < 0.05; Table 2).

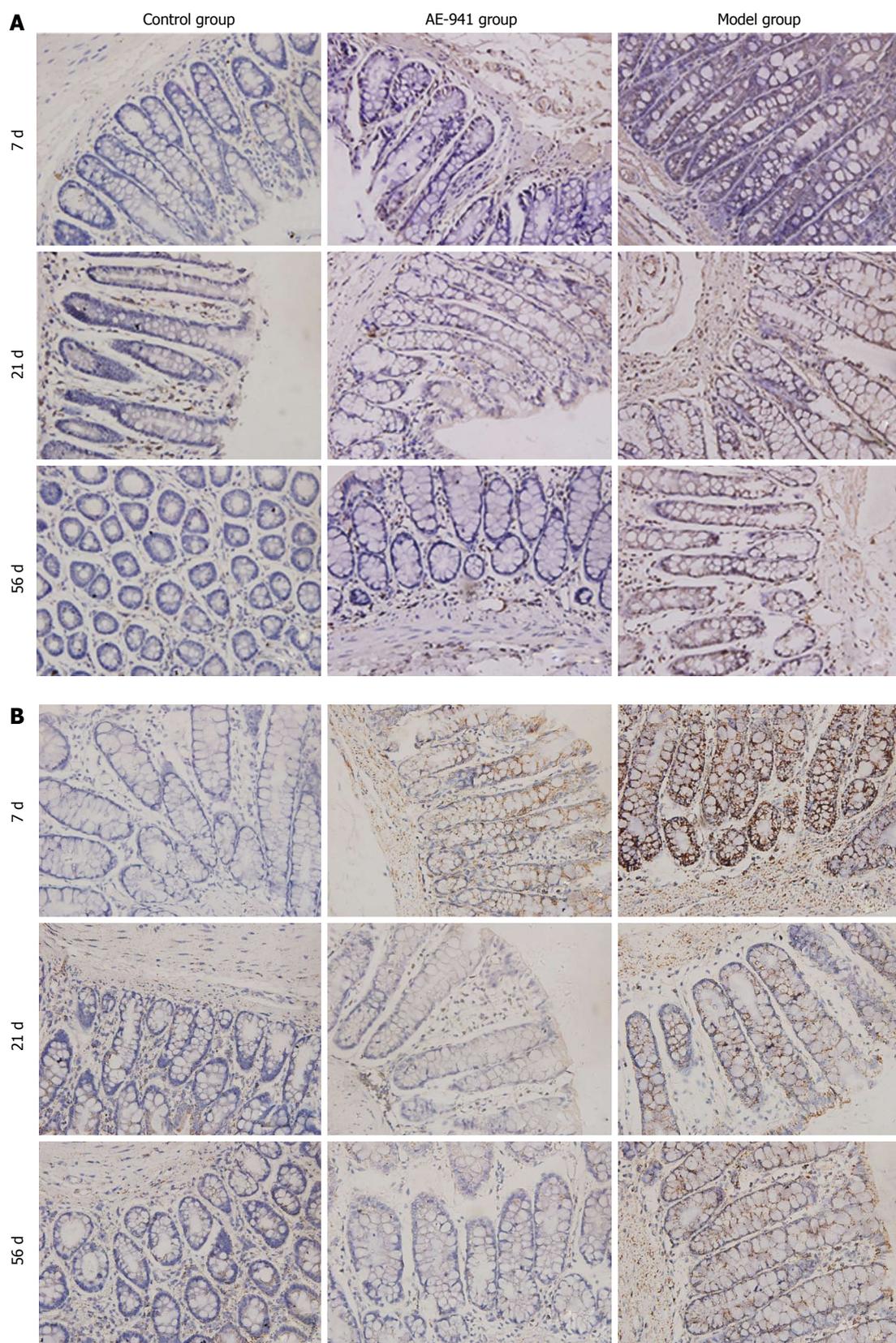


Figure 2 Colonic mucosal matrix metalloproteinase-2 and -9 protein expression in control, AE-941, and ulcerative colitis model groups at different time-points. The expression of matrix metalloproteinases (MMP)-2 (A) and MMP-9 (B) protein in the colonic mucosa of the ulcerative colitis model group was significantly higher than in the control group ($P < 0.05$). By days 21 and 56 expression of MMP-2 and MMP-9 in the AE-941 treatment group was significantly less than in the model group ($P < 0.05$).

Table 4 Colonic mucosal protein expression of matrix metalloproteinase-2 and matrix metalloproteinase-9 (mean \pm SD)

Time	Control group		Model group		AE-941 group	
	MMP-2	MMP-9	MMP-2	MMP-9	MMP-2	MMP-9
7th day	0.0053 \pm 0.0037	0.0073 \pm 0.0015	0.0986 \pm 0.0084 ^a	0.0916 \pm 0.0077 ^a	0.0923 \pm 0.0071	0.0893 \pm 0.0075
21st day	0.0047 \pm 0.0027	0.0067 \pm 0.0011	0.0773 \pm 0.0052 ^a	0.0748 \pm 0.0067 ^a	0.0536 \pm 0.0034 ^c	0.0554 \pm 0.0061 ^c
56th day	0.0057 \pm 0.0026	0.0070 \pm 0.0013	0.0453 \pm 0.0036 ^a	0.0537 \pm 0.0035 ^a	0.0324 \pm 0.0025 ^c	0.0364 \pm 0.0025 ^c

^a $P < 0.05$ vs control group; ^c $P < 0.05$ vs model group. MMP: Matrix metalloproteinase.

Results of reverse transcription polymerase chain reaction

Expression of MMP-2 and MMP-9 mRNA in the colonic mucosa was significantly higher in the UC model group compared to the control group ($P < 0.05$). However, compared to the UC model group, MMP-2 and MMP-9 mRNA expression in the AE-941 treatment group was reduced by day 7, although not significantly ($P > 0.05$). On days 21 and 56, the two index values in the AE-941 treatment group were significantly lower than that of the model group ($P < 0.05$, Table 3, Figure 1).

Results of immunohistochemistry

The expression of MMP-2 and MMP-9 protein in the colonic mucosa of the UC model group was significantly higher than in the control group ($P < 0.05$). Compared with the model group, expression of MMP-2 and MMP-9 protein in the AE-941 treatment group was reduced, although not significantly, by day 7 ($P > 0.05$). In contrast, by days 21 and 56 expression of MMP-2 and MMP-9 in the AE-941 treatment group was significantly lower than in the model group ($P < 0.05$) (Figure 2, Table 4).

DISCUSSION

Our previous research indicated that MMPs may be involved in the occurrence and development of UC^[8,9]. MMPs are a family of calcium- or zinc-dependent proteolytic enzymes that can remodel the ECM^[10]. At least 28 different MMP enzymes have been identified to date^[11]. MMP-2 and MMP-9 are the main matrix metalloproteinases that function to degrade collagen subtypes, including type IV and V, in the matrix^[12]. The study of von Lampe demonstrated that MMP-2 was highly expressed in the mucosa of patients with UC^[13]. Pirilä *et al.*^[14] identified MMP-2 overexpression in the colonic mucous membrane surrounding areas of inflammation in patients with UC. Medina also found that the expression and activity of MMP-9 was upregulated in colonic mucosa in the UC rat model^[15]. These results suggest that overexpression of MMP-2 and MMP-9 is related to mucosal membrane injury, inflammation of the epithelium and tissue destruction. We therefore tested MMP-2 and MMP-9 expression levels in colonic mucosa using RT-PCR and immunohistochemistry. Our results showed that expression of two indices, DAI and CMDI were significantly higher in

UC model animals than in the control group, and that decreased MMP-2 and MMP-9 expression is associated with an improvement in both DAI and CMDI. These data suggest that overexpression of MMP-2 and MMP-9 is related to mucosal injury and is particularly important for inflammation.

TIMPs are endogenous secretory proteins that can regulate the activity of MMPs^[16]. A disequilibrium of MMPs and TIMPs can lead to a synthetic and degradative imbalance of the ECM, which induces a series of pathological reactions^[17]. Our previous research showed that hyperdegradation combined with insufficient synthesis of colonic ECM resulted in mucosal injury, necrosis and ulceration that were attributed to unbalanced colonic mucosal expression of MMPs and TIMPs in patients with UC^[18,19].

AE-941, also known as Neovastat, is a natural MMP inhibitor derived from shark cartilage, and is a potent inhibitor of MMP-2 and MMP-9 that can dissolve gelatin and elastic protein^[20,21]. Béliveau and Falardeau *et al.*^[22,23] have shown that the activity of MMP-2, MMP-9 and MMP-12 is strongly inhibited by AE-941. Neovastat is a naturally occurring anti-angiogenic compound with multiple mechanisms of action that provide a broad therapeutic potential for a number of diseases^[23]. This drug is currently in international Phase II trials for multiple myeloma, renal cell carcinoma, and non-small-cell lung cancer^[24-26], and its inhibitory function may represent an innovative approach to the treatment of these diseases. The potent inhibition of MMP-2 and MMP-9 by AE-941 was used in our study of TNBS-induced UC in rats. Compared to the UC model group, the DAI and CMDI in the colon of AE-941-treated rats were significantly decreased at the 21- and 56-d timepoints. We found that expression of MMP-2 and MMP-9 mRNA and protein in the colonic mucosa of AE-941-treated rats was significantly lower than in the UC model group. Our results indicated that AE-941 is a potent MMP-2 and MMP-9 inhibitor that can relieve the intensity of chronic inflammation by decreasing the activity and expression of MMP-2 and MMP-9. We found no significant difference in the DAI, the CMDI, and colonic mucosal expression of MMP-2 and MMP-9 between the UC model animals and the AE-941 treatment group by day 7. A possible reason for this is that rats suffering the worst reaction to the TNBS enema at the day 7 evaluation were included in the UC model group. However,

AE-941 did not achieve a response at the shorter (7-d) treatment time.

By comparing the colonic mucosa expression of MMP-2 and MMP-9 in UC model animals at different timepoints, we found that MMP-2 and MMP-9 expression decreased as the DAI and CMDI improved. Using Spearman analysis, we identified a positive correlation between the expression of both MMP-2 and MMP-9 and the severity of UC.

In conclusion, our study confirmed that colonic mucosa in UC expressed MMP-2 and MMP-9 excessively and this overexpression correlated with the severity of UC. Treatment with AE-941 decreased the DAI and CMDI of UC, as well as colonic mucosal expression of MMP-2 and MMP-9, which indicates that AE-941 can protect the colon by inhibiting the excessive expression of MMP-2 and MMP-9 in an UC model. Therefore, MMP inhibitors may become a new measure to treat UC.

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COMMENTS

Background

Ulcerative colitis (UC) is a type of nonspecific inflammatory bowel diseases for which the pathogenesis is not completely clear and lacking of effective treatments. The aberrant expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) can cause a synthetic and degradative imbalance of the extracellular matrix, which then leads to colonic mucosa inflammation, erosion and ulceration. Therefore, in this study, AE-941, a natural MMP inhibitor (MMPI), was employed to verify its protective effect on the rat model of UC.

Research frontiers

In recent years, intense and extensive studies have shown that MMPs, TIMPs play an important role in the development of UC, while studies on the effects of exogenous MMPI on UC are not well documented. Therefore, studies are needed to verify the protective and therapeutic effects of MMPI on UC.

Innovations and breakthroughs

AE-941, a natural MMPI derived from shark cartilage, has been used in experimental and clinical treatment of tumors due to its potent inhibition of MMP-2 and MMP-9. However, its protective effects on UC remain largely unknown. Therefore, in this study, the authors verified the protective effects of AE-941 on UC in rats and provided a new therapeutic approach to UC.

Applications

Up till now, no satisfactory therapy for UC has been available. MMPI targeting MMPs may become a new and effective treatment modality for UC.

Peer review

This concise study reports interesting features on the role of metalloproteinase in ulcerative colitis. The study is well done, and the results are clearly presented.

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Connective tissue growth factor is overexpressed in human hepatocellular carcinoma and promotes cell invasion and growth

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Abstract

AIM: To determine the expression characteristics of connective tissue growth factor (CTGF/CCN2) in human hepatocellular carcinoma (HCC) in histology and to elucidate the roles of CCN2 on hepatoma cell cycle progression and metastasis *in vitro*.

METHODS: Liver samples from 36 patients (who underwent hepatic resection for the first HCC between 2006 and 2011) and 6 normal individuals were exam-

ined for transforming growth factor β 1 (TGF- β 1) or CCN2 mRNA by *in situ* hybridization. Computer image analysis was performed to measure integrated optical density of CCN2 mRNA-positive cells in carcinoma foci and the surrounding stroma. Fibroblast-specific protein-1 (FSP-1) and E-cadherin were examined to evaluate the process of epithelial to mesenchymal transition, α -smooth muscle actin and FSP-1 were detected to identify hepatic stellate cells, and CD34 was measured to evaluate the extent of vascularization in liver tissues by immunohistochemical staining. CCN2 was assessed for its stimulation of HepG2 cell migration and invasion using commercial kits while flow cytometry was used to determine CCN2 effects on HepG2 cell-cycle.

RESULTS: *In situ* hybridization analysis showed that TGF- β 1 mRNA was mainly detected in connective tissues and vasculature around carcinoma foci. In comparison to normal controls, CCN2 mRNA was enhanced 1.9-fold in carcinoma foci (12.36 ± 6.08 vs 6.42 ± 2.35) or 9.4-fold in the surrounding stroma (60.27 ± 28.71 vs 6.42 ± 2.35), with concomitant expression of CCN2 and TGF- β 1 mRNA in those areas. Epithelial-mesenchymal transition phenotype related with CCN2 was detected in 12/36 (33.3%) of HCC liver samples at the edges between carcinoma foci and vasculature. Incubation of HepG2 cells with CCN2 (100 ng/mL) resulted in more of the cells transitioning into S phase (23.85 ± 2.35 vs 10.94 ± 0.23), and induced a significant migratory (4.0-fold) and invasive (5.7-fold) effect. TGF- β 1-induced cell invasion was abrogated by a neutralizing CCN2 antibody showing that CCN2 is a downstream mediator of TGF- β 1-induced hepatoma cell invasion.

CONCLUSION: These data support a role for CCN2 in the growth and metastasis of HCC and highlight CCN2 as a potential novel therapeutic target.

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Key words: Connective tissue growth factor; Hepatocellular carcinoma; Hepatoma cell line; Migration; Invasion

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide and the third most common cause of cancer death^[1]. Although HCC resection plays an important role in improving prognosis of HCC, short survival has been observed in these patients due to high rate of recurrence and metastasis after surgery^[2,3]. The mechanisms regulating tumor growth and metastasis of HCC are very complicated and still not fully understood.

HCC consists of a tumor parenchyma comprised of hepatoma cells and a surrounding microenvironment composed of various cell types such as hepatic stellate cells (HSCs), fibroblasts or inflammatory cells that produce a variety of tumor stroma components, including extracellular matrix (ECM) proteins, growth factors and cytokines^[4,5]. In normal liver, HSCs are typically located in the perisinusoidal space of Disse which lies between endothelial cells of sinusoids and hepatocytes. Following liver injury, HSCs become activated, undergo a phenotypic transformation into fibroblast-like cells or myofibroblasts, and migrate to sites of injury where they produce a provisional ECM to support parenchymal repopulation. In settings of chronic injury, this process proceeds unabated, leading to deposition of excessive amounts of ECM proteins and fibrillar collagens, resulting in fibrosis and scarring. This fibrotic environment supports the growth of hepatoma cells and HCC progression is dependent on an intricate cross-talk between the tumor and its surrounding environment^[6-8]. Recent literature has highlighted a fundamental role of the tumor microenvironment in modulating the process of liver fibrosis, hepatocarcinogenesis, epithelial-mesenchymal transition (EMT), tumor invasion and metastasis^[9,10]. A growing body of evidence from animal models and *in vitro* studies suggests that growth factors and matricellular proteins as well as resident HSCs play a key role in this stromal-tumor interaction^[5,10-12].

Connective tissue growth factor (CTGF/CCN2) is a cysteine-rich matricellular protein that has been implicated in regulating diverse processes *in vivo*, including angiogenesis, embryogenesis, chondrogenesis, fibro-

genesis, and tumorigenesis^[13]. CCN2 is transcriptionally activated by transforming growth factor β 1 (TGF- β 1) through a mechanism that involves activation of Smad binding elements and a unique TGF- β response element in the CCN2 promoter. Enhanced expression of CCN2 by TGF- β 1 has been demonstrated in many cell types including HSCs and hepatoma cells^[7,14]. Numerous studies have demonstrated that CCN2 is a downstream mediator of TGF- β 1-induced ECM production in HSCs, pancreatic stellate cells and osteoblasts^[15-17]. Recently, down-regulation of CCN2 by either the TGF- β 1 inhibitor LY2109761 or CCN2 siRNA appeared to inhibit the growth of HCC in culture or xenograft models^[7,18].

In this study, we have characterized CCN2 expression in human HCC, its association with EMT, and its effects on hepatoma cell migration, invasion or cell cycle progression.

MATERIALS AND METHODS

Clinical data

Thirty-six HCC specimens were obtained by surgical resection from May 2006 to June 2011. Samples obtained from the peripheral areas of the tumors were selected for this study. The patients with HCC consisted of 26 men and 10 women, mean age 56.4 years (range: 27-71 years). According to Pittsburgh modified TNM criteria, the patients were classified as small hepatocellular carcinoma ($n = 20$), solitary large hepatocellular carcinoma ($n = 10$) and nodular hepatocellular carcinoma ($n = 6$). Six normal liver specimens were obtained from patients undergoing hepatic resection due to a single cavernous hemangioma and the normal liver tissues were taken at the greatest distance from the location of the angiocavernoma. The tissue samples used as controls were histologically analyzed and clearly shown to have no inflammation or fibrosis. All specimens were examined under a light microscope after haematoxylin and eosin (HE) staining or Masson's trichrome staining.

In situ hybridization

ISH was performed using digoxigenin-labeled sense or anti-sense probes for CCN2 or TGF- β 1 (Boster Biotechnology Co. Ltd., Wuhan, China). In brief, liver samples from HCC patients were formaldehyde-fixed and paraffin-embedded. The tissue sections (5 μ m) were deparaffinized, rehydrated with phosphate buffered solution (PBS), digested with pepsin (30 μ g/mL) for 10 min at 37 $^{\circ}$ C, fixed in 4% paraformaldehyde in PBS and washed in 3 \times SSC. The samples were pre-hybridized at 42 $^{\circ}$ C for 2 h, and hybridization was performed overnight at 42 $^{\circ}$ C with sense or anti-sense probes. After hybridization, unbound probe was removed by washing in 2 \times SSC, 0.5 \times SSC and then 0.2 \times SSC at 37 $^{\circ}$ C for 2 h. The tissue sections were incubated at 37 $^{\circ}$ C for 1 h with biotinylated mouse anti-digoxigenin, followed by addition of streptavidin-biotin-peroxidase complex for

20 min. The slides were then developed with 3-amino-9-ethylcarbazole (Boster Biotechnology). Ten random images (original magnification $\times 400$) of each slide underwent computer image analysis using Image-Pro Plus 6.0 software to assess the integrated optical density (A) of TGF- β 1 or CCN2-positive cells in liver tissues.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections ($5 \mu\text{m}$) were de-waxed and re-hydrated. Sections were incubated overnight at 4°C with rabbit anti-human E-cadherin polyclonal antibody (Boster Biotechnology Co. Ltd., Wuhan, China), mouse anti-human cluster of differentiation 34 monoclonal antibody (Maixin Bio, Fuzhou, China), rabbit anti-human CCN2 polyclonal antibody (Santa Cruz, Heidelberg, Germany) or rabbit anti-human fibroblast-specific protein-1 (FSP-1) polyclonal antibody (Millipore Corporation, Billerica, MA, United States). Sections were washed in PBS and incubated at room temperature for 10 min with biotinylated goat anti-mouse and anti-rabbit IgG (Maixin Bio, Fuzhou, China). After washing with PBS, sections were incubated with streptavidin-peroxidase (Maixin Bio, Fuzhou, China) for 10 min and then developed with diaminobenzidine or 3-amino-9-ethylcarbazole.

Flow cytometry

Two milliliter of $1 \times 10^5/\text{mL}$ HepG2 cell suspension were placed to each well of 6-well tissue culture plates in 12% FBS-DMEM and incubated for 12 h. The cells were then cultured in 0.5% FBS-DMEM for 24 h. The cell culture medium was exchanged with 0.5% FBS-DMEM and the cells were incubated for another 24 h in the absence or presence of 100 ng/mL CCN2 (Biovendor, Modrice, Czech Republic) or 20 ng/mL TGF- β 1 (Peprotech, Rehovot, Israel). The cells were then trypsinized with 0.25% trypsin and washed in PBS. HepG2 cell-cycle progression was determined by resuspending the cells at 1×10^6 cells/mL in PBS, fixing the cells with 75% ethanol overnight, and then staining the cells with 0.1 mg/mL propidium iodide in a 0.1% sodium citrate/0.1% Triton X-100 solution in the presence of 0.2 mg/mL Rnase for 30 min at room temperature in the dark. Analysis of cellular DNA content after cell staining with propidium iodide was performed by flow cytometry at an excitation wavelength of 488 nm. The distribution of cells in three major phases of the cycle (G0/G1, S, G2/M) was analyzed using CellQuest software (BD Biosciences, San Jose, CA, United States).

Migration assay

HepG2 migration studies were performed using a 12-well companion plate into which inserts were placed that had an uncoated membrane with $5\text{-}\mu\text{m}$ pores (Millipore, Billerica, MA). HepG2 cells, detached from stock dishes with 1 mmol/L EDTA, were suspended in DMEM and placed at 3×10^4 cells per insert for 6

h in the absence or presence of CCN2 (100 ng/mL) or TGF- β 1 (20 ng/mL) in the lower chamber of the well. Cells that adhered to the membrane were fixed with 100% methanol and stained with May-Grunwald's Giemsa. The numbers of HepG2 cells on the upper or lower surfaces were individually counted in 10 randomly selected microscopic fields at a magnification of $400 \times$. The rate of migration of HepG2 cells were expressed as a migration index (%) defined as follows: (number of cells on the undersurface of membrane/total number of cells on both surface of the membrane) $\times 100$.

Invasion assay

A HepG2 cell invasion assay was performed in a 24-well plate with ECMatrixTM cell culture inserts (Chemicon, United States). The inserts contained an ECMatrix-coated polycarbonate membrane with $8 \mu\text{m}$ pores which was rehydrated with 300 μL DMEM for 2-h at room temperature. 300 μL Hep G2 cells in DMEM (5×10^4 cells/mL) were added to each insert while 500 μL DMEM containing CCN2 (100 ng/mL) or TGF- β 1 (20 ng/mL) were added to the lower chamber. After 24 h, adherent cells were fixed with 100% methanol and stained with staining solution. The numbers of non-invaded cells on the upper surface and those of invaded cells on the under surface were counted in 10 randomly selected microscopic fields at a magnification of $400 \times$. The cell invasion index (%) was defined as follows: (number of cells on the undersurface of membrane/total number of cells on both surface of the membrane) $\times 100$.

Ethical approval

This work was approved by First Hospital of Jilin University and was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. Informed consent was obtained from all patients prior to sample collection.

Statistical analysis

Statistical analysis of the data was performed using SPSS 13.0 for Windows (SPSS Inc, Chicago, IL, United States). The values reported represent the mean \pm SD of the measurements. Differences were analyzed statistically with paired sample Student's t -test and $P < 0.05$ was considered significant.

RESULTS

Localization of CCN2 mRNA in HCC

At the light microscopic level, all HCC samples tested in this study had multiple foci of carcinoma by HE or Masson's trichrome staining. The carcinoma foci were separated or wrapped by their surrounding stroma. Collagen bundles were present in the stroma (Figure 1A) while CD34, a specific marker of vascular endothelial cells, was detected in the vasculature surrounding the stroma and in a few small blood vessels within tumor foci (Fig-

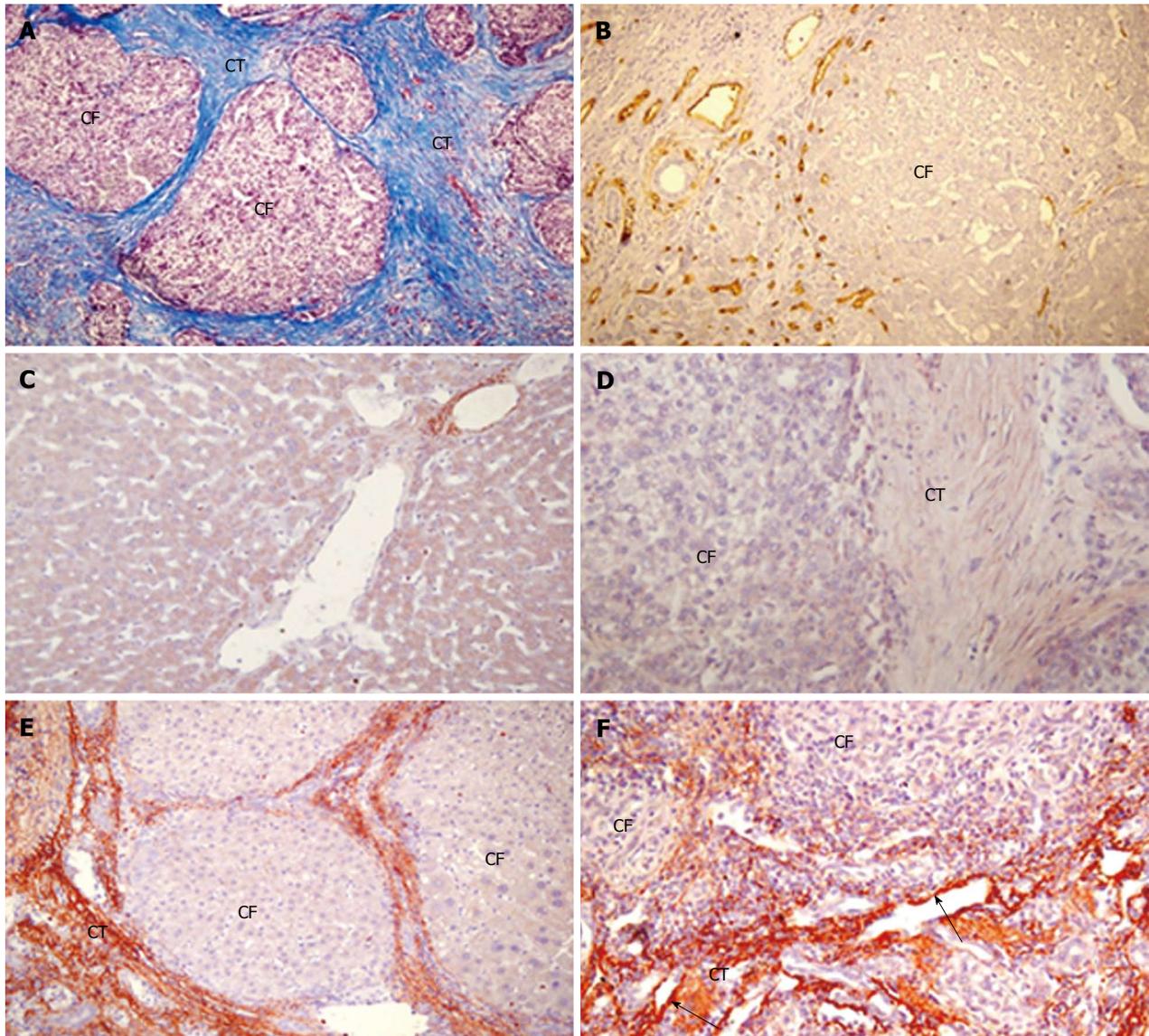


Figure 1 Expression and distribution characteristics of transforming growth factor β 1 or connective tissue growth factor mRNA in human hepatocellular carcinoma. A: Masson's trichrome stain for collagen (blue) in hepatocellular carcinoma (HCC); B: Immunohistochemical detection of CD34 in vascular endothelial cells (brown) in HCC; C: Normal liver showing connective tissue growth factor (CCN2) mRNA expressed in connective tissue around the veins; D: Absence of staining of HCC when the *in situ* hybridization probes were omitted; E: HCC stained for transforming growth factor β 1 mRNA, showing reactivity in connective tissue and around the carcinoma foci; F: Over-expression of CCN2 mRNA in connective tissue and vascular endothelial cells (black arrow) in HCC. Original magnification, $\times 100$ in A and E, $\times 200$ in B, C, D and F. CF: Carcinoma foci; CT: Connective tissue.

ure 1B). *In situ* hybridization showed that CCN2 mRNA positive cells were mainly distributed in connective tissues surrounding the carcinoma foci and its associated vasculature. However, only mild CCN2 mRNA staining was detected in veinal vasculature in normal liver (Figure 1C) and there was no staining in HCC liver tissue when the *in situ* hybridization probe was omitted (Figure 1D). We next examined the expression and distribution of hepatic TGF- β 1 mRNA in HCC patients since TGF- β stimulates and interacts co-operatively with CCN2 to drive fibrosis and tumor progression^[7,13]. As expected, TGF- β 1 mRNA was detected in the connective tissues surrounding carcinoma foci where it was expressed concomitantly with CCN2 mRNA (Figure 1E and F).

Computer image analysis of CCN2 mRNA expression in comparison to normal controls revealed a 9.4-fold increase in stromal expression (60.27 ± 28.71 vs 6.42 ± 2.35 , $P < 0.01$) and a 1.9-fold increase in expression in carcinoma foci (12.36 ± 6.08 vs 6.42 ± 2.35 , $P < 0.01$, Table 1).

Relationship between CCN2 expression and EMT

Having shown the expression and localization of CCN2 mRNA, we next investigated the relationship between CCN2 and the occurrence of EMT, the latter of which was assessed by staining for either (1) E-cadherin which is expressed in normal epithelial cells but is absent or only weakly expressed in high-metastatic cancer cells^[8] or (2) FSP-1 which is a marker of fibroblasts and epithelial

Table 1 Expression levels of transforming growth factor β 1 or connective tissue growth factor mRNA in human hepatocellular carcinoma (mean \pm SD)

	<i>n</i>	TGF- β 1 mRNA (A)	CCN2 mRNA (A)
Normal control	6	6.24 \pm 2.35	6.42 \pm 2.35
Carcinoma foci	36	15.02 \pm 4.43 ^b	12.36 \pm 6.08 ^b
Stroma around carcinoma foci	36	47.56 \pm 7.31 ^d	60.27 \pm 28.71 ^d

^b*P* < 0.01 *vs* normal control; ^d*P* < 0.01 *vs* carcinoma foci. TGF- β 1: Transforming growth factor β 1; CCN2: Connective tissue growth factor.

cancer cells with EMT features. CCN2 mRNA (Figure 2A) or protein (Figure 2B) were detected in hepatoma cells at the edge of carcinoma foci by ISH or immunohistochemistry respectively. E-cadherin was present in areas that were adjacent to the carcinoma foci but it was only very weakly detectable in hepatoma cells within the carcinoma foci (Figure 2C). At the edges between carcinoma foci and the vasculature, hepatoma cells were FSP-1-positive in 33.3% (12/36) of HCC sections (Figure 2D). As CCN2 has been proposed as a key regulatory cytokine in tumor-stroma crosstalk in an animal model of HCC^[7], this aspect was investigated in our clinical specimens. As expected, CCN2 mRNA expression occurred in (1) fibroblast-like or myofibroblast-like HSC marked by expression of FSP-1 or α -smooth muscle actin (α -SMA) (Figure 2D-F); (2) hepatoma cells (Figure 2G) located along the border between tumor foci and vasculature; Since CCN2 mRNA was detected in hepatoma cells that were FSP-1-positive (Figure 2D and H), this finding supports a role for CCN2 in autocrine or paracrine regulation of EMT in human hepatoma cells.

Effect of CCN2 on hepatoma cell cycle distribution

Cell cycle analysis was assessed by flow cytometry of propidium iodide-stained HepG2 cells after treatment of the cells with 100 ng/mL CCN2 for 24 h. As shown in Table 2, approximately 53% of HepG cells were in G0/G1, 11% were in the S, and 34% were in G2/M after serum starvation of the cells in medium containing 0.5% FCS. After CCN2 stimulation, a relatively lower proportion of cells (41%) were in the G0/G1 phase (41.01 \pm 4.45 *vs* 53.56 \pm 2.51, *P* < 0.05), and a higher proportion (23.8%) were in S phase (23.85 \pm 2.35 *vs* 10.94 \pm 0.23, *P* < 0.05), as compared to control cells. However, TGF- β 1 stimulation did not significantly alter the distribution of phases of the cell cycle as compared to control cells.

CCN2 induces hepatoma cell migration and invasion

To determine effects of CCN2 on HepG2 cell migration, we used a chemotaxis assay in which the migration of HepG2 cells from the upper chamber of a culture insert was assessed following addition of CCN2 to the lower chamber. As shown in Figure 3A, CCN2 induced HepG2 cell migration across the polyethylene membrane in the culture insert. Cell migration was also promoted

Table 2 Effect of connective tissue growth factor on HepG2 cell cycle progression

Groups	G0-G1	S	G2-M
Normal control	53.56 \pm 2.51	10.94 \pm 0.23	34.17 \pm 1.29
CCN2	41.01 \pm 4.45 ^a	23.85 \pm 2.35 ^a	34.17 \pm 1.29
TGF- β 1	54.97 \pm 1.88	12.07 \pm 2.44	33.30 \pm 0.73

^a*P* < 0.05 *vs* normal control. TGF- β 1: Transforming growth factor β 1; CCN2: Connective tissue growth factor.

by TGF- β 1.

Since CCN2 expression was associated with EMT in hepatoma cells (see above), we further examined whether CCN2 played a role in HepG2 cell invasion through ECM using an ECMatrix culture insert. As shown in Figure 3B, HepG2 invasion across the ECM layer was stimulated by either CCN2 or TGF- β 1, the latter of which was blocked by anti-CCN2 antibody but not normal IgG. These data indicate that HepG2 cell invasion is stimulated by CCN2 directly or by TGF- β 1-mediated CCN2 production.

DISCUSSION

In this study, we determined the expression and distribution of CCN2 in HCC, its relationship to HCC-associated EMT, and the role of CCN2 in stimulating hepatoma migration, invasion, or cell cycle progression *in vitro*. Our findings can be summarized as follows: (1) In comparison to normal controls, CCN2 mRNA expression was enhanced, respectively, 9.4- or 1.9-fold in tumor stroma or carcinoma foci; (2) A concomitant expression of CCN2 and TGF- β 1 mRNA was found in these areas; (3) CCN2 expression in the stroma, hepatoma cells or HSCs was correlated with expression of markers of EMT; (4) CCN2 promoted HepG2 cell migration, invasion or cell cycle progression; and (5) CCN2 neutralizing antibody blocked TGF- β 1-induced HepG2 cell invasion.

The initiation, growth and progression of HCC are dependent on an intricate crosstalk between tumor and stroma^[7]. Components of the microenvironment that surround hepatoma cells, including ECM protein and growth factors as well as its constituent non-tumor cells, are critical for HCC growth and metastasis^[6,19]. It has previously been demonstrated that TGF- β 1 plays an important role in HCC by stimulating fibrogenic remodeling of the liver and contributing to tumor progression^[20]. Produced downstream of TGF- β 1, CCN2 participates in a variety of pathophysiological processes, including formation of fibrous scar, angiogenesis, tumor growth^[21-24]. Expression of CCN2 is related to recurrence, metastasis and poor prognosis in human HCC and pancreatic cancer^[25,26]. Increasing evidence supports a role for CCN2 in mediating the matrigenic actions of TGF- β 1 in numerous cell types, especially those that have specialized role in ECM production such as HSC, pancreatic stellate cells and osteoblasts^[14-17]. In experimental HCC models,

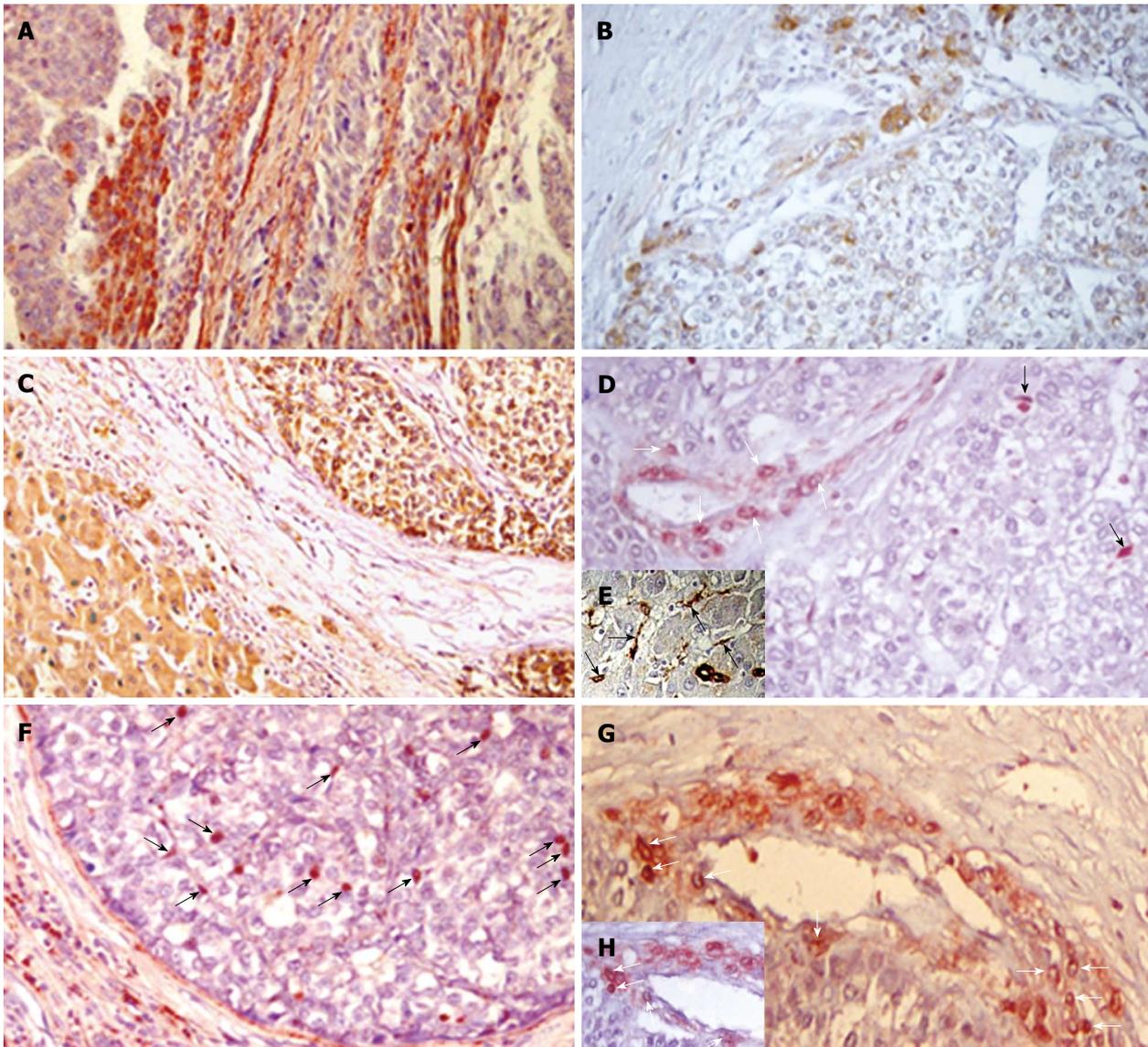


Figure 2 Expression of connective tissue growth factor mRNA and its association with epithelial-mesenchymal transition in hepatocellular carcinoma. Connective tissue growth factor (CCN2) mRNA (A) or protein (B) were detected in hepatoma cells at the edge of carcinoma foci by *in situ* hybridization or immunohistochemistry respectively; C: E-cadherin was only weakly detectable in carcinoma foci but stained more intensely in normal hepatocytes; D, E: Fibroblast-specific protein-1 (FSP-1)-positive hepatoma cells (white arrow) were located at a position between the edge of carcinoma foci and vascular wall; FSP-1-positive (black arrows) (D) or (E) α -smooth muscle actin-positive (black arrows) fibroblast-like or myofibroblast-like hepatic stellate cell (HSC) were found within carcinoma foci; F-H: CCN2 mRNA was detected in either fibroblast-like or myofibroblast-like HSC (black arrows); CCN2 mRNA (G) or (H) FSP-1 were found in some hepatoma cells. Original magnification, $\times 400$ in A, B, C, D, F and G, $\times 200$ in E and H. Examples of positively stained cells or structures in each panel are arrowed.

LY2109761, an inhibitor of TGF- β 1, was shown to inhibit neo-angiogenesis, tumor growth and progression by down-regulation of CCN2 production^[6,7]. Our results show that human HCC is characterized by enhanced CCN2 expression in tumor stroma and tumor cells, as well as its co-expression with TGF- β 1. Collectively these data suggest that CCN2 is an important component of the tumor-stroma axis and likely plays an important role in the development and progression of HCC.

EMT is a cellular program characterized by loss of cell adhesion, repression of E-cadherin expression, rearrangement of the cellular cytoskeleton, upregulation of matrix remodeling factors and increased cell mobility^[27]. EMT is essential for numerous developmental processes

including embryo implantation, embryogenesis, organ development and fibrosis^[28-30]. Over past decade, the EMT process has been increasingly recognized to occur during the progression of various carcinomas such as HCC or pancreatic adenocarcinoma^[31,32]. Recent studies have shown that TGF- β 1 or hepatocyte growth factor induces EMT in HCC cell lines or in animal models^[27,32-34]. CCN2 has been demonstrated to promote human renal tubular epithelial cells to mesenchymal transition characterized by down-regulation of E-cadherin and up-regulation of α -SMA^[35]. In this study we showed that hepatoma cells with EMT characteristics (downregulation of E-cadherin and upregulation of FSP-1) were located at the tumor border and were positive for CCN2,

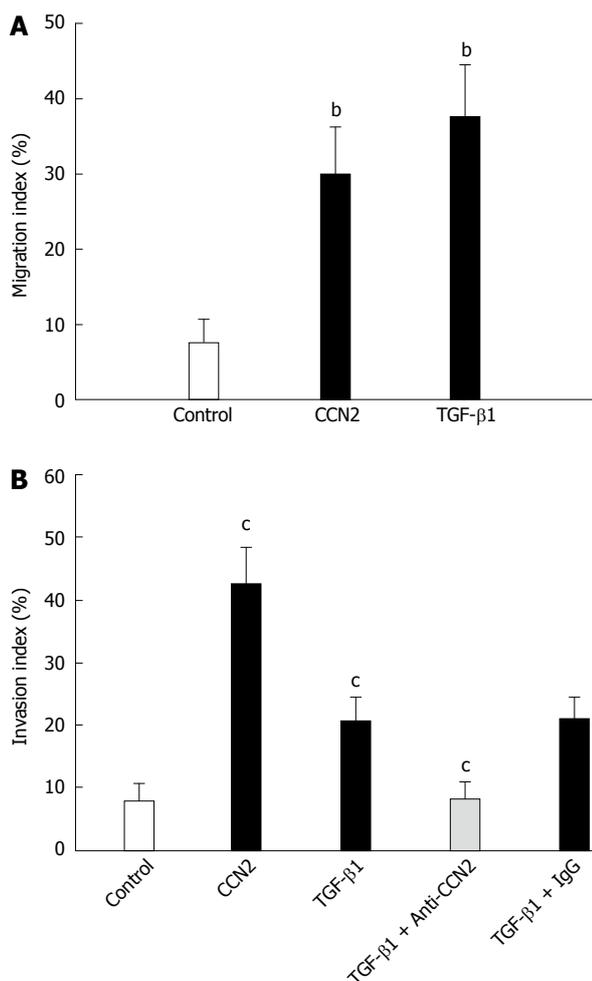


Figure 3 Connective tissue growth factor induces HepG2 cell migration or invasion *in vitro*. A: HepG2 cell migration assays were performed by placing the cells in culture inserts followed by incubation in a 12-well companion plate for 6 h in the absence (control) or presence of connective tissue growth factor (100 ng/mL) or transforming growth factor β1 (TGF-β1) (20 ng/mL) in the lower chamber; B: HepG2 cell invasion assays were performed by placing the cells in culture inserts followed by incubation in a 24-well companion plate for 24 h in the absence (control) or presence of connective tissue growth factor (100 ng/mL) or TGF-β1 (20 ng/mL) in the lower chamber. For inhibitory studies, HepG2 cells were incubated with 25 μg/mL anti-connective tissue growth factor (CCN2), or normal mouse immunoglobulin G during treatment with TGF-β1. ^a*P* < 0.01 vs control or CCN2 group; ^b*P* < 0.05 vs TGF-β1 group.

which was also detected in tumor- or stroma- associated HSCs. Taken together, these results support a role for HSC- or HCC-derived CCN2 in the process of EMT.

Tumor cell invasion and metastasis are complex and multistage processes that occur after cancer cells have undergone genotypic and phenotypic alterations resulting in the acquisition of a matrix-degrading and migratory phenotype^[36]. Tumor cell migration is necessary at the initiation of the metastatic cascade, at which time the tumor cells leave the primary site and migrate in response to, and toward, specific external chemotactic factors such as growth factors and ECM^[36,37]. In mouse models, proliferation and invasion of breast or pancreatic cancer cells are increased by CCN2, while the growth and metastasis of pancreatic adenocarcinoma

are attenuated by neutralizing anti-CCN2 antibody^[38,39]. Increasing evidence from *in vitro* studies demonstrates that CCN2 regulates cell motility, migration and invasion in glioma, melanoma or gastric cancer cells^[40-43]. Specifically, CCN2 induces gastric cancer cell migration through downregulation of E-cadherin *via* the nuclear factor κB (NF-κB) pathway while glioma tumor initiation or tumor stem cell invasion are promoted by CCN2 *via* its binding with integrin β1 and tyrosine kinase receptor type A, and activation NF-κB signal pathway^[40,43]. Downregulation of matrix metalloproteinase (MMP)-2 and MMP-9 contributed to the reduced invasion of gastric cancer cells in which CCN2 expression was knocked down^[41]. Moreover, down-regulation of CCN2 by either the TGF-β1 inhibitor LY2109761 or CCN2 siRNA diminished hepatoma growth, intravasation and metastatic dissemination in an *in vivo* xenograft model or in cell culture systems^[7,18]. Thus, these findings are consistent with our observations that exogenous CCN2 induces hepatoma cell migration, invasion and cell cycle progression. These functional properties in hepatoma cells coupled with the pattern of CCN2 over-expression in HCC, support a role of CCN2 in the growth and metastasis of human HCC.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is the most common form of liver malignancy worldwide. HCC consists of a tumor parenchyma comprised of hepatoma cells and a surrounding microenvironment composed of various cell types such as hepatic stellate cells (HSCs), fibroblasts, and inflammatory cells that produce a variety of tumor stroma components including extracellular matrix (ECM) proteins, growth factors and cytokines. A growing body of literature has highlighted a fundamental role of the tumor microenvironment in modulating the process of hepatocarcinogenesis, epithelial-mesenchymal transition, tumor invasion and metastasis. Connective tissue growth factor (CTGF/CCN2) is downstream mediator of transforming growth factor β1 (TGF-β1)-induced ECM production in HSCs and hepatoma cells. CCN2 has been previously implicated in cancer metastasis and invasion in various tumors. However, the role of CCN2 in human HCC remains largely unknown.

Research frontiers

Recently, down-regulation of CCN2 by either the TGF-β1 inhibitor LY2109761 or CCN2 siRNA appeared to inhibit the growth of HCC in culture or xenografted models. High intratumoral CCN2 expression was associated with vascular invasion and poor survival after curative resection of hepatocellular carcinoma. The preliminary results suggest that CCN2 might play a crucial roles in regulating the growth and metastasis of HCC.

Innovations and breakthroughs

To date, there have been a limited number of HCC studies using *in vitro* cell culture, animal models or human clinical specimens to address the roles of CCN2 in regulation of tumor growth and metastasis. In this study, the authors determined the expression characteristics of CCN2 in human HCC by histology and elucidated the roles of CCN2 on hepatoma cell migration, invasion and cell cycle progression. Furthermore, the authors described the relationship between the overexpression of CCN2 in human HCC and its association with epithelial-mesenchymal transition (EMT).

Applications

These studies suggest that CCN2 plays important roles in tumor growth and metastasis in human HCC and highlight CCN2 as potential novel therapeutic target.

Peer review

This paper provided some aspects on understanding EMT of HCC and mecha-

nisms on HCC migration and invasion. The result is interesting and suggest that a role for CCN2 in the growth and metastasis of HCC and highlight CCN2 as a potential novel therapeutic target.

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Astragalus polysaccharides can regulate cytokine and P-glycoprotein expression in H22 tumor-bearing mice

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Abstract

AIM: To investigate the adjunct anticancer effect of Astragalus polysaccharides in H22 tumor-bearing mice.

METHODS: To establish a solid tumor model, 5.0×10^6 /mL H22 hepatoma cells were inoculated subcutaneously into the right armpit region of Kunming mice (6-12 wk old, 18-22 g). When the tumors reached a size of 100 mm³, the animals were treated as indicated, and the mice were randomly assigned to seven groups ($n = 10$ each). After ten days of treatment, blood samples were collected from mouse eyes, and serum was harvested by centrifugation. Mice were sacrificed, and the whole body, tumor, spleen and thymus were weighed immediately. The rate of tumor inhibition and organ indexes were calculated. The expression levels of serum cytokines, P-glycoprotein (P-GP) and multidrug resis-

tance (*MDR1*) mRNA in tumor tissues were detected using enzyme-linked immunosorbent assay, Western blotting, and quantitative myeloid-derived suppressor cells reverse transcription-polymerase chain reaction, respectively.

RESULTS: The tumor inhibition rates in the treatment groups of Adriamycin (ADM) + Astragalus polysaccharides (APS) (50 mg/kg), ADM + APS (100 mg/kg), and ADM + APS (200 mg/kg) were significantly higher than in the ADM group (72.88% vs 60.36%, $P = 0.013$; 73.40% vs 60.36%, $P = 0.010$; 77.57% vs 60.36%, $P = 0.001$). The spleen indexes of the above groups were also significantly higher than in the ADM group (0.65 ± 0.22 vs 0.39 ± 0.17 , $P = 0.023$; 0.62 ± 0.34 vs 0.39 ± 0.17 , $P = 0.022$; 0.67 ± 0.20 vs 0.39 ± 0.17 , $P = 0.012$), and the thymus indexes of the ADM + APS (100 mg/kg) and ADM + APS (200 mg/kg) groups were significantly higher than in the ADM group (0.20 ± 0.06 vs 0.13 ± 0.04 , $P = 0.029$; 0.47 ± 0.12 vs 0.13 ± 0.04 , $P = 0.000$). APS was found to exert a synergistic anti-tumor effect with ADM and to alleviate the decrease in the sizes of the spleen and thymus induced by AMD. The expression of interleukin-1 α (IL-1 α), IL-2, IL-6, and tumor necrosis factor- α (TNF- α) was significantly higher in the ADM + APS (50 mg/kg), ADM + APS (100 mg/kg) and ADM + APS (200 mg/kg) groups than in the ADM group; and IL-10 was significantly lower in the above groups than in the ADM group. APS could increase IL-1 α , IL-2, IL-6, and TNF- α expression and decrease IL-10 levels. Compared with the ADM group, APS treatment at a dose of 50-200 mg/kg could down-regulate *MDR1* mRNA expression in a dose-dependent manner (0.48 ± 0.13 vs 4.26 ± 1.51 , $P = 0.000$; 0.36 ± 0.03 vs 4.26 ± 1.51 , $P = 0.000$; 0.21 ± 0.04 vs 4.26 ± 1.51 , $P = 0.000$). The expression level of P-GP was significantly lower in the ADM + APS (200 mg/kg) group than in the ADM group (137.35 ± 9.20 mg/kg vs 282.19 ± 20.54 mg/kg, $P = 0.023$).

CONCLUSION: APS exerts a synergistic anti-tumor

effect with ADM in H22 tumor-bearing mice. This may be related to its ability to enhance the expression of IL-1 α , IL-2, IL-6, and TNF- α , decrease IL-10, and down-regulate *MDR1* mRNA and P-GP expression levels.

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Key words: Astragalus polysaccharides; Tumor inhibition rate; Cytokines; P-glycoprotein; Adjunct anticancer

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INTRODUCTION

Cancer has become a major public health problem globally^[1]. The World Health Organization predicts that by 2030 an estimated number of 21.4 million new cases of cancer and 13.2 million cancer deaths will occur annually around the world^[2]. Surgery, radiotherapy, chemotherapy and endocrine therapy remains the classic cancer therapies^[3]. For advanced tumors, chemotherapy is still the treatment of choice, and although these drugs are effective, they are associated with severe adverse events and drug resistance, especially multidrug resistance (MDR)^[4].

Severe adverse events affect patients' compliance. Drug resistance, especially MDR, is the leading cause of treatment failure in cancer therapy. Once the MDR occurs, chemotherapy is no longer effective even with doses of drugs high enough to overcome the resistance; toxic effects are observed and resistance mechanisms can be further stimulated^[5]. One of the underlying mechanisms of MDR is cellular overproduction of P-glycoprotein (P-GP) which acts as an efflux pump for various anticancer drugs. P-GP is encoded by the *MDR1* gene and its over-expression in cancer cells has become a therapeutic target for circumventing MDR. A potential therapeutic strategy is to co-administer efflux pump inhibitors, although such reversal agents might actually increase the side effects of chemotherapy by blocking physiological anticancer drug efflux from normal cells. Although great efforts have been made to overcome MDR with the first- and second-generation reversal agents available in current clinical use for other indications (e.g., verapamil, cyclosporine A and quinidine) or analogues of the first-generation drugs (e.g., dexverapamil, valsopodar and cinchonine), few significant advances have been achieved. Clinical trials with the third-generation modulators (e.g., biricodar, zosuquidar and laniquidar) specifically for MDR reversal are being developed. The results however are not encour-

aging possibly because that the perfect reverser does not exist^[6].

Traditional Chinese medicine (TCM) and herbal medicines in particular have been used in the treatment of cancer for thousands of years in China, Japan, South Korea and other Asian countries. These medicines are widely accepted as current forms of adjuvant therapy in cancer treatment in the United States and Europe^[7,8]. TCM has been shown to play an adjunct anticancer role by inducing apoptosis and differentiation, enhancing the immune system, inhibiting angiogenesis and reversing MDR^[9]. As adjunct anticancer agents, TCM has great advantages in terms of increasing the sensitivity of chemotherapeutics, reducing the side effects and complications associated with chemotherapy, and improving patient quality of life and survival time^[10]. In the search for new cancer therapeutics with lower toxicity and fewer side effects, TCM has shown promise^[11].

The dried root of *Astragalus membranaceus* has a long history of medicinal use in TCM. *Astragalus* has demonstrated a wide range of potential therapeutic applications in immunodeficiency syndromes, as an adjunct cancer therapy, and for its adaptogenic effect on the heart and kidneys^[12]. *Astragalus* extract inhibits destruction of gastric cancer cells by mesothelial cells through its anti-apoptosis effects^[13]. The active pharmacological constituents of *Astragalus membranaceus* include various polysaccharides, saponins and flavonoids as well as L-arginine or L-canavanine^[14,15]. Among these, *Astragalus* polysaccharides (APS) have been most widely studied. APS plays its adjunct anticancer role by improving immune function^[16-19], counteracting the side effects of chemotherapeutic drugs^[12,15,20,21] and increasing the sensitivity of chemo-therapeutics^[13,17,18,22-25]. However, the mechanism underlying the adjunct anticancer property of APS, especially whether or not it involves the regulation of cytokines and reversal of MDR, is not completely clear.

Thus, the present study focuses on investigating the effect of APS on the expression of cytokines and P-GP in H22 tumor-bearing mice.

MATERIALS AND METHODS

Main reagents

APS (20 000-60 000 mol/L) was purchased from Shanxi Undersun Biomedtech Co. Ltd., China). Adriamycin (ADM), verapamil, and rifampicin (RFP) were purchased from the National Institutes for Food and Drug Control. Mouse interleukin-1 α (IL-1 α) enzyme-linked immunosorbent assay (ELISA) kit, mouse IL-2 ELISA kit, mouse IL-6 ELISA kit, mouse IL-10 ELISA kit and mouse tumor necrosis factor- α (TNF- α) ELISA kit, TaKaRa-RNA PCR Kit (AMV) Ver3.0, and TRIZOL reagent were purchased from Sigma Corporation, MO, United States. Goat anti-mouse IgG and fluorescein-affinity pure goat anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories. Oligonucleotides and reagents for polymerase chain reaction (PCR) assay were

purchased from Sigma Corporation.

Cell lines and culture

H22 hepatoma cells lines (purchased from Beijing Cowin Biotech Co. Ltd., Beijing, China) were cultured in cell culture vessels *in vitro*. The H22 cells were harvested and inoculated intraperitoneally for 9 d to the eight Kunming mice (KM). To establish the tumor-bearing mouse model, cells with ascites were harvested and inoculated subcutaneously into the right armpit region of of the mice.

Animals and trial groups

Male KM (age, 6-12 wk; weight, 18-22 g) were purchased from the Animal Center of the Third Xiangya Hospital. These animals were maintained at 25 ± 1 °C and $60\% \pm 5\%$ humidity under a 12 h light-dark cycle. All experimental animals were housed under specific-pathogen-free conditions for 1 wk to get accustomed to the surroundings before initiation of the experiment. They were allowed free access to food and water throughout the study. All experimental protocols described in this study were approved by the Ethics Review Committee for Animal Experimentation of Central South University.

Mice were randomly assigned to one of the seven groups ($n = 10$ each): ADM group, ADM + RFP group, normal saline (NS) group, ADM + APS (50 mg/kg) group, ADM + APS (100 mg/kg) group, ADM + APS (200 mg/kg) group, ADM + VER group. All the agents were administered by intraperitoneal injection (*ip*): ADM 1.25 mg/kg, 0.2 mL, *qd* \times 5 d; RFP 40 mg/kg, 0.2 mL, *qd* \times 10 d; NS 0.4 mL, *qd* \times 10 d; APS 50 mg/kg, 100 mg/kg, 200 mg/kg 0.2 mL, *qd* \times 10 d; and VER 1 mg/kg, 0.2 mL, *qd* \times 10 d. After ten days of treatment, the organ indexes and tumor inhibition rate were calculated, and compared with those of control group.

Modeling of the tumor-bearing mice

This model was created by subcutaneous injection of H22 cells as previously described^[26-28]. Briefly, the H22 cells with ascites were harvested, diluted to a concentration of 5.0×10^6 /mL with sterilized NS, and inoculated subcutaneously into the right armpit region of the mice. Each mouse was weighed immediately after inoculation. During this period, growth rate and tumor size were measured every two days by determining two perpendicular dimensions. Then the volume of each tumor was calculated using the following formula: volume = $1/2 \times \text{length} \times \text{width}$ ^[28,29]. When the tumors reached a size of 100 mm³ (excluding maximum and minimum values) the animals were treated as indicated, and were randomly assigned to one of the groups mentioned above. Forty-eight hours after the final administration of tested drug on the 10th day of the experiment, blood samples were collected from the mice's eyes and serum was harvested by centrifugation. Mice were killed by pulling and breaking of the cervical vertebra, and the whole body, tumor, spleen, and thymus were weighed immediately. Sera were stored at -70 °C and other specimens were stored in liq-

uid nitrogen for further analysis^[28,29].

Tumor inhibition rate and immune organ index

The inhibitory effect of experimental treatment on tumor growth was evaluated by tumor inhibition rate, and the influence of different drugs on the immune organs was evaluated by the immune organ index. The inhibitory rates of tumor growth were calculated as follows: inhibitory rate = $(1 - \text{average tumor weight in the experimental group} / \text{average tumor weight of control group}) \times 100\%$ ^[28,29]. The organ indexes of spleen and thymus were calculated as follows: organ index (%) = average weight of organ / (average body weight) \times 100%.

Measurement of cytokines

The serum levels of cytokines were determined by ELISA according to the manufacturer's instructions (eBioscience, United States). ELISA kits were employed for the measurement of the levels of IL-1 α , IL-2, IL-6, IL-10, and TNF- α .

Western blotting analysis

RFP (P-GP inducer) and VER (P-GP antagonist) were used as positive controls. Western blotting analysis was performed as previously described^[30-32]. Briefly, 200 mg of each tumor was frozen with liquid nitrogen and crushed in a mortar. The tumor samples were homogenized in a lysis buffer. Cells were washed twice with ice-cold PBS and total cell lysates were collected in sodium dodecyl sulfate (SDS) sample buffer (50 mmol Tris-HCl, pH 6.8, 100 mmol dithiothreitol (DTT), 2% SDS, 0.1% bromophenol blue, 10% glycerol). Cell lysates containing equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. After blocking in 5% non-fat milk in Tris-buffered saline with 0.1% Tween 20 (pH 7.6), membranes were incubated with the appropriate primary antibodies (goat anti-mouse IgG) at 4 °C, overnight, and exposed to the appropriate secondary antibody (goat anti-rabbit IgG) for 3 h at 37 °C. Immunoreactive proteins were visualized using the enhanced chemiluminescence system from Pierce (Rockford, IL, United States).

Quantitative real time reverse transcription-PCR

The *MDR1* mRNA expression level in the H22 tumor-bearing mice was measured using quantitative reverse transcription (QRT)-PCR. Briefly, total RNA was extracted using the TRIzol reagent following the manufacturer's instructions and reverse transcribed to cDNA using the Gene Amp RNA PCR kit in a DNA thermal cycler (Bio-Rad). QRT-PCR was performed with SYBR green PCR master mix in an ABI Prism 7700 real time PCR machine (Applied Biosystems, Foster City, CA, United States). The synthesized cDNA served as a template in a 25 μ L reaction. A non-template control was included in all experiments. Primer sequences were as follows: P-GP GenBank, sense: 5'-TAATGCGACAGG AGATAGGCT-3',

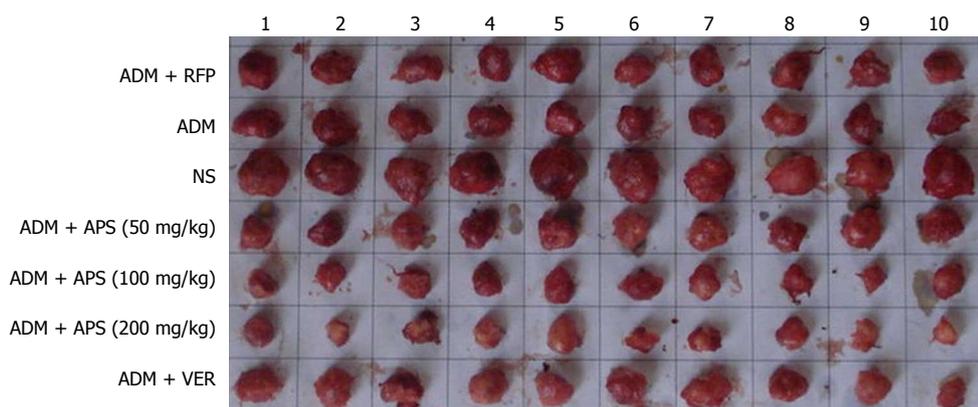


Figure 1 Solid tumors from tumor-bearing mice. ADM: Adriamycin; RFP: Rifampicin; NS: Normal saline; APS: Astragalus polysaccharides; VER: Verapamil.

Table 1 Tumor inhibition rate and immune organ index (n = 10)

Group	Tumor weight (g)	Inhibition rate (%)	Spleen index	Thymus index
ADM + RFP	5.37 ± 1.31 ^a	60.00	0.51 ± 0.28 ^a	0.15 ± 0.04
ADM	5.32 ± 2.03 ^a	60.36	0.39 ± 0.17 ^a	0.13 ± 0.04
NS	13.42 ± 1.03	-	0.74 ± 0.21	0.15 ± 0.09
ADM + APS (50 mg/kg)	3.64 ± 1.54 ^{a,c}	72.88 ^c	0.65 ± 0.22 ^c	0.15 ± 0.05
ADM + APS (100 mg/kg)	3.57 ± 1.66 ^{a,c}	73.40 ^c	0.62 ± 0.34 ^c	0.20 ± 0.06 ^c
ADM + APS (200 mg/kg)	3.01 ± 1.95 ^{a,c}	77.57 ^c	0.67 ± 0.20 ^c	0.47 ± 0.12 ^{a,c}
ADM + VER	4.75 ± 1.86 ^a	69.45	0.50 ± 0.17 ^a	0.13 ± 0.03

^aP < 0.05 vs normal saline (NS) group; ^cP < 0.05 vs adriamycin (ADM) group. RFP: Rifampicin; APS: Astragalus polysaccharides; VER: Verapamil.

and antisense: 5'-CCGCCATTTGA CTGAAAGAA-CAT-3'; GAPDH GenBank: sense: 5'-GAGTCAACGGA TTTGGTTCG-3', and antisense: 5'-CGGAAGATG-GTGATGGGATT-3'. QRT-PCR was performed at 94 °C for 4 min, followed by 40 cycles at 94 °C for 15 s, at 60 °C for 25 s, and at 72 °C for 25 s. Data were analyzed with Sequence Detector software (v1.9, Applied Biosystems, Foster City, CA, United States). The mean Ct value for duplicate measurements was used to detect the expression of target genes, which were normalized to a housekeeping gene, which was used as an internal control [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] according to the 2^{-ΔΔCt} formula.

Statistical analysis

For statistical analysis of cytotoxicity, results from P-GP and *MDR1* mRNA expression assays were analyzed using SPSS 14.0 software (v14, SPSS Inc. Chicago, IL, United States). Differences among the experimental groups were analyzed by one-way analysis of variance. The real-time PCR data were analyzed using the SDS software on the ABI PRISM[®]7700 sequence detection system at a confidence limit of 95%. A P < 0.05 was considered statistically significant.

RESULTS

General health status of mice

There was no abnormality detected in the daily behavior,

autonomic activities, ingestion, hydropsia, pelage, feces, or urine of any of the mice in each experimental group after drug administration. In addition, there was no abnormal secretion from the eyes, ears, noses, or mouths of the mice.

Tumor inhibition rates and the immune organ index

After mice were killed, the solid tumors were removed from tumor-bearing mice and are shown in Figure 1. The tumor inhibition rate and immune organ index are shown in Table 1. The mean weight of tumors in the treatment groups was significantly lower than in the NS group (P < 0.05). The mean weight of tumors in ADM + APS (50 mg/kg), ADM + APS (100 mg/kg), and ADM + APS (200 mg/kg) groups were significantly lower than in the ADM group (P < 0.05). The tumor inhibition rates in these groups were significantly higher than in the ADM group (P < 0.05). The spleen indexes in the ADM + RFP, ADM, and ADM + VER groups were significantly lower than that of the NS group (P < 0.05) and the thymus index of the ADM + APS (200 mg/kg) group was significantly higher than that of the NS group (P < 0.05). The spleen indexes of the ADM + APS (50 mg/kg), ADM + APS (100mg/kg), and ADM + APS (200mg/kg) groups were significantly higher than that of the ADM group (P < 0.05), and the thymus indexes of the ADM + APS (100 mg/kg), ADM + APS (200 mg/kg) group were significantly higher than the ADM group (P < 0.05). Collectively, these results showed that APS can act syner-

Table 2 Expression of interleukin-1 α , interleukin-2, interleukin-6, tumor necrosis factor- α , and interleukin-10 ($n = 10$)

Group	mean \pm SD (pg/mL)				
	IL-1 α	IL-2	IL-6	TNF- α	IL-10
ADM + RFP	5.89 \pm 2.12	6.11 \pm 2.9 ^a	10.99 \pm 2.09 ^b	9.00 \pm 1.21	55.98 \pm 2.43
ADM	4.63 \pm 3.2	5.23 \pm 2.12 ^a	10.78 \pm 3.13 ^a	8.01 \pm 1.22	54.01 \pm 2.33
NS	13.21 \pm 2.01 ^c	11.35 \pm 2.09 ^c	29.55 \pm 8.97 ^c	23.1 \pm 1.83 ^c	56.67 \pm 4.32
ADM + APS (50 mg/kg)	14.34 \pm 1.78 ^c	12.11 \pm 3.08 ^c	41.57 \pm 6.42 ^{a,c}	29.97 \pm 4.09 ^c	41.23 \pm 3.12 ^{a,c}
ADM + APS (100 mg/kg)	25.31 \pm 2.98 ^{a,c}	19.98 \pm 3.21 ^{a,c}	38.82 \pm 5.88 ^{a,c}	33.23 \pm 3.99 ^{a,c}	27.23 \pm 7.68 ^{a,c}
ADM + APS (200 mg/kg)	22.45 \pm 4.01 ^{a,c}	26.67 \pm 7.21 ^{a,c}	40.99 \pm 5.54 ^{a,c}	44.78 \pm 3.98 ^{a,c}	24.87 \pm 5.78 ^{a,c}
ADM + VER	7.34 \pm 2.11	5.77 \pm 2.11	9.34 \pm 1.75 ^a	9.11 \pm 2.01	50.66 \pm 1.12

^a $P < 0.05$ vs normal saline (NS) group; ^c $P < 0.05$ vs adriamycin (ADM) group. IL: Interleukin; TNF- α : Tumor necrosis factor- α ; RFP: Rifampicin; APS: Astragalus polysaccharides; VER: Verapamil.

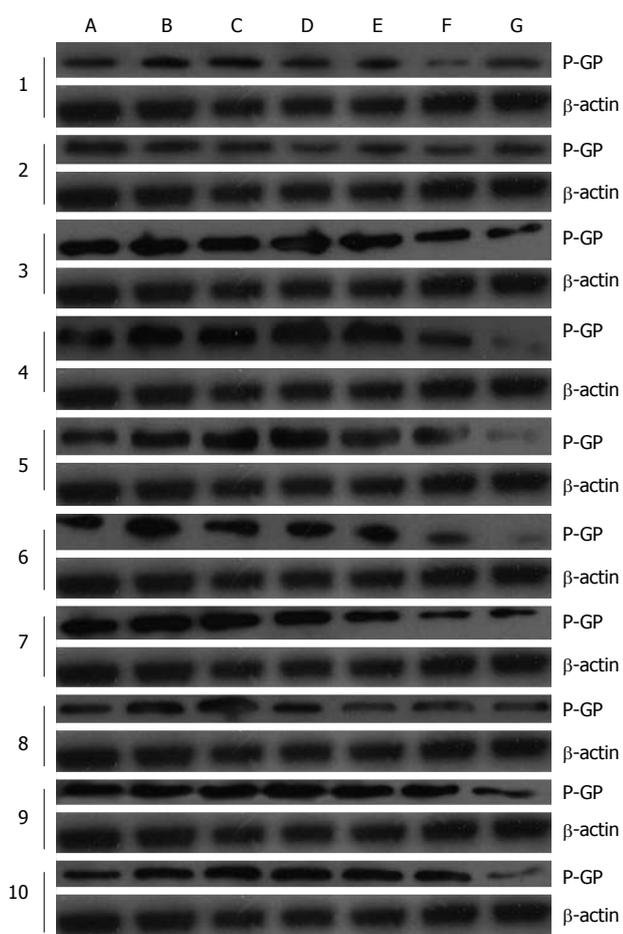


Figure 2 P-glycoprotein expression in tumor tissues after different chemotherapeutic treatment. A: Normal saline group (No.1-5); B: Adriamycin (ADM) group (No.1-5); C: ADM + rifampicin (RFP) group (No.1-5); D: ADM + astragalus polysaccharides (APS) (50 mg/kg) group (No.1-5); E: ADM + APS (100 mg/kg) group (No.1-5); F: ADM + APS (200 mg/kg) group (No.1-5); G: ADM + verapamil (VER) group (No.1-5); H: NS group (No. 6-10); I: ADM group (No. 6-10); J: ADM + RFP group (No. 6-10); K: ADM + APS (50 mg/kg) group (No. 6-10); L: ADM + APS (100 mg/kg) group (No. 6-10); M: ADM + APS (200 mg/kg) group (No. 6-10); N: ADM + VER group (No. 6-10). P-GP: P-glycoprotein.

gistically with AMD on tumor inhibition and can alleviate the decreased size of the spleen and thymus induced by AMD.

Expression levels of IL- α , IL-2, IL-6, TNF- α and IL-10

The expression levels of serum cytokines are shown in Table 2. The expression levels of IL-1 α , IL-2, IL-6, and TNF- α were significantly higher in the ADM + APS (50 mg/kg), ADM + APS (100 mg/kg), and ADM + APS (200 mg/kg) groups than in the NS group ($P < 0.05$), particularly in the medium- and high-dose groups. Cytokine levels were lower in the ADM group than in the NS group. The expression levels of IL-1 α , IL-2, IL-6, and TNF- α were also significantly higher ($P < 0.05$) in the ADM + APS (50 mg/kg), ADM + APS (100 mg/kg) and ADM + APS (200 mg/kg) groups than in the ADM group. The expression level of IL-10 was significantly lower ($P < 0.05$) in the ADM + APS (50 mg/kg), ADM + APS (100 mg/kg), and ADM + APS (200 mg/kg) groups than in either the NS or ADM groups. In summary, APS increased expression levels of IL-1 α , IL-2, IL-6, and TNF- α and decreased IL-10 levels.

Expression of P-GP in tumor tissue

The expression of P-GP in tumor tissue is shown in Figure 2. As assessed by computer-assisted gel analysis (Figure 3A), the ADM + RFP group exhibited a significantly higher P-GP expression than other groups. Different concentrations of APS and verapamil were found to down-regulate P-GP expression. APS down-regulated less P-GP expression than verapamil did. Also, APS (50-200 mg/kg) was found to down-regulate P-GP expression in a dose-dependent manner. The expression level of P-GP was significantly lower ($P < 0.05$) in the ADM + APS (200 mg/kg) and verapamil groups than in the ADM or ADM + RFP groups.

QRT-PCR detection of multidrug resistance 1 mRNA in tumor tissue

The expression of *MDR1* mRNA in tumor tissue is shown in Table 3 and Figure 3B. The ADM and ADM + RFP group showed significantly higher *MDR1* mRNA expression than the other groups ($P < 0.05$). Compared to the ADM group, the RFP group showed increased *MDR1* mRNA expression, and the VER group and APS groups showed decreased *MDR1* mRNA expression (P

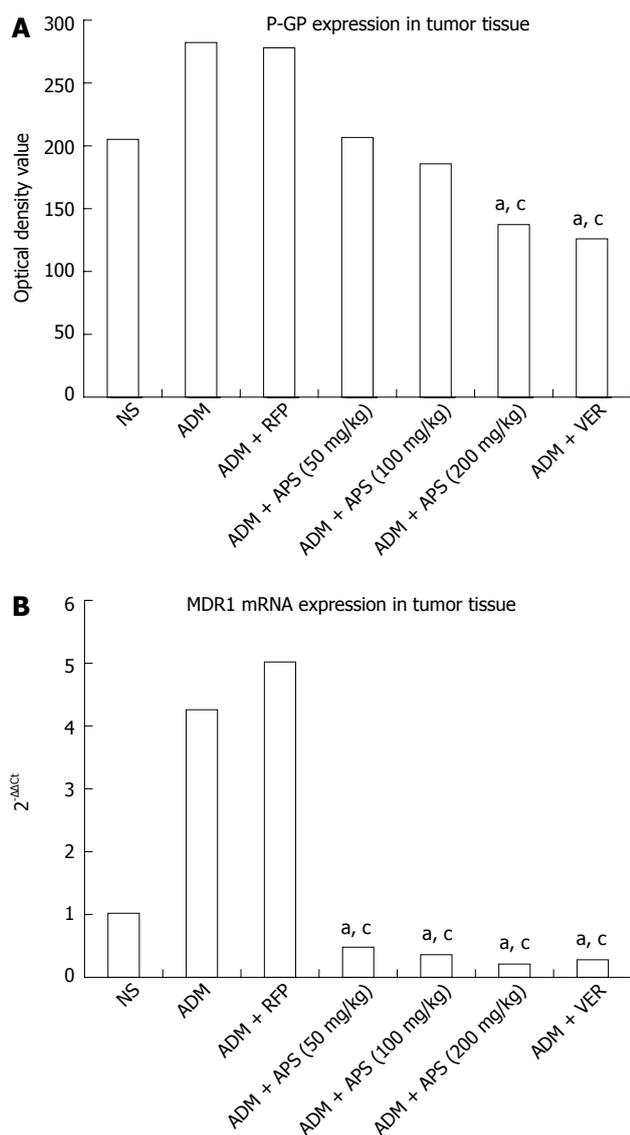


Figure 3 P-glycoprotein optical density values and *MDR1* mRNA expression in tumor tissues after different chemotherapeutic treatment. A: P-glycoprotein (P-GP) optical density values; B: *MDR1* mRNA expression. ^a*P* < 0.05 vs adriamycin (ADM) group; ^c*P* < 0.05 vs ADM + rifampicin (RFP) group. NS: Normal saline; APS: Astragalus polysaccharides; VER: Verapamil.

< 0.05). *MDR1* mRNA expression decreased with increasing concentrations of APS (50-200 mg/kg). *MDR1* mRNA expression was reduced in a dose-dependent manner.

DISCUSSION

Guo *et al.*^[24] reported that treatment with APS along with vinorelbine and cisplatin significantly improves quality of life in patients with advanced non-small-cell lung cancer over vinorelbine and cisplatin alone. Cui *et al.*^[22] reported that hepatocarcinogenesis could be prevented in rats fed with the aqueous extract of Astragalus, which is mainly composed of Astragalus polysaccharides. There are also reports that APS act as an adjunct anticancer agent^[13,18,23,25].

These results led the researchers to speculate that

Table 3 Multidrug resistance 1 mRNA expression in tumor tissue (2^{-ΔΔCt}) (*n* = 10)

Group	2 ^{-ΔΔCt} (mean ± SD)
ADM + RFP	5.02 ± 1.82
ADM	4.26 ± 1.51
NS	1.02 ± 0.05 ^{a,c}
ADM + APS (50 mg/kg)	0.48 ± 0.13 ^{a,c}
ADM + APS (100 mg/kg)	0.36 ± 0.03 ^{a,c}
ADM + APS (200 mg/kg)	0.21 ± 0.04 ^{a,c}
ADM + VER	0.28 ± 0.09 ^{a,c}

^a*P* < 0.05 vs adriamycin (ADM) group; ^c*P* < 0.05 vs ADM + rifampicin (RFP) group. NS: Normal saline; APS: Astragalus polysaccharides; VER: Verapamil.

the adjunct anticancer role of APS might be related to immune function enhancement. However, the mechanism underlying these effects remains to be determined. In particular, it is not completely clear whether APS is involved in the regulation of cytokines and reversal of MDR.

Our conclusions regarding the sensitivity to chemotherapy drugs were partially supported by the results of these studies. Compared with the ADM group, the mean weight of tumors was significantly decreased (*P* < 0.05) and the inhibition rates of tumors were significantly increased (*P* < 0.05) within the APS treatment range of 50 to 200 mg/kg. The spleen and thymus index were also significantly increased. Collectively, these results show that APS exerts a synergistic anti-tumor effect with ADM, and alleviates the decreased sizes of the spleen and thymus induced by ADM (Table 1). The cytokines IL-1α, IL-2 and IL-6 are capable of inducing the proliferation of responsive T-cells. TNF-α has been proven to be an effective anticancer agent in *in vitro* and *in vivo* preclinical studies, by inducing apoptotic cell death and tumor necrosis. IL-10 inhibits the synthesis of IL-2 and TNF-α produced by activated macrophages and by helper T cells^[33]. In the present study, APS was found to induce increase in IL-1α, IL-2, IL-6, and TNF-α expression and decrease in IL-10 expression (Table 2). APS effect on cytokine levels may be one of its adjunct anticancer mechanisms.

However, tumor immunology is a complex biological phenomenon, with the secretion, function and regulation of cytokines occurring through multiple mechanisms, and is mediated by a wide variety of cell populations. How APS induces expression of these cytokines merits further study.

It has been shown that drug resistance in tumor cells are related to *MDR1* upregulation and P-GP over expression^[34-36]. As a P-GP substrate, ADM can induce P-GP expression and consequently reduce its efficacy. In the present study, APS was found to enhance the chemosensitivity to ADM of H22 tumor-bearing mice. To determine whether APS is involved in P-GP expression, the P-GP inducer rifampin and P-GP inhibitor verapamil were used as positive controls. Western blotting analysis of P-GP expression and real-time PCR detection

of *MDR1* mRNA expression in tumor tissue revealed that APS (50 to 200 mg/kg) reduced P-GP protein and *MDR1* mRNA expression in a dose-dependent manner (Figures 2, 3 and Table 3). The expression levels of P-GP and *MDR1* were significantly decreased ($P < 0.05$) in the ADM + APS (200 mg/kg) treatment compared with the ADM or ADM + RFP treatment groups ($P < 0.05$). In the present study, APS was found to down-regulate *MDR1* mRNA and P-GP expression levels, thereby increasing the intracellular concentration of chemotherapeutic drugs. This may be the mechanism behind its secondary anti-cancer effects.

It has been reported that APS exhibits several therapeutic advantages in terms of increasing sensitivity to chemo-therapeutics, reducing the side effects and complications associated with chemotherapy^[12,15,20,21] and improving patient quality of life and survival time^[24]. In addition, our present study reveals that APS can regulate cytokine and P-GP expression levels. Thus, APS is a promising candidate for therapeutics that exhibit a low toxicity and few side effects.

In summary, APS was found to exert a synergistic anti-tumor effect with Adriamycin in H22 tumor-bearing mice *in vivo*. This may be related to its ability to enhance the expression of IL-1 α , IL-2, IL-6, and TNF- α , decrease IL-10, and down-regulate *MDR1* mRNA and P-GP expression levels.

COMMENTS

Background

Cancer has been a major public health problem globally. Traditional Chinese medicine has great advantages in terms of increasing sensitivity to chemotherapeutics, reducing side effects and complications associated with chemotherapy, and improving patient quality of life and survival. Astragalus membranaceus is widely accepted as a complementary and alternative medicine in cancer treatment. Astragalus polysaccharides (APS) are active constituents of Astragalus membranaceus.

Research frontiers

APS has been most widely studied mainly for its immunopotentiating properties, ability to counteract the side effects of chemotherapeutic drugs, and adjunct anticancer agent properties. However, the mechanism underlying the adjunct anticancer property of APS, specifically whether or not it involves the regulation of cytokines and reversal of multidrug resistance (MDR), is not completely clear. The present study focused on investigating the effect of APS on the expression of cytokines and P-glycoprotein (P-GP) in H22 tumor-bearing mice *in vivo*.

Innovations and breakthroughs

To date, there have been a limited number of studies regarding the adjunct anticancer property of APS. In the present study, APS was found to exert a synergistic anti-tumor effect with Adriamycin in H22 tumor-bearing mice. This may be related to its ability to enhance the expression of interleukin-1 α (IL-1 α), IL-2, IL-6, and tumor necrosis factor- α , decrease IL-10, and down-regulate *MDR1* mRNA and P-GP expression. This is the first study to report that the adjunct anticancer property of APS is partly through regulation of cytokines and P-GP expression in H22 tumor-bearing mice.

Applications

Traditional Chinese medicine has great advantages in terms of increasing sensitivity to chemo-therapeutics, reducing side effects and complications associated with chemotherapy, and improving patient quality of life and survival. APS could regulate cytokines and P-GP expression, and in the search for new cancer therapeutics with a lower toxicity and few side effects, APS is a promising candidate.

Terminology

APS, extracted and purified from Chinese medicinal herb Astragalus membranaceus roots, consist of several classes of neutral and acidic polysaccharides and glycoproteins. APS has also been observed to possess anti-tumor and anti-virus activities. Cytokine is a small protein released by cells and can exert a specific effect on the interactions between cells, on communications between cells or on the behavior of cells. P-GP is a plasma membrane protein which acts as a localized drug transport, actively exporting drugs out of the cell. Adjunct anticancer agents are a variety of medications that complement the chemotherapy regimen, help alleviate side effects of the treatment or symptoms of the cancer, or help potentiate the effect and activity of chemotherapy drugs.

Peer review

The manuscript reports on a possible adjunct anticancer effect of APS in tumor-bearing mice, using an *in vivo* animal model. Data on tumor inhibition were provided, and its possible effects on cytokines, *MDR1* mRNA and P-GP expression were elucidated. APS may be a promising candidate for cancer therapeutics that exhibits a low toxicity and few side effects.

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Establishment of an orthotopic transplantation tumor model of hepatocellular carcinoma in mice

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Abstract

AIM: To improve the outcome of orthotopic transplantation in a mouse model, we used an absorbable gelatin sponge (AGS) in nude mice to establish an orthotopic implantation tumor model.

METHODS: MHCC-97L hepatocellular carcinoma (HCC)

cells stably expressing the luciferase gene were injected into the subcutaneous region of nude mice. One week later, the ectopic tumors were harvested and transplanted into the left liver lobe of nude mice. The AGS was used to establish the nude mouse orthotopic implantation tumor model. The tumor suppressor gene, paired box gene 5 (*PAX5*), which is a tumor suppressor in HCC, was transfected into HCC cells to validate the model. Tumor growth was measured by bioluminescence imaging technology. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) and histopathology were used to confirm the tumorigenicity of the implanted tumor from the MHCC-97L cell line.

RESULTS: We successfully developed an orthotopic transplantation tumor model in nude mice with the use of an AGS. The success rate of tumor transplantation was improved from 60% in the control group to 100% in the experimental group using AGS. The detection of fluorescent signals showed that tumors grew in all live nude mice. The mice were divided into 3 groups: AGS-, AGS+/*PAX5*- and AGS+/*PAX5*+. Tumor size was significantly smaller in *PAX5* transfected nude mice compared to control mice ($P < 0.0001$). These fluorescent signal results were consistent with observations made during surgery. Pathologic examination further confirmed that the tissues from the ectopic tumor were HCC. Results from RT-PCR proved that the HCC originated from MHCC-97L cells.

CONCLUSION: Using an AGS is a convenient and efficient way of establishing an indirect orthotopic liver transplantation tumor model with a high success rate.

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Key words: Hepatocellular carcinoma; Orthotopic transplantation tumor model; Absorbable gelatin sponge; Nude mice; Bioluminescence imaging

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common types of malignant tumors^[1-3]. Thus, establishing appropriate animal models is critical to promote our understanding of the molecular, cellular and pathophysiological mechanisms of HCC, and is essential for the development of new therapeutic strategies. Most HCC patients are diagnosed at an advanced stage when available treatments are ineffective. The ideal HCC animal model should display similarities to the human disease and accurately recapitulate the disease with a high success rate^[4]. Currently, the most commonly employed models of HCC are xenograft models, including subcutaneous and orthotopic transplantation in nude mice^[5-9]. Orthotopic transplantation of liver tumors allows the development of tumor metastases and provides relevant sites for host-tumor interaction and a microenvironment. Although this method is well accepted, its use is still quite limited. This is because construction of the model is complex, and a high level of technical skill is required. This model would be more popular if the surgical procedure could be simplified.

The goal of this study was to investigate the value of using an absorbable gelatin sponge (AGS) in HCC orthotopic transplantation, compared with the routine procedure. Previous research has shown that paired box gene 5 (*PAX5*) acts as a tumor suppressor in the development of HCC^[10]. Therefore, in this experiment, we also over-expressed *PAX5* in the xenograft tumor to validate the efficiency of the liver orthotopic transplantation tumor model in real time.

MATERIALS AND METHODS

Cell line

The human hepatocellular carcinoma cell line MHCC97L (a kind gift from the Liver Cancer Institute, Fudan University) was used in this study. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Rockville, MD, United States) with 10% fetal bovine serum (Life Technologies, Carlsbad, CA, United States), 100 mg/mL penicillin G, and 50 µg/mL streptomycin (Life Technologies) at 37 °C in a humidified atmosphere containing 5% CO₂. MHCC97L cells stably

expressing the luciferase gene were constructed.

PAX5 expression plasmid construction and transfection

Complementary DNA corresponding to full-length *PAX5* was obtained by reverse transcription-polymerase chain reaction (RT-PCR) amplification of normal human stomach cDNA with primers specific to *PAX5*. The PCR aliquots were subcloned into the mammalian expression vector pcDNA3.1-TOPO TA (Life Technologies)^[11]. Either pcDNA3.1 or pcDNA3.1-*PAX5* was transfected into MHC-C97L cells using Lipofectamine 2000 (Life Technologies). Positive clones were selected in DMEM supplemented with G418 at a concentration of 500 µg/mL.

Animal model

MHCC97L cells (1×10^7 cells in 0.1 mL PBS) transfected with *PAX5* or pcDNA3.1 were subcutaneously injected into the dorsal left flank of 4-wk-old male Balb/c nude mice (1/group); the body weight of all mice was between 12 and 15 g. Six-week-old athymic male Balb/c nude mice weighing 16-18 g were used for xenograft tumor transplantation. The nude mice were obtained from the Chinese University of Hong Kong Laboratory Animal Service Center and maintained under specific pathogen free (SPF) conditions. All experimental procedures were approved by the Animal Ethics Committee of the Chinese University of Hong Kong.

Establishment of an orthotopic transplantation tumor model of HCC in nude mice

An orthotopic HCC mouse model was established to determine intrahepatic tumorigenicity. Subcutaneous tumors were harvested when they reached 1 cm in diameter and were cut into pieces under aseptic conditions. After washing with PBS, the tumors were rinsed in DMEM. Only intact pieces were chosen for further study. After removing thanatosis tissues, the tumors were cut into cubes 1 mm³ in size. One piece was then implanted into the left liver lobe of each mouse. The mice were divided into 3 groups. The ten mice in group I were operated without an AGS. The wounds were sutured using VICRYL Plus Suture 5-O (Ethicon Inc, Somerville, NJ, United States) Absorbable gelatin sponges were used in groups II and III. The mice in groups II ($n = 6$) and III ($n = 6$) underwent tumor transplantation with or without *PAX5* expression. Intraperitoneal anesthesia was administered using 20 g/L pentobarbital sodium at 0.04 mL per gram body weight. Once anesthetized, the mice were fixed on an experiment board in the supine position. After sterilizing with 70% alcohol, a 2 mm transverse incision was made below the xiphoid, which was perpendicular to the median line and was 1-1.5 cm long. The left liver lobes were carefully pulled out of the abdominal cavity with a sterile cotton swab. A 0.2 cm incision was made in Glisson's capsule to serve as the transplantation site. The tumor tissue mentioned above was transplanted into recipient mouse livers using extra-fine forceps.

In group I, a routine procedure was carried out to

suture the incision in the liver and stop bleeding, and homeostasis was achieved by compression. In group II and group III, in addition to compression, the transplantation site was further covered by a 5 mm × 5 mm × 1.5 mm AGS (Nanjing Jinglin Biopharming Co. Ltd., Nanjing, Jiangsu, China). The liver was placed back into the enterocoelia using a sterile cotton swab, and shifting of the AGS was not allowed. If neither bleeding nor tumor tissue leakage occurred, then the skin was sterilized with 70% alcohol and the wound sutured with a Plus 5-0 suture line. The average operation time was 10 min. Following surgery, the mice were kept warm using a heat lamp and allowed to recover. The recovery period was reduced from 30 ± 5 min to 20 ± 5 min at room temperature. Following recovery, the mice were put back into their cages and raised under SPF conditions. All mice had free access to sterilized food and autoclaved water. We inspected the living conditions of the mice daily.

Mouse tumor bioluminescence imaging

For bioluminescence imaging, the animals were injected with the luciferase substrate D-luciferin at a dose of 150 mg/kg in 0.2 mL sterile isotonic saline.

Liver tumor growth rates were monitored using the Xenogen International Veterinary Information Service (IVIS) imaging system every week for 3 wk. All images were obtained after intraperitoneal injection of luciferin (100 mg/kg body weight; Synchem, Elk Grove Village, IL, United States). Ten minutes after injection of luciferin, nude mice were placed onto the Xenogen IVIS 200 imaging stage and were continuously sedated during image acquisition. Image analysis and bioluminescence quantification were performed using Living Image software (Caliper Life Sciences, Hopkinton, MA, United States). Once imaging was complete, each animal was removed from the Xenogen IVIS 200 imaging stage, placed on a heated platform in its original cage and allowed to recover. After the animals had fully recovered from the anesthesia, they were returned to the specific investigator.

After 3 wk, the mice were sacrificed. Following tumor excision from the euthanized mice, tumor weights were measured the same day. A portion of the tumor tissue was fixed in 10% formalin for subsequent histological examination, and the remaining tissue was snap-frozen in liquid nitrogen and stored at -70 °C for molecular studies.

Semi-quantitative RT-PCR

Total RNA was extracted from tissues using QIAzol reagent (Qiagen, Valencia, CA, United States). cDNA was synthesized from 2 µg total RNA using Transcriptase Reverse Transcriptase (Roche Applied Sciences, Indianapolis, IN, United States). Semi-quantitative RT-PCR analyses were performed using *PAX5* forward primer 5'-GTC-CATTCCATCAAGTCCTG-3' and *PAX5* reverse primer 5'-TTGCTGACACAACCATGGCT-3'. β -actin was used as an internal control for mRNA expression (5'-GTCTTCCCCTCCATCGTG-3' and 5'-AGGGT-GAGGATGCCTCTCTT-3').

Histologic evaluation

Formalin-fixed tumor was embedded in paraffin, and 4 µm sections were cut and stained with hematoxylin and eosin (HE) as previously described^[12].

Statistical analysis

Data were presented as mean ± SD. A repeated measures analysis of variance was used to compare the differences between groups. All analyses were performed using SPSS software (version 13.0, Chicago, IL, United States). Statistical significance was accepted at the level of $P < 0.05$.

RESULTS

One day after surgery, only six of ten mice in group I (without AGS) were alive after transplantation. The survival rate using the routine suture method was only 60%. In contrast, all mice in groups II and III were alive and recovered well. From the 7th day after surgery, the nude mice were scanned in the Xenogen IVIS 100 imaging system for a total of 3 wk. Fluorescence signals showed that transplanted tumors grew in all nude mice (Figure 1).

The fluorescence signal intensity was related to tumor size. The signal intensity steadily increased from the second week to the fourth week. Tumor size was significantly smaller in the *PAX5*-transfected nude mice from group III compared to the vector control mice from group II ($P < 0.0001$) (Figure 2). This indicated that *PAX5* gene expression inhibited tumor growth in nude mice (Figure 3). The area around the tumor and the surrounding tissues was euangiotic, and adjacent organs were strongly adhered.

HE staining further confirmed tumorigenesis of the liver tissue (Figure 4). Tumor tissue characteristics included atypia, big core, dark color, different cell size, irregular form, and nuclear division. Many of these characteristics were similar to those of the original cancer cells.

RT-PCR results showed that no *PAX5* expression was observed in the tissue from group II, and all the tissues from group III had a high *PAX5* mRNA level. This demonstrated that the HCC cells originated from MHCC-97L cells transfected with or without the *PAX5* expression plasmid (Figure 5), and the orthotopic liver tumor model was successfully established. We did not detect any intrahepatic or abdominal metastases.

DISCUSSION

Currently, the most common way of producing liver orthotopic tumor models is either by implantation of an established subcutaneous tumor harvested from another nude mouse, or by injecting tumor cells directly into the liver^[6,7]. Both strategies require surgery following liver exposure. The tumor cell injection animal model is more relevant to clinical practice; thus, it can be a useful research model for lymph node metastasis^[13]. However, the technical difficulty associated with this model is that it is

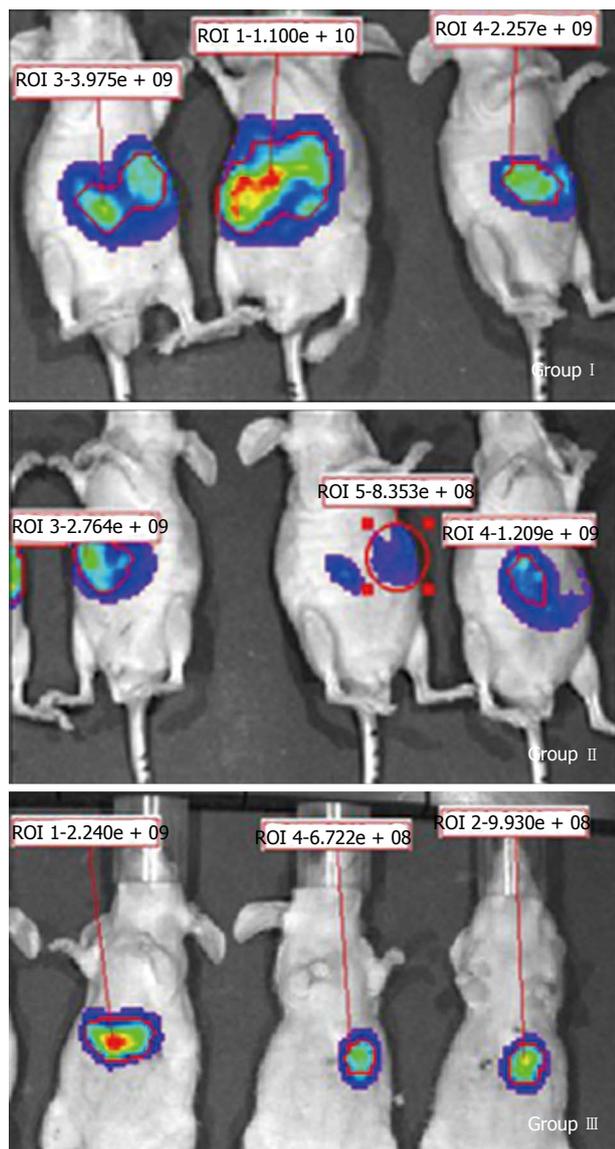


Figure 1 Representative images of fluorescence signals in nude mice detected by the International Veterinary Information Service system. The fluorescence signal intensity shows xenograft tumor size.

hard to control tumor cell leakage during injection. Analyses are complicated by the fact that apparent metastasis may just be due to leakiness.

In the second method, the goal is to establish a subcutaneous model of human HCC in nude mice, and then to inoculate the liver in order to establish the orthotopic transplantation model. The principle is to make the HCC cells adapt to the environment and grow into tumor tissues, then implant the tumor into the host's liver. Thus, the survival rate of the transplanted tumor is supposed to increase.

Compared with subcutaneous and intraperitoneal xenografts in nude mice, the properties of invasion and metastasis in the orthotopic liver tumor model are better^[14-16]. Use of this model is limited due to the need for a highly complex surgical technique, especially in bleeding control. Routine surgery using incision suture takes

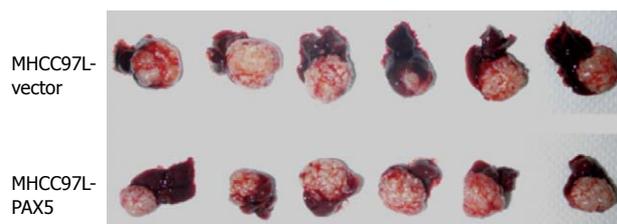


Figure 2 Harvesting orthotopic liver transplantation tumors. Tumors were harvested from the mice in groups II and III. Tumor size and weight in the paired box gene 5 (PAX5) group were smaller than those in the control group ($P < 0.0001$).

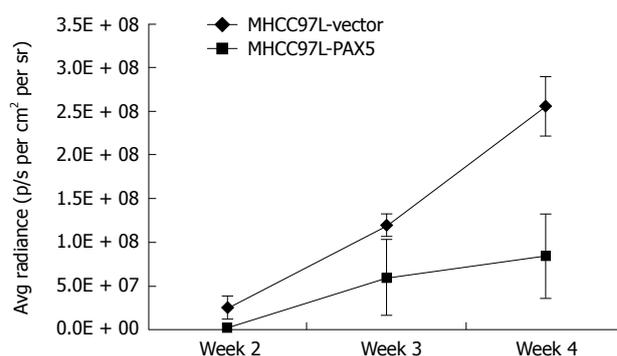


Figure 3 Tumor growth curve determined by the International Veterinary Information Service system. The P value was calculated using repeated measures analysis of variance. PAX5: Paired box gene 5.

a long time. This is one of the reasons why the mice in group I had a high mortality rate. If post-operative hemorrhage occurred, the mice would probably die, leading to failure of the long-term experiment. Thus the efforts to obtain the cell culture, establish a subcutaneous tumor and orthotopic tumor implantation would be in vain. An alternative method of achieving homeostasis is by suturing Glisson's capsule at the transplantation site. However, the mouse liver has an abundant blood supply, and the tissue is fragile and easily crushed. The method is further complicated by the possibility of bleeding at the suture site. Therefore, not many laboratory staff are capable of performing this procedure. To circumvent these difficulties, we used the AGS. The AGS is commonly used in surgery to stop minor bleeding at the surgical site. It is an aseptic solid material that has good deformability. In our experiment, covering the transplantation site with an AGS was an easy and effective way of maintaining homeostasis. The sponge was slowly absorbed and the cost was minimal. In addition, the sponge prevented the implanted tumor from moving out of the host's liver, increasing the success rate of the experiment. This procedure was safe, and none of the nude mice experienced post-operative complications or mortality, compared with the high post-operative mortality of 40% in group I. Importantly, adverse reactions to the sponge were not observed in normal tissues, as evaluated by HE staining. The gelatin was gradually absorbed and was absent when the mice were sacrificed.

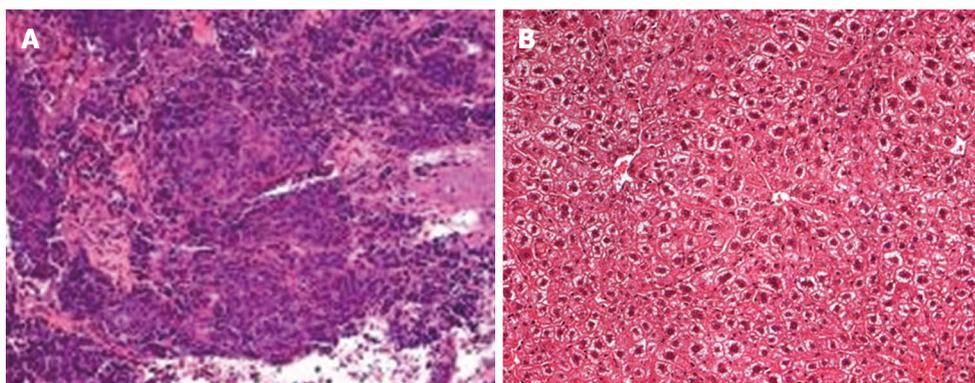


Figure 4 Liver tissues from nude mice were detected by hematoxylin and eosin staining. A: Liver tumor, $\times 200$; B: Normal liver, $\times 200$.

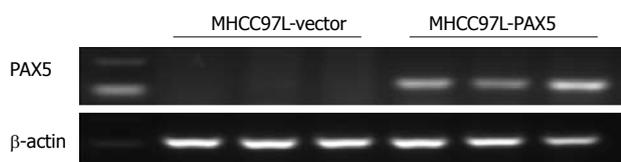


Figure 5 Gene expression of paired box gene 5 in liver tumor tissues of mice *in situ* which was detected by reverse transcription-polymerase chain reaction. The expression of β -actin which acted as the internal control was determined. PAX5: Paired box gene 5.

The horizontal incision made during the operation should neither be too low nor too small. The optimal incision should be as small as possible to fully expose the left liver lobe. If it is too small, the liver could be crushed when pulled out, or there may not be enough space to place the AGS into the abdominal cavity. The size of the sponge should be appropriate-5 mm \times 5 mm \times 1.5 mm, enough to efficiently stop bleeding and be easily absorbed.

It should be noted that although we observed a high post-operative mortality in group I, all mice achieved homeostasis before closure of the abdominal cavity. One of the reasons for this high mortality may have been due to the fast post-operative recovery and frequent activities caused by pain. Administration of post-operative analgesics may reduce pain, thus might lead to a higher survival rate^[17-19]. However, analgesics were not administered in this study. We would also like to point out that in models established by the direct injection of cultured human HCC cells, an AGS may not prevent leakage. In such models, HCC cells are suspended in medium rather than forming a solid tumor. The sponge is hygroscopic, thus, if leakage occurs, the sponge would only aggravate the leak.

Post-operative evaluation of orthotopic tumors is also a challenge. Monitoring subcutaneous liver transplantation tumors may be performed either by touch or by visual inspection. Orthotopic tumors can be monitored by laparotomy, computed tomography (CT)/magnetic resonance imaging (MRI)/positron emission tomography (PET)-CT scanning, or using a high frequency ultrasound mini-probe. However, there are drawbacks associated

with each method. Frequent laparotomy may cause severe injury and death in mice. CT/MRI/PET-CT scanning is non-invasive, but the cost is high and is difficult to perform^[20-23]. Ultrasonic examination is less expensive, but analysis of the data requires a skilled operator^[24-26]. Finally, only some of the above-mentioned methods are quantitative.

Using the IVIS imaging system, bioluminescence was non-invasively measured in transplanted subcutaneous tumors using the IVIS imaging system (Xenogen Corp, Alameda, CA, United States) following injection of the luciferase substrate luciferin. Tumor growth was continuously monitored. According to the bioluminescence data, tumor growth conditions were analyzed in real time. The results showed successful tumor transplantation. Histopathology and PCR analyses showed that the transplanted tumor had the same phenotype as the liver tumor cell line MHCC-97L. Thus, the orthotopic transplantation tumor model was successfully established from the subcutaneous model of human HCC.

Based on our procedure, this operation has been simplified and can be completed in a very short period of time. The success rate is high, and reproducibility is good. Importantly, the model maintained the characteristics of the original cell line. Inspections were quantified in real time, and the model can be adapted for general use.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors and is the third leading cause of cancer-related death. The establishment of appropriate animal models of this disease could help people to understand the mechanisms driving HCC, leading to potential therapeutics and improvements in patient survival. An absorbable gelatin sponge was used as an efficient way of establishing an indirect orthotopic liver transplantation tumor model with a high success rate.

Research frontiers

It is reported that the conventional surgical method involves the liver capsule being sutured using microsurgery. Compared with the microsurgical method, the authors used an absorbable gelatin sponge which prevented bleeding and tissues being crushed during the experiment.

Innovations and breakthroughs

This is the first report on the use of an absorbable gelatin sponge to cover the transplantation site in the liver capsule. This experiment showed that the

absorbable gelatin sponge can be used to constrict the liver and stop bleeding, and to efficiently cover tumor tissue, thus, avoiding the tumor tissue moving out of the placement site.

Applications

Using the absorbable gelatin sponge to establish the HCC model is a convenient method for researchers to study HCC. The establishment of an animal model could provide a new way to perform more successful, convenient and efficient experiments.

Terminology

An absorbable gelatin sponge is a sterile, absorbable, water-insoluble, gelatin-base material which absorbs blood and provides an area for clot formation, and is used for hemostasis during surgery.

Peer review

Authors found a new technique to do HCC animal model experiment. It is easy to perform and has a high success rate. The study has been well conducted and provided further validation.

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Associations between interleukin-1 polymorphisms and gastric cancers among three ethnicities

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Abstract

AIM: To investigate the associations between interleukin (IL)-1B and IL-1RN polymorphisms and gastric cancers among the Tibet, Hui and Han ethnicities.

METHODS: Genomic DNA was extracted from peripheral blood of 210, 205, and 202 healthy volunteers and from 155, 158, and 197 gastric cancer patients from the Tibet, Hui, and Han populations, respectively. Polymorphisms in *IL-1B* and *IL-1RN* were analyzed by denaturing high-performance liquid chromatography.

RESULTS: Carriers of the *IL-1B*-31 CC genotype had an increased risk of intestinal type gastric cancer [odds ratio (OR) = 2.17, $P = 0.037$] in the Tibet ethnicity.

Carriers of the *IL-1B* 2/L genotype had an increased risk of both intestinal and diffuse types of gastric cancer (OR = 2.08, 2.31, $P = 0.007$, 0.016, respectively) in the Hui ethnicity. In the Han population, carriers of the *IL-1B*-31 CC, *IL-1B*-511CT, TT genotypes had increased risk of intestinal type gastric cancer (OR = 2.51, 2.74, 5.66, $P = 0.005$, 0.002, 0.000, respectively).

CONCLUSION: *IL-1B* and *IL-1RN* genotypes may differentially contribute to gastric cancer among the Tibet, Hui, and Han ethnicities in the Qinghai area of China.

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Key words: Gastric cancer; Interleukin-1B; Interleukin-1RN; Polymorphism; Risk of gastric cancer

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Zhao JD, Geng PL, Li ZQ, Cui S, Zhao JH, Wang LJ, Li JZ, Ji FX, Li GY, Shen GS, Lin MZ, Shen CF, Cao CZ. Associations between interleukin-1 polymorphisms and gastric cancers among three ethnicities. *World J Gastroenterol* 2012; 18(47): 7093-7099 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i47/7093.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i47.7093>

INTRODUCTION

Gastric cancer (GC) is the second leading cause of cancer-related death worldwide, and approximately 42% of these deaths occur in China^[1,2]. Mortality of GC patients in China is the highest in the world, especially in the northwestern part of the country, which includes Qing-

hai province^[3]. There are 56 different ethnicities living in China. The Han ethnicity represents the major ethnicity within Qinghai province, while the Tibet and Hui are minority nationalities. The incidence of GC in the Tibet and Hui populations is higher than that in the Han ethnicity. However, the study of minority ethnicities is not advanced due to fewer individuals and poorer economic conditions^[4].

Interleukin (IL)-1B and IL-1RN belong to the *IL-1* gene cluster. The *IL-1* gene encodes both the glycoprotein IL-1 β , which is a pro-inflammatory cytokine, and the *IL-1* receptor antagonist (IL-1Ra), which is an anti-inflammatory cytokine. *IL-1B* is a potent inhibitor of gastric acid secretion and plays a major role in both initiating and amplifying the inflammatory response to *Helicobacter pylori* (*H. pylori*) infection^[5-7]. *IL-1RN* encodes the *IL-1Ra*, an anti-inflammatory cytokine that competitively binds to IL-1 receptors and modulates the potentially damaging effects of IL-1^[7,8]. Two biallelic polymorphisms in the *IL-1B* gene have been described, both C-T base transitions found at positions-511 (C>T) and -31 (T>C) bp from the translation initiation codon. IL-1RN has a variable number of identical tandem repeats polymorphism of 86 bp in intron 2. To date, over 50 studies have reported on the association between *IL-1B* and *IL-1RN* polymorphisms and GC risk^[5]. While some studies have reported that *IL-1B* and *IL-1RN* polymorphisms are associated with increased GC risk in both Caucasians and Asians^[5,6,9,10], other studies have shown inverse associations, especially in Asians^[11,12]. Two studies chose a single ethnicity population in two different regions with different prevalence rates of GC as their subjects^[10,13]. However, no study has examined several different ethnicities in a single geographical area at the same time. Thus, our study is the first to report such an examination.

Here, we investigated the associations between *IL-1B-31*, *IL-1B-511* and *IL-1RN* polymorphisms and GC risk among the Tibet, Hui and Han ethnicities in the Qinghai area of China.

MATERIALS AND METHODS

Study subjects

Three ethnicities (Tibet, Hui and Han) from the Qinghai province of China were included in this study. Healthy controls included 210 Tibet, 205 Hui and 202 Han individuals who were enrolled from the Hainan Tibet Ethnicity Autonomous Prefecture, Minhe Hui Ethnicity Autonomous County, and Xining city in Qinghai province, respectively. Between December 2008 and October 2011, 155, 158 and 197 Tibet, Hui and Han individuals, respectively, with GC were enrolled from the Affiliated Hospital of Qinghai University. All recruited healthy controls were from families that had lived for a long time in that locality, did not marry other ethnicities for at least three generations, and were not related to each other. Both the age and sex of the healthy controls were matched to the patients and are shown in Table 1. None of these

subjects had a history of systemic lupus erythematosus, diabetes mellitus, rheumatoid arthritis, or inflammatory bowel disease. Subjects with a family history of any cancer were excluded. All patients were histologically confirmed as having noncardiac GC. Patients and controls were interviewed with regard to smoking status. Individuals who smoked once a day for over 1 year were defined as smokers. The presence of *H. pylori* infection in the sera of patients and controls was measured using an enzyme-linked immunosorbent assay (Anti-*H. pylori* enzyme immunoassay, Huamei Biotech Inc., China). This study was approved by the Clinical Research Ethics Committee of the Qinghai University of Medical Sciences, and all patients provided signed informed consent.

Analysis of the *IL-1B-31*, *IL-1B-511* and *IL-1RN* polymorphisms

Genomic DNA was isolated from 5 mL of venous blood by the conventional proteinase K digestion and phenol/chloroform extraction method. Polymorphisms were analyzed by polymerase chain reaction (PCR)-based denaturing high-performance liquid chromatography (DHPLC). The corresponding primers have been described by Lu *et al.*^[14] and are shown together with the PCR conditions, PCR annealing temperatures and DHPLC detection methods in Table 2. PCR was performed with a 25 mL reaction mixture containing 100 ng of genomic DNA, 1.0 mmol/L of primer, 0.2 mmol/L of dNTP, 2.0 mmol/L of MgCl₂, and 1.0 U Taq DNA polymerase in 1 \times reaction buffer (Promega, Madison, WI, United States). DHPLC analysis was performed on a Transgenomic WAVE System. The detailed genotyping process has been previously described^[14]. The PCR products were applied to the DHPLC column at an optimal oven temperature and eluted with a linear acetonitrile gradient at a flow rate of 0.9 mL/min (Figure 1A). The genotypes identified by DHPLC analysis were further confirmed by DNA sequencing using the ABI Prism 377 DNA Sequencer. The sizes of *IL-1RN* PCR products were analyzed by DHPLC based on the relationship between elution time and base pair number of the fragment (Figure 1B).

H. pylori antibody assays

Enzyme-linked immunosorbent assay for detection of *H. pylori* was performed according to the manufacturer's instructions. After termination of the enzyme reaction, the absorbance at 630 nm was measured. Absorbance ratios (sample/negative control) equal to or greater than 2.1 were considered positive, and those below 2.1 were considered negative.

Statistical analysis

The data were analyzed using SPSS software (Version 13.0, SPSS, Chicago, IL, United States). The significance of the difference in the distribution of the polymorphisms among the different groups was calculated using the χ^2 test. All allelic distributions were examined for deviations from their corresponding Hardy-Weinberg

Table 1 Primer sequences, polymerase chain reaction and denaturing high-performance liquid chromatography conditions for detection of gene polymorphisms

Gene	Primer sequence	PCR annealing temperature (°C)	PCR product size (bp)	DHPLC application type	Oven temperature (°C)
<i>IL-1B-31</i>	F: AGAAGCTCCACCAATACTC	60	240	Mutation	59
	R: AGCACCTAGTTGTAAGGAAG				
<i>IL-1B-511</i>	F: TGGCATIGATCTGGTTCATC	58.5	306	Mutation	60.5
	R: GTTTAGGAATCTTCCCACCTT				
<i>IL-1 RN</i>	F: CCCCTCGAGCAACATCC	59	270-442	VNTR	50.0
	R: GGTCAGAAGGCAGAGA				

DHPLC: Denaturing high-performance liquid chromatography; VNTR: Variable number of identical tandem repeats; PCR: Polymerase chain reaction; IL: Interleukin.

Table 2 Selective characteristics and risk factors in patients with gastric cancer and controls from the Tibet, Hui and Han ethnicities *n* (%)

Variable	Tibet				Hui				Han				
	Cases (<i>n</i> = 155)	Controls (<i>n</i> = 210)	χ^2	<i>P</i> value	Cases (<i>n</i> = 158)	Controls (<i>n</i> = 205)	χ^2	<i>P</i> value	Cases (<i>n</i> = 197)	Controls (<i>n</i> = 202)	χ^2	<i>P</i> value	
Age, yr	< 35	3 (1.94)	5 (2.38)	0.006	0.940	2 (1.27)	2 (0.980)	0.069	0.793	5 (2.54)	5 (2.48)	0.602	0.437
	35-60	82 (52.90)	110 (52.38)	0.010	0.921	75 (47.46)	102 (49.76)	0.187	0.665	98 (49.75)	106 (52.48)	0.297	0.586
	≥ 60	70 (45.16)	95 (45.24)	0.000	0.988	81 (51.27)	101 (49.24)	0.142	0.706	94 (47.71)	91 (45.04)	0.285	0.593
Gender	Male	116 (74.84)	154 (73.33)	0.105	0.746	116 (73.42)	148 (71.20)	0.067	0.795	146 (74.62)	151 (74.75)	0.003	0.952
	Female	39 (25.16)	56 (26.67)			42 (26.58)	57 (28.80)			50 (25.38)	51 (25.25)		
Smoking	Yes	99 (63.87)	135 (64.29)	0.007	0.935	28 (17.71)	40 (19.51)	0.188	0.665	131 (66.50)	129 (63.86)	0.200	0.655
	No	56 (36.13)	75 (35.71)			130 (82.29)	165 (80.49)			66 (33.50)	73 (36.14)		
Hp	Positive	101 (65.16)	112 (53.33)	5.134	0.023	100 (63.29)	95 (46.34)	10.311	0.001	124 (62.94)	105 (51.98)	4.903	0.027
	Negative	54 (34.84)	98 (46.67)			58 (36.71)	110 (53.66)			73 (37.06)	97 (48.02)		

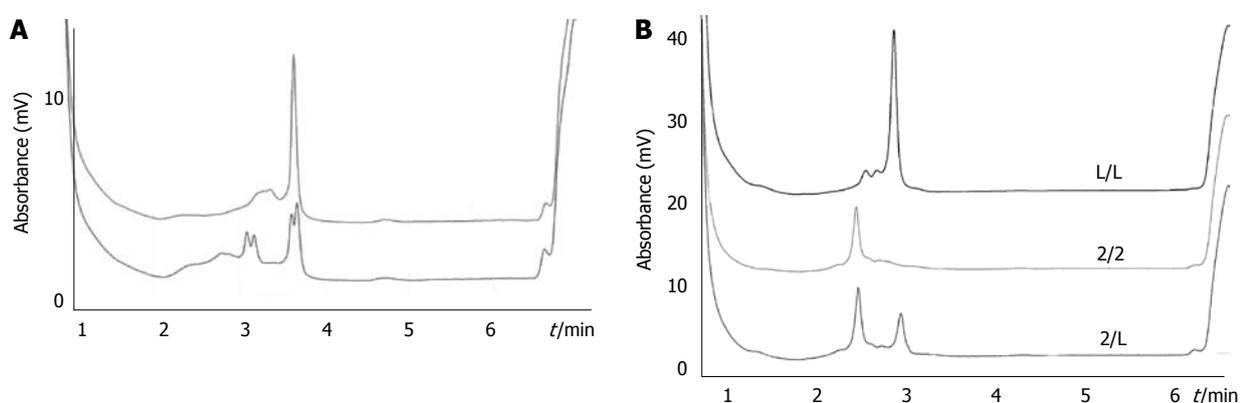


Figure 1 Typical denaturing high-performance liquid chromatography elution profiles for different genotypes. A: Representative denaturing high-performance liquid chromatography (DHPLC) profiles for different allelic polymerase chain reaction products containing the *interleukin-1B* (*IL-1B*)-311 C/T polymorphism site. In the first DHPLC, the CT genotype (lower panel) was discriminated from homozygous (upper panel). To determine the CC or TT genotype, the second DHPLC was run for the homozygous DNA mixed with a DNA sample known as the CC genotype. The profile of the CC genotype was unaltered, while that of the TT genotype changed into the same as the lower panel; B: DHPLC elution profiles of *IL-1RN* and *IL-1RN* variable number of identical tandem repeats were determined by elution time and base pair number of the fragment.

equilibrium. Multivariate logistic regression was used to obtain odds ratios (ORs) and 95%CI, adjusting for age, sex, smoking status and *H. pylori* infection. $P < 0.05$ (two-tailed) was considered statistically significant.

RESULTS

Clinical characteristics

The study population consisted of 210, 205 and 202 healthy controls and 155, 158 and 197 GC patients from the Tibet, Hui and Han ethnicities, respectively. Age, sex,

smoking status, and *H. pylori* infection in the GC patients and control subjects are shown in Table 1. There were no statistically significant differences between the cases and controls with regard to age, sex, and smoking status in each ethnicity group. However, *H. pylori* infection was significantly higher in the cases compared to the controls ($P = 0.023$, 0.001 and 0.027, respectively) in each ethnicity group. The genotype frequencies of *IL-1B-31*, *IL-1B-511* and *IL-1RN* in the controls in each ethnicity group were in agreement with the Hardy-Weinberg equilibrium ($P > 0.05$ for all).

Table 3 Genotype distributions of *interleukin-1B-31*, *interleukin-1B-511* and *interleukin-1RN* gene polymorphisms among gastric cancer cases and controls from the Tibet, Hui and Han ethnicities *n* (%)

Genotype	Tibet				Hui				Han			
	Cases	Controls	OR (95%CI) ¹	<i>P</i> value	Cases	Controls	OR (95%CI) ¹	<i>P</i> value	Cases	Controls	OR (95%CI) ¹	<i>P</i> value
<i>IL-1B-31</i>												
TT	23 (14.84)	47 (22.38)	1		46 (29.11)	59 (28.78)	1		40 (20.30)	65 (32.18)	1	
CT	81 (52.26)	112 (53.33)	1.47 (0.81-2.66)	0.208	79 (50.00)	105 (51.22)	0.97 (0.58-1.62)	0.896	102 (51.77)	98 (48.52)	1.69 (1.03-2.76)	0.036
CC	51 (32.90)	51 (24.29)	2.10 (1.09-4.04)	0.027	33 (20.89)	41 (20.00)	1.03 (0.55-1.95)	0.919	55 (27.92)	39 (19.31)	2.29 (1.28-4.10)	0.005
<i>IL-1B-511</i>												
CC	34 (21.94)	55 (26.19)	1		33 (20.89)	43 (20.98)	1		31 (15.74)	65 (32.17)	1	
CT	80 (51.61)	93 (44.29)	1.37 (0.80-2.35)	0.210	88 (55.70)	110 (53.66)	1.04 (0.59-1.83)	0.888	101 (51.27)	99 (49.09)	2.16 (1.28-3.65)	0.004
TT	41 (26.45)	62 (29.52)	1.02 (0.56-1.86)	0.945	37 (23.41)	52 (25.37)	0.96 (0.50-1.84)	0.891	65 (32.99)	38 (18.81)	3.53 (1.49-8.33)	0.004
<i>IL-1RN</i>												
L/L	129 (83.22)	185 (88.10)	1		94 (59.49)	156 (76.10)	1		166 (84.26)	171 (84.66)	1	
2/L	26 (16.78)	25 (11.90)	1.50 (0.80-2.79)	0.206	64 (40.50)	49 (23.90)	2.11 (1.31-3.40)	0.002	31 (15.28)	31 (14.44)	1.03 (0.59-1.79)	0.924

¹Adjusted for age, sex, smoking status and *Helicobacter pylori* infection. IL: Interleukin; OR: Odds ratio.

***IL-1B-31*, *IL-1B-511* and *IL-1RN* polymorphisms and GC risk**

The frequencies of genotypes *IL-1B-31*, *IL-1B-511* and *IL-1RN* among the Tibet, Hui and Han ethnicities are summarized in Table 3. In the Tibet ethnicity group, the *IL-1B-31* CC genotype was significantly more frequent in GC patients (32.90%) compared with controls (24.29%) ($\chi^2 = 4.98$, $P = 0.026$). The risk of developing gastric cancer with this genotype was significantly increased (adjusted OR = 2.10, 95%CI: 1.09-4.04, $P = 0.027$).

In the Hui nationality group, the *IL-1RN* L/2 genotype was significantly more frequent in GC patients (40.50%) compared with controls (23.90%) ($\chi^2 = 11.47$, $P = 0.001$). The risk of developing GC with this genotype was significantly increased (adjusted OR = 2.11, 95%CI: 1.31-3.40).

Unlike the results obtained for individuals belonging to either the Tibet or Hui populations, two genotype sites were associated with GC in the Han ethnicity. For the *IL-1B-31* CT genotype, there was a significant difference between GC patients (51.77%) and controls (48.52%) ($\chi^2 = 4.61$, $P = 0.032$). There was also a significant difference in the CC genotype between GC patients (27.92%) and controls (19.31) ($\chi^2 = 8.29$, $P = 0.004$). The risk of developing GC in patients with *IL-1B-31*CT or CC genotypes was significantly increased (adjusted OR = 1.69, 2.29; 95%CI: 1.03-2.76, 1.28-4.10; $P = 0.036$, 0.005, respectively). In addition, for the *IL-1B-511* genotypes, there was a statistically significant difference in the CT genotype distribution between GC patients (50.50%) and controls (49.09%) ($\chi^2 = 8.70$, $P = 0.003$). Additionally, there was also a statistically significant difference in TT genotype distribution between GC patients (32.67%) and controls (18.81%) ($\chi^2 = 18.90$, $P = 0.000$). The risk of developing GC in patients with the *IL-1B-511* CT or TT genotypes was significantly increased (adjusted OR = 2.16, 3.53; 95%CI: 1.28-3.65, 1.49-8.33; $P = 0.004$, 0.004, respectively).

***IL-1B-31*, *IL-1B-511* and *IL-1RN* polymorphisms and intestinal type GC risk**

The identification of a genetic risk outline for GC could

help the populations most at risk. Therefore, the prevalence of *IL-1B-31*, *IL-1B-511* and *IL-1RN* polymorphisms in different GC subtypes were analyzed. In this study, there were 109, 106 and 139 cases (70.32%, 67.09% and 70.56%) of intestinal-type GC, and 46, 52 and 58 cases (29.68%, 32.91% and 29.44%) of diffuse or mixed-type GC in the Tibet, Hui and Han ethnicities, respectively. The frequencies of genotypes *IL-1B-31*, -511, and *IL-1RN* among intestinal-type GC and diffuse or mixed-type GC in all three populations are summarized in Table 4.

For individuals belonging to the Tibet ethnicity group, the *IL-1B-31* CC genotype was only associated with intestinal type GC ($P = 0.037$) with an adjusted OR of 2.17 (95%CI: 1.05-4.51).

In the Hui ethnicity group, the *IL-1RN* 2 genotype was associated with both intestinal and diffuse types of GC ($P = 0.007$, 0.016, respectively) with adjusted ORs of 2.08 and 2.31 (95%CI: 1.22-3.56, 1.17-4.56, respectively).

In the Han ethnicity group, the *IL-1B-31* CC genotype was only associated with intestinal type GC ($P = 0.005$) with an adjusted OR of 2.51 (95%CI: 1.32-4.76). However, compared to the TT genotype, the GC risk in *IL-1B-31* CT carriers did not achieve the threshold of statistical significance ($P = 0.067$) with an adjusted OR of 1.68 (95%CI: 0.97-2.90). Moreover, both the *IL-1B-511* CT and TT genotypes were only associated with intestinal type GC ($P = 0.002$, 0.000) with adjusted ORs of 2.74 and 5.66 (95%CI: 1.44-5.22, 2.82-11.33, respectively).

No other significant associations were found when GC patients were sorted according to age, sex, and presence of *H. pylori* infection (data not shown).

DISCUSSION

Like many other malignancies, GC develops as a result of complex interactions between environmental risk factors (e.g., unhealthy lifestyle, smoking, uncontrolled over-drinking, unhealthy diet, and *H. pylori* infection) and genetic alterations^[15]. This gene-environment interaction can alter gene expression and promote cell growth and carcinogenesis. The *IL-1B* and *IL-1RN* polymorphisms

Table 4 Genotype distributions of the interleukin-1B-31, interleukin-1B-511 and interleukin-1RN polymorphisms among different subtypes of gastric cancer in patients from the Tibet, Hui and Han ethnicities *n* (%)

Genotype	Tibet						Hui						Han									
	Intestinal cases			Diffuse cases			Intestinal cases			Diffuse cases			Intestinal cases			Diffuse cases						
	Controls	Cases	OR (95%CI)	P value	Controls	Cases	OR (95%CI)	P value	Controls	Cases	OR (95%CI)	P value	Controls	Cases	OR (95%CI)	P value	Controls	Cases	OR (95%CI)	P value		
<i>IL-1B-31</i>																						
TT	47 (22.38)	16 (14.68)	1		7 (15.22)	1		13 (25.00)	1	65 (32.18)	28 (20.14)	1	12 (20.69)	1			65 (32.18)	28 (20.14)	1			
CT	112 (53.33)	56 (51.38)	1.46 (0.75-2.84)	0.271	25 (54.35)	1.48 (0.59-3.70)	0.407	105 (51.22)	51 (48.11)	0.87 (0.49-1.54)	0.618	28 (53.85)	1.11 (0.51-2.39)	0.792	98 (48.52)	70 (50.36)	1.68 (0.97-2.90)	0.067	32 (55.17)	2.00 (0.82-4.88)	0.126	
CC	51 (24.29)	37 (33.94)	2.17 (1.05-4.51)	0.037	14 (30.43)	1.86 (0.68-5.09)	0.228	41 (20.00)	22 (21.28)	0.93 (0.46-1.90)	0.845	11 (21.15)	1.18 (0.46-3.01)	0.734	39 (19.31)	41 (29.50)	2.51 (1.32-4.76)	0.005	14 (24.14)	1.79 (0.85-3.77)	0.126	
<i>IL-1B-511</i>																						
CC	55 (26.19)	22 (20.18)	1		12 (25.53)	1		10 (19.23)	1	65 (32.17)	16 (11.51)	1	15 (25.86)	1			65 (32.17)	16 (11.51)	1			
CT	93 (44.29)	54 (49.54)	1.48 (0.76-2.60)	0.277	26 (55.32)	1.28 (0.59-2.79)	0.535	110 (53.66)	58 (54.72)	1.00 (0.53-1.89)	0.998	30 (57.69)	1.13 (0.48-2.62)	0.784	99 (49.09)	69 (49.64)	2.74 (1.44-5.22)	0.002	32 (55.17)	1.45 (0.72-2.93)	0.301	
TT	62 (29.52)	33 (30.28)	1.25 (0.64-2.42)	0.518	9 (19.15)	0.62 (0.24-1.63)	0.336	52 (25.37)	25 (23.58)	0.91 (0.43-1.92)	0.811	12 (23.08)	0.92 (0.34-2.50)	0.872	38 (18.81)	54 (38.85)	5.66 (2.82-11.33)	0.000	11 (18.97)	1.23 (0.50-3.02)	0.645	
<i>IL-1RN</i>																						
L/L	185 (88.10)	93 (83.22)	1		36 (84.78)	1		156 (76.10)	64 (60.38)	1	171 (84.66)	116 (83.45)	1	50 (86.21)	1		171 (84.66)	116 (83.45)	1			
2/L	25 (11.90)	16 (14.68)	1.32 (0.66-2.66)	0.439	10 (16.78)	1.87 (0.79-4.43)	0.158	49 (23.90)	42 (39.62)	2.08 (1.22-3.56)	0.007	22 (42.31)	2.31 (1.17-4.56)	0.016	31 (14.44)	23 (16.55)	1.09 (0.59-1.97)	0.807	8 (13.79)	1.02 (0.43-2.39)	0.971	

¹Adjusted for age, sex, smoking status and *Helicobacter pylori* infection. IL: Interleukin; OR: Odds ratio.

are implicated in cancer risk through their influences on *IL-1B* transcription.

Since El-Omar *et al*⁵ reported that carriers of *IL-1B-511 T* or *IL-1B-31 C* were more susceptible to GC than other genotypes in 2000, other studies have reported on the associations between *IL-1B* and *IL-1RN* polymorphisms and GC risk in various populations but with mixed, or even conflicting results^[6,7,10-14]. To date, at least four meta-analyses on the associations between *IL-1B* and *IL-1RN* polymorphisms and GC have been reported, however, their outcomes were different and even opposite^[16-19]. In fact, the genetic/environmental interactions among different ethnic groups are quite complex. Thus, *IL-1B* and *IL-1RN* polymorphisms may play different roles in different populations or geographical areas.

Two studies have been performed to evaluate the differences in *IL-1B* and *IL-1RN* polymorphisms in patients of the same ethnicity - Chinese Han and Italian, respectively. Zeng *et al*¹⁰ found that the *IL-1B-511 T/T* genotype frequency was significantly higher in patients with GC than in control subjects (25.0% vs 12.5%, $\chi^2 = 6.7, P = 0.01$) in the low GC prevalence region (Guangdong), but was similar (23.0% vs 23.0%) in the high prevalence region (Shanxi). Perri *et al*¹³ did not find any association between GC occurrence and either *IL-1B-511T* or *IL-1RN* polymorphism when dividing the subjects between geographic areas displaying high prevalence rates (the North) or low prevalence rates (the South) in Italy.

To the best of our knowledge, this is the first study to examine the associations between *IL-1* polymorphisms and GC risk among several ethnicities in one area at the same time. We examined these polymorphisms and their potential association with GC risk among the Tibet, Hui and Han ethnicities in the Qinghai area of China. We found associations between *IL-1B-511*, *IL-1B-31* and *IL-1 RN* polymorphisms and GC risk among Tibet, Hui and Han ethnicities in the Qinghai area, which were not identical.

In the Tibet ethnicity group, the *IL-1B-31 CC* genotype was associated with GC. Additionally, our study showed that the *IL-1B-31 CC* genotype was only associated with intestinal type GC. Our results are consistent with those from several studies in Chinese and Caucasian populations, where *IL-1B-31 CC* polymorphisms were associated with an increased risk of GC^[10,20,21]. However, these results are inconsistent with those from other studies in Asian and Caucasian populations^[14,22,23].

In the Hui ethnicity group, the *IL-1RN2* was associated with GC, and further study supported that the *IL-1RN2* polymorphism was associated with both intestinal and diffuse types of GC. These findings were in agreement with those previously reported, including studies from Caucasian, Arab and Asian (including Chinese) populations^[13,20,22,24]. However, our findings also differ from those reported in several other studies^[14,23,26].

In the Han ethnicity group, the *IL-1B-31 CT* and *CC* genotypes and the *IL-1B-511 CT* and *TT* genotypes were associated with GC compared with the *IL-1B-31 TT* genotype

and the *IL-1B-511* CC genotype, respectively. Moreover, the *IL-1B-31* CC genotype was also only associated with intestinal type GC. Importantly, although *IL-1B-31* CT carriers displayed a trend in risk, they did not achieve the threshold of statistical significance. The *IL-1B-511* CT and TT genotypes were only associated with intestinal type GC, compared with the CC genotype. While this is the same in Caucasian and Chinese populations^[11,18,27,28], it differs from the findings observed in South Korean and Japanese populations in Asia^[10,20]. This study also revealed that *IL-1RN* polymorphisms were not associated with increased risk of GC in the Han ethnicity group, which is similar to that seen in Japanese and South Korean populations^[20].

It is interesting and important that the *IL-1B-31*, *IL-1B-511* and *IL-1RN* polymorphisms and GC risk among the three ethnicities examined were not identical. Other studies have drawn similar conclusions. We believe that the differences among the ethnicities are related to different inherited gene backgrounds. This is supported by other studies which showed differential genetic/environmental interactions in different ethnic groups resulting in altered gene expression and altered effects on cell growth and tumorigenesis^[29]. The gene distributions of *IL-1B-31*, *IL-1B-511* and *IL-1RN* among the Tibet, Hui and Han ethnicities in Qinghai were different. Despite this, they were at least somewhat related to other Asian populations. The *IL-1B-31* TT genotype was less frequent in the Tibet population (22.38%) than in the Han population (32.18%). The *IL-1B-511* TT genotype was more frequent in the Tibet population (29.52%) than in the Han population (18.81%). The *IL-1B-511* TT genotype was significantly lower in the Hui population (20.98%) than in the Han population (32.17%). In addition, the *IL-1RN* L was significantly lower in the Hui (76.10%) population compared to both the Tibet population (88.10%) and the Han population (84.66%). Ethnic origin is a crucial determinant of the frequency of genetic markers in all populations. Our data may reflect the influence of past selective pressures on the genotypes of Tibet, Hui and Han ethnicities over a long period of time. Han, Tibet and Hui ethnicities have different origins. The Han population is the major ethnicity, while the Tibet and Hui ethnicities are considered to be minorities in the Qinghai province of China. The selected healthy controls of the Tibet ethnicity were living in the Hainan Tibet Ethnicity Autonomous prefecture in Qinghai province, which belongs to the Tibet Anduo area. Shi *et al.*^[30] studied more than 5000 male samples from 73 East Asian populations and reconstructed the phylogenetic geography of the D-M174 lineage. The suggested frequency of D-M174 in Tibet (41.31%) was close to Japan (35.08%) but different from the Han ethnicity (< 5%). Thus, the Tibet gene feature is more similar to the Han ethnicity, but also has its own characteristics. The Hui ethnicity migrated from Central Asia, Persia and the Arab world. Yao *et al.*^[31] analyzed M*, N* and R* mtDNAs and found that the western Eurasian specific haplogroup frequency in the Hui population was 6.7%, but no western Eurasian type was found in Han Chinese samples from the same place.

Since both the Tibet and Hui ethnicities practice endogamy, they tend to be ethnically homogeneous. Therefore, we believe that the Tibet, Hui, and Han ethnicities in the Qinghai area of China have different origins leading to different associations between *IL-1B-31*, *IL-1B-511* and *IL-1RN* polymorphisms and GC risk.

Our study has some limitations. First, we did not consider education, consumption of alcohol, fresh fruits, and vegetables in the controls which could influence GC risk. Second, the altitude at which healthy controls lived was not considered. In this study, all of the Tibet controls and most of the Tibet patients lived in the plateau above 2800 meters of one another. For the Hui and Han populations, all controls and most patients lived within 2200 meters of each other. Finally, different eating habits among the groups were not investigated.

In conclusion, the present study shows different associations between the *IL-1B-31*, *IL-1B-511* and *IL-1RN* polymorphisms and GC among the Tibet, Hui and Han ethnicities in the Qinghai area of China. No significant association was observed when GC patients were sorted by age, sex and the presence of *H. pylori* infection.

COMMENTS

Background

Studies suggest that polymorphisms in interleukin (IL)-1B and IL-1RN are associated with a differential risk of developing gastric cancer. However, there does not seem to be a consensus regarding these polymorphisms, since in some populations the polymorphisms are associated with increased disease occurrence, whereas in other populations, they are associated with a protective effect.

Research frontiers

There is no consensus regarding the associations between IL-1B, IL-1RN polymorphisms and gastric cancers in different areas or ethnicities. The authors investigated the associations between IL-1B and IL-1RN polymorphisms and gastric cancers among the Tibet, Hui and Han ethnicities in China.

Innovations and breakthroughs

The outcomes of previous studies on the associations between *IL-1B* and *IL-1RN* polymorphisms and gastric cancer were different and even opposite. The genetic/environmental interactions among different ethnic groups are quite complex. Thus, *IL-1B* and *IL-1RN* polymorphisms may play different roles in different populations or geographical areas. This study suggests that carriers of the *IL-1B-31* CC genotype had an increased risk of intestinal type gastric cancer in the Tibet ethnicity, carriers of the *IL-1B* 2/L genotype had an increased risk of both intestinal and diffuse types of gastric cancer in the Hui ethnicity, while carriers of the *IL-1B-31* CC, *IL-1B-511* CT, TT genotypes have an increased risk of intestinal type gastric cancer in the Han population.

Applications

The study results suggest that *IL-1B* and *IL-1RN* genotypes may differentially contribute to gastric cancer among the Tibet, Hui and Han nationalities in the Qinghai area of China, and that *IL-1B* and *IL-1RN* polymorphisms may play different roles in different populations or geographical areas.

Terminology

Single nucleotide polymorphisms (SNPs) are short polymorphisms in the human DNA. SNPs occur once in every 300 nucleotides on average and can act as biological markers. When SNPs occur within a gene or in a regulatory region near a gene, they may play a more direct role in disease by affecting the gene's function. SNPs can also be used to track the inheritance of disease genes within families.

Peer review

This is a good descriptive study in which authors investigated *IL-1B* and *IL-1RN* polymorphisms in Han, Tibetan and Hui ethnic populations in Qinghai Province of China and their associations with gastric cancer risk in these populations. The topic is interesting and of potential clinical implications.

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Inflammatory myofibroblastic tumor successfully treated with chemotherapy and nonsteroidals: A case report

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Abstract

Inflammatory myofibroblastic tumor (IMT) occurring at retroperitoneal sites has rarely been reported. We report the case of a previously well 14-year-old girl with no history of abdominal disease whose past medical history and family tumor history were unremarkable. She complained of intermittent abdominal pain for one month. An abdominal mass was found on physical examination and abdominal contrast-enhanced computed tomography (CT) showed a hypodense soft mass, the size and location of which suggested a well delineated retroperitoneal tumor surrounding the superior mesenteric vessels measuring 3.3 cm × 4.5 cm × 4.5 cm with enlarged lymph nodes. The patient underwent an exploratory laparotomy followed by biopsy and was subsequently diagnosed with retroperitoneal IMT. She was successfully treated with postoperative chemotherapy and oral diclofenac sodium. Following completion of therapy the mass was no longer palpable and no longer visible on CT scanning. The use of methotrexate and cisplatin for aggressive myofibroblastic tumors is also reviewed.

INTRODUCTION

Inflammatory myofibroblastic tumor (IMT) is a rare soft-tissue tumor with a clinical resemblance to malignant neoplasm^[1]. It is an unusual solid tumor commonly seen in children and young adults, and can occur at any site in the body, the lung being the most common site^[2]. Extrapulmonary IMTs account for 5% of all IMTs and retroperitoneal IMTs are relatively rare^[3]. The retroperitoneum is an unusual site of presentation in these rare tumors, which share the neoplastic characteristics of aggressive local tissue infiltration, recurrence after resection, and occasionally distant metastasis^[2,4]. Due to disease progression, lack of alternative therapeutic regimens, recurrence and poor prognosis, almost all patients with IMT have been treated with radical surgical resection or single nonsteroidal anti-inflammatory drugs. Herein, we present a patient with a retroperitoneal IMT, who was successfully treated with chemotherapy and oral diclofenac sodium.

CASE REPORT

A 14-year-old girl was admitted to our hospital in June

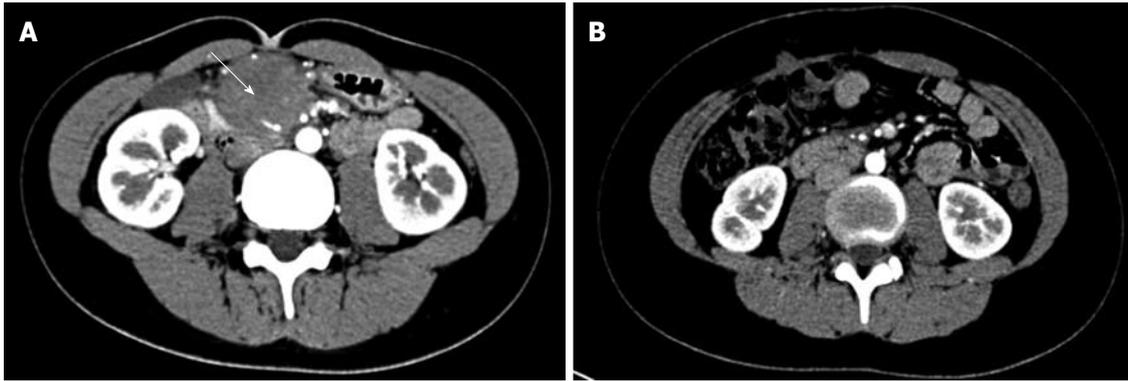


Figure 1 Photograph of abdominal computer tomography. A: The complex soft tissue involved including the mesenteric vessels (arrow); B: Disappearance of the mass.

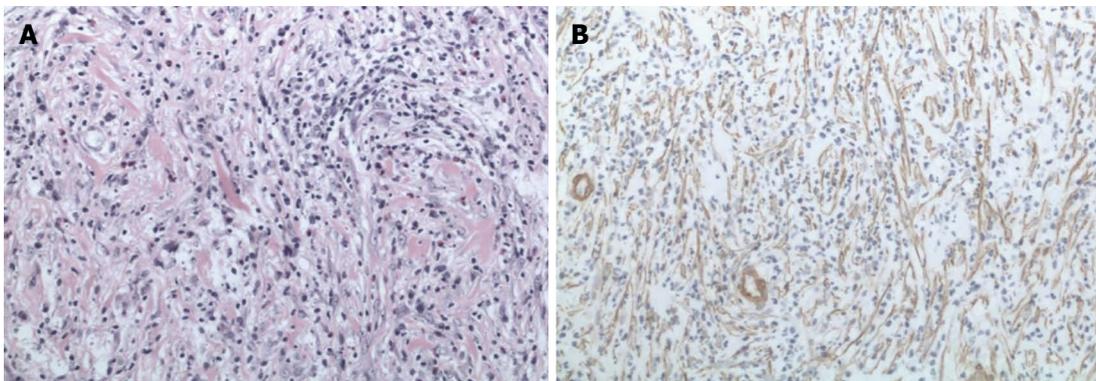


Figure 2 Photomicrograph of the tumor cells. A: A proliferation of spindle tumor cells (hematoxylin and eosin stain, $\times 200$); B: Positivity for smooth muscle actin ($\times 200$).

2009 with the chief complaint of intermittent abdominal pain for one month. She experienced intermittent abdominal pain without bloating every two days and all symptoms were in remission after she buckled both legs, however, the pain returned a few minutes later. The patient did not have symptoms of fever, nausea, vomiting, weight loss, bowel obstruction or other discomfort and she did not receive treatment to relieve these symptoms prior to admission. This 14-year-old girl was previously well with no history of abdominal disease, and her past medical history and family tumor history were unremarkable. Physical examinations showed a 7 cm abdominal mass in the right side of the umbilicus, which was hard, without tenderness and firm but mobile on palpation. Blood count analysis and tumor markers such as carcinoembryonic antigen and CA19-9 were negative. Abdominal contrast-enhanced computed tomography showed a hypodense soft mass, the size and location of which suggested a well delineated retroperitoneal tumor surrounding the superior mesenteric vessels which measured 3.3 cm \times 4.5 cm \times 4.5 cm with enlargement of the surrounding lymph nodes (Figure 1A). Given that the preoperative diagnosis was unconfirmed and radical surgery was the most efficient therapeutic option, after a multidisciplinary discussion, an exploratory laparotomy

was performed. During the operation, a 10 cm round solid mass was found at the root of the mesentery. The tumor reached the uncinate process of the pancreas and the horizontal section of the duodenum. Complete excision of the mass was difficult, because the tumor surrounded most of the superior mesenteric artery branches. Therefore, biopsy of the mass was undertaken. Pathologic examination confirmed the diagnosis of IMT with a proliferation of spindle cells and dense polymorphic infiltration of mononuclear inflammatory cells (Figure 2A). On immunohistochemical staining, spindle cells were positive for vimentin, smooth muscle actin (Figure 2B) and CD68, but negative for Cluster of differentiation 34 (CD34), Cluster of differentiation 117 (CD117) and anaplastic lymphoma kinase-1 (ALK-1).

The postoperative course was uneventful and the patient was discharged from the hospital without complications. Three weeks after surgery, the patient received chemotherapy which consisted of 6 courses of middle dose methotrexate (20 mg/m²) and cisplatin (30 mg/m²) administered once a month, associated with oral administration of slow-release diclofenac sodium until cessation of chemotherapy. The patient was re-examined after completing chemotherapy; the mass was no longer palpable and was no longer visible on (computer tomog-

raphy) CT scanning (Figure 1B). Currently, the patient is alive without recurrence after 3 years of follow-up.

DISCUSSION

IMT was originally described in the lungs in 1937, and since then has been reported to occur throughout the body including the mesentery, stomach, abdomen, liver, mediastinum, retroperitoneum, omentum and bladder of children and adolescents^[5]. Retroperitoneal IMTs are extremely rare. This tumor was previously described as an inflammatory pseudotumor, inflammatory myofibroblastoma, lymphoplasmacytic, histiocytoma, and fibrous pseudotumor until 1994 when myofibroblastic tumor was established as a distinct low grade malignancy by the World Health Organization classification^[6-8]. The exact etiology of the disease is still not completely known. In a review of the medical literature, researchers have suggested that trauma, surgery, inflammation and infection by the Epstein-Barr virus or Human Herpes virus could result in the development of IMT^[9]. Others have insisted that a chromosomal rearrangement involving the *ALK* gene results in the activation of a tyrosine kinase receptor and could lead to abnormal expression^[10]. In the present case, the patient had no past medical history related to this disease, therefore, there is no evidence of a direct relationship between the development of the disease and susceptible factors.

The clinical presentation of the tumor often depends on its anatomical site. Retroperitoneal tumors usually grow slowly and always present as a solid abdominal palpable mass, accompanied by abdominal pain or weight loss. It is only when the retroperitoneal tumor increases in size resulting in obstructive symptoms, that intestinal obstruction may be diagnosed. As shown in our report, the patient presented with an atypical clinical manifestation of intermittent abdominal pain accompanied by a localized mass and no significant abnormal laboratory tests. The CT features in our patient showed varied degrees of enhancement on the complex soft tissue involving the mesenteric vessels with nonspecific findings, which was mostly indistinguishable from a malignant process. It is difficult to confirm this disease using these atypical clinical manifestations pre- or intra-operatively. A definite diagnosis therefore depends on histopathological evaluation after biopsy or resection.

When necessary, surgical resection could be considered the preferred treatment for IMT, which can also be used to confirm this disease. The recurrence rate has been reported to range from 18% to 40%, and only precise removal of the tumor will avoid recurrence^[11]. In our patient, the location of the tumor made it difficult to cure by means of a complete radical resection which may have led to intestinal ischemia and even postoperative short bowel syndrome. Steroids and radiotherapy as well as chemotherapy as adjuvant therapy are feasible alternatives to surgery. Chemotherapy, radiotherapy, and

even immunomodulation have not been reported to be consistently effective against this aggressive tumor^[12]. No other efficient treatment regimens have been reported for this tumor. Consequently, in patients with unresectable IMTs, treatment options are limited, and better recommendations have not been defined.

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been successful in the treatment of IMTs when resectability is limited due to the tumor invading vital structures^[13]. Przkora *et al.*^[14] reported that two patients with nonresectable intra-abdominal IMTs had a satisfactory outcome after receiving treatment with nonsteroidal anti-inflammatory drugs. There is no evidence to prove that chemotherapy is effective when used singly, however, it may play a role following complete resection. As shown in our case, patients with unresectable retroperitoneal IMTs receiving conventional-dose methotrexate/cisplatin chemotherapy and oral NSAIDs can be symptom-free after 3 years of follow-up without relapse or metastasis and achieve a favorable prognosis. Therefore, chemotherapy combined with oral NSAIDs may be an effective therapeutic option for patients with unresectable IMT.

Our experience with the patient in this report might provide another treatment choice for patients with unresectable, residual or recurrent IMT.

In summary, our findings suggest that middle-dose chemotherapy and NSAIDs may be a feasible therapeutic choice in patients with unresectable retroperitoneal IMT.

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Management of surgical splenorenal shunt-related hepatic myelopathy with endovascular interventional techniques

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Abstract

We present a case with hepatic myelopathy (HM) due to a surgical splenorenal shunt that was successfully treated by endovascular interventional techniques. A 39-year-old man presented with progressive spastic paraparesis of his lower limbs 14 mo after a splenorenal shunt. A portal venogram identified a widened patent splenorenal shunt. We used an occlusion balloon catheter initially to occlude the shunt. Further monitoring of the patient revealed a decrease in his serum ammonia level and an improvement in leg strength. We then used an Amplatzer vascular plug (AVP) to enable closure of the shunt. During the follow up period of 7 mo, the patient experienced significant clinical improvement and normalization of blood ammonia, without any complications. Occlusion of a surgically created splenorenal shunt with AVP represents an alternative therapy to surgery or coil embolization that can help to relieve shunt-induced HM symptoms.

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Key words: Hepatic myelopathy; Shunts; Portosystemic; Hepatic encephalopathy; Embolization; Endovascular

INTRODUCTION

Hepatic myelopathy (HM) is a rarely reported disorder characterized by progressive spastic paraparesis of the lower extremities due to impaired corticospinal tract function in the setting of cirrhosis or portosystemic shunting^[1-3]. Over 85% of reported HM cases have been associated with surgical, angiographic, or spontaneous portosystemic shunting. Both HM and hepatic encephalopathy (HE) after a surgical portosystemic shunt are thought to be secondary to the increased shunting of portal venous toxins to the systemic circulation and from hypoperfusion and ischemia of the hepatocytes. However, unlike HE, conservative treatment of HM is usually considered inefficient.

Recently, outcomes for a small number of patients who have undergone liver transplantation (LT) for HM suggest a potential neurological benefit, especially with earlier transplantation^[4-6]. However, for patients with normal liver function or Child-Pugh A grade cirrhosis, the choice of LT vs other treatments (i.e., narrowing or occlusion of the shunt) is debatable^[3,4]. In addition, the limited donor organ supply remains a major issue. In the present study, we report a case with HM due to a surgical splenorenal shunt that was successfully occluded by

endovascular interventional techniques, which resulted in significant clinical improvement and normalization of blood ammonia.

CASE REPORT

Patient's history

A 39-year-old man presented in April 2010 with an 11 mo history of slowly progressive spastic paraparesis of his lower extremities. The patient in question had a history of hepatitis B disease. In March 2008, he underwent a surgical splenorenal shunt and splenectomy due to recurrent esophagogastric variceal bleeding and thrombocytopenia. In May 2009, fourteen months after his surgical splenorenal shunt, gait impairment was first noticed. An episode of HE was not seen during this period. The patient received conservative medical management, including protein restriction, non-absorbable antibiotics, lactulose and physical therapy, after the onset of gait impairment. However, these measures did not prevent the progressive decline in his mobility and this necessitated the use of a cane because of marked leg weakness, gait imbalance, and instability.

Physical examination

Physical examination revealed normal mental status and cranial nerve function, and the absence of asterixis. Kayser-Fleischer rings were absent, upper extremity strength and tendon reflexes were normal, and Hoffman's sign was absent. In the lower extremities, the legs were spastic, the tone was more noticeably increased with brisk tendon reflexes and there was clonus at the ankles. Plantar reflexes were extensor and the patient was unable to move his legs. There was no atrophy or any fasciculations, nor any evidence of ascites or peripheral edema.

Laboratory studies

Laboratory investigations showed normal serum levels of electrolytes, glucose, vitamin B12, and creatinine. He had mild anemia (hemoglobin level of 112 g/L; normal range, 120-160 g/L) and thrombocytopenia ($85 \times 10^3/\text{mm}^3$; normal range, 100×10^3 - $300 \times 10^3/\text{mm}^3$). Prothrombin time was 16 s (normal range, 11-14.5 s). Other values were as follows: 28 $\mu\text{mol/L}$ total bilirubin (normal range, 3-20 $\mu\text{mol/L}$); 15 $\mu\text{mol/L}$ conjugated bilirubin (normal range, 0-7 $\mu\text{mol/L}$); 156 $\mu\text{mol/L}$ ammonia (normal range, 18-45 $\mu\text{mol/L}$); 56 U/L alanine aminotransferase (normal range, 5-40 U/L); 65 U/L aspartate aminotransferase (normal range, 15-45 U/L); 126 U/L alkaline phosphatase (normal range, 30-115 U/L); 36 g/L albumin (normal range, 35-50 g/L); 45 g/L globulin (normal range, 21-40 g/L); international normalized ratio 1.3 (normal range, 0.9-1.2). His liver was graded as Child-Pugh class A.

Serum examinations for hepatitis B surface antigen and hepatitis B e-antigen were positive, but a test for hepatitis B core antigen was negative. Antibodies to hepatitis C virus and human immunodeficiency virus were nega-

tive, syphilis serology was negative, and an analysis of the cerebrospinal fluid was normal.

Magnetic resonance imaging of the brain and spine was normal, and an abdominal computed tomography (CT) revealed a cirrhotic liver. An abdominal ultrasound examination showed features of cirrhosis of liver and a Doppler study showed a widely-open splenorenal shunt. Electro-encephalography revealed no definite abnormality and an endoscopic examination revealed no esophagogastric varices.

Diagnosis and general management

Given the aforementioned extensive neurological evaluation, which did not identify any alternative explanation for the patient's spastic paraparesis of the lower extremities, the diagnosis of HM secondary to the surgical splenorenal shunting was made. After admission, with protein restriction and medication with lactulose, neomycin, B-vitamins, physical therapy, and antispastic agents for 3 wk, the patient showed no improvement in his spastic paraparesis, and his hyperammonemia remained within the range of 140-180 $\mu\text{mol/L}$.

After discussion with hepatologists, gastroenterologists, and vascular surgeons at the authors' hospital, we decided to occlude the surgical splenorenal shunt to prevent further neurologic deterioration. Informed consent was obtained from the patient.

Angiographic procedure

Under local anesthesia (2% lidocaine) and fluoroscopic guidance, venous access was obtained through the right internal jugular vein, and a 9 Fr with an angled-tip 80-cm long introducer sheath (Cook, Bloomington, Indiana) was advanced into the inferior vena cava. The splenorenal shunt was then catheterized with a 5 Fr Cobra catheter (Terumo Corporation, Tokyo, Japan) and an angled-tip 0.035-inch hydrophilic guidewire (Terumo). Initial portography and pressure measurements were performed with a 5 Fr multiple-side-hole catheter (Cook) positioned in the superior mesenteric vein. At the portal venogram, a widened patent shunt was seen (Figure 1), with a portacaval pressure gradient of 8.0 mmHg. The portal vein pressure was 12.5 mmHg.

Temporary balloon occlusion of the surgical splenorenal shunt

We initially placed an occlusion balloon catheter into the splenorenal shunt tract to test if the patient could be tolerable to the full embolization procedure. Following the angiographic procedure, over a 0.035-inch Amplatz wire (Cook), the 9 Fr introducer sheath advanced into the splenorenal shunt. A 7 Fr latex balloon catheter, with a maximum outer diameter of 16 mm (Boston Scientific, Watertown, Mass) was then inserted into the splenorenal shunt through the sheath. Under the inflated balloon, injection of contrast material through the sheath showed complete occlusion of the surgical splenorenal shunt. After the balloon occlusion, heparin infusion was given *via*



Figure 1 Digital subtraction portogram obtained via a catheter inserted through the distal splenorenal shunt into the superior mesenteric vein (arrows) using the right jugular vein approach. The wide patent splenorenal shunt (arrow heads) is shown. Note the inferior vena cava (curved arrow).

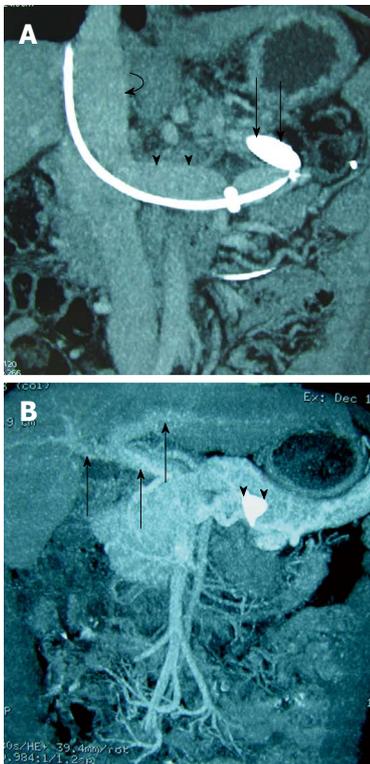


Figure 2 Coronal maximum intensity projection reconstruction of a contrast enhanced 64-slice helical computed tomography scan of the abdomen. A: Obtained 5 d after placement of an occlusion through the splenorenal shunt and shows the occlusion balloon placed within the shunt tract (arrows), note the inferior vena cava (curved arrow) and the left renal vein (arrow heads); B: Obtained 3 mo after the Amplatzer vascular plugs (AVP) occlusion procedure and shows the correct AVP position (arrow heads), opacification of the portal system (arrows), and no opacification of the surgical shunt, as well as the varices.

a peripheral vein, at a dosage of 120 mg/24 h, to avoid catheter-related thrombosis.

Following the balloon occlusion procedure, the patient's plasma ammonia level decreased to 110 $\mu\text{mol/L}$, 70 $\mu\text{mol/L}$, 60 $\mu\text{mol/L}$ and 50 $\mu\text{mol/L}$, on post-procedural days 2, 3, 5 and 6, respectively. He reported a

mild improvement in his leg strength and balance, while the other HE-related symptoms remained unchanged. Follow-up CT on post-procedural day 5 showed the balloon positioned appropriately (Figure 2A). Repeat liver function tests were normal, and repeat endoscopic examination confirmed no induction of esophagogastric varices. There was no evidence of ascites. Six days after the balloon occlusion procedure, we decide to occlusion the splenorenal shunt permanently with endovascular embolization.

Embolization of the surgical splenorenal shunt with an Amplatzer vascular plug

Given the relatively larger diameter of the splenorenal shunt, we decided to use an Amplatzer vascular plug (AVP) instead of conventional embolization materials (i.e., coils, glue, and particles).

The diameter of the surgical splenorenal shunt was approximately 13 mm in diameter, calculated on the angiography workstation. Given the diameter of the shunt, we used an 18-mm AVP (AGA Medical Corp., Golden Valley, MN, United States) with the 30%-50% oversizing recommended by the manufacturer, to prevent plug migration. For deploying the AVP, the 9 Fr introducer sheath advanced over a 0.035-inch Amplatz wire (Cook) into the most distal extreme of the splenorenal shunt. The AVP device was then advanced through the 9 Fr sheath and initially deployed by retracting the sheath, but without detaching the device. A control injection was performed, to ensure satisfactory positioning of the device and to enable repositioning. When in a satisfactory position, the AVP was detached by anticlockwise rotation of the AVP guide wire to unscrew it from the AVP device proper. Within minutes of deployment, the shunt had completely stopped. Injection of contrast material through the sheath showed complete occlusion of the surgical shunt.

Following AVP embolization, an indirect digital subtraction portography, carried out during the same procedure and obtained with a 4 Fr catheter (Terumo) inserted into the superior mesenteric artery (SMA) *via* the right femoral artery approach, was used to evaluate portal vein system. Digital subtraction SMA angiogram at the portal venous phase demonstrated a marked improvement in the opacification of the portal vein in comparison with that of the portography pre-deployment of the AVP (Figure 3), indicating an increase in antegrade intrahepatic portal vein perfusion. No contrast material passed through the surgical splenorenal shunt.

Follow-up

No complication was noted during and after the procedure. Five days after the AVP embolization, the patient's serum ammonia level had normalized to 40 $\mu\text{mol/L}$.

The patient was discharged 6 d post-AVP embolization. Doppler US confirmed complete occlusion of the splenorenal shunt. In June 2010, one month after the embolization, the patient reported a gradual improvement



Figure 3 After deploying the Amplatzer vascular plugs, indirect digital subtraction portography, obtained through the catheter inserted into the superior mesenteric artery using the right femoral artery approach, demonstrates opacification of the portal vein (arrows). Note the correct Amplatzer vascular plugs position (arrow heads) and no visualization of varices.



Figure 4 Axial image of contrast enhanced 64-slice helical computed tomography scan of the abdomen, showing the correct Amplatzer vascular plug position (curved arrow), opacification of the portal system (arrow heads), and a thrombosed shunt tract (arrows).

in strength; he was still moderately weak but was able to walk short distances (50-100 m) with crutches.

By 3 mo after the procedure, he was able to walk about 300-500 m with crutches. He still complained of stiffness and spasms in his legs, but improved compared to 3 mo ago. A follow-up contrast enhanced CT scan at the venous phase confirmed that the AVP position was correct, that there was opacification of the intrahepatic portal veins, that the splenorenal shunt tract was thrombosed, and that there was no opacification of the surgical shunt or varices (Figures 2B and 4).

The patient continued to show improvement in his HM symptoms over the next few months. In December 2010, 7 mo after the splenorenal shunt embolization, his strength improved significantly, he was able to walk 1 to 2 km aided by crutches, and had only mild stiffness in both legs. Esophagogastrosocopy showed no evidence of esophagogastric varices.

DISCUSSION

HM responds poorly to conservative medical therapy and it has a poor prognosis because of its progressive and irreversible nature^[2,3]. Currently, treatment options in HM include surgical ligation, liver transplantation, shunt reduction, or occlusion by interventional procedures. Surgical ligation has been reported to be effective, but is used only occasionally^[1]. LT remains a potentially definitive treatment for HM in patients with decompensated cirrhosis of Child-Pugh B and C grade^[4-6]. However, for patients with normal function liver or Child-Pugh A grade cirrhosis, the choice of LT *vs* other treatments (i.e., narrowing or occlusion of the shunt) is debatable^[1,2]. Our patient had no history of HE, and his laboratory studies showed no liver dysfunction, with the exception of an increase in his serum ammonia level. Thus, occlusion of the surgical splenorenal shunt may be an alternative therapeutic option.

Interventional endovascular shunt occlusion has been used previously to treat post-surgical shunt HE and post-

transjugular intrahepatic portosystemic shunt (TIPS) HE^[2,7]; however, the usefulness of the technique for post-surgical shunt HM has not yet been determined. In the present case, the patient's gait impairment was noticed 14 mo after his surgical splenorenal shunt, with a progressive decline in his mobility afterward. We successfully occluded the large surgical splenorenal shunt using an AVP. Following AVP embolization, the patient reported a gradual improvement in leg strength and balance. Seven months later the patient was able to walk 1 to 2 km aided by crutches, with only mild residual spasticity of his lower extremities. To our knowledge, reversal of HM by occlusion of a surgical splenorenal shunt with AVP has not been reported.

Possible embolizing materials for the embolization of the portosystemic shunt are coils, a detachable balloon, and an AVP. We chose AVP implantation for our patient due to the relatively large size of the surgical splenorenal shunt. In this situation, a number of coils would be needed to achieve adequate vessel closure. In addition, coil migration may occur when used in short shunt tracts^[7-10]. AVPs has recently been shown to be effective in the occlusion of internal iliac arteries^[9], the treatment of pulmonary arteriovenous malformations^[10], and as an occlusion system for a splenorenal shunt arising after TIPS^[8,11]. The advantage of the AVP, compared to coils, is that it can be more precisely placed within the vessel and that it can be repositioned or removed, if necessary.

There is some doubt as to whether portal pressure could rise after portosystemic shunt embolization. This would constitute a critical complication for the patient, resulting in the aggravation of esophageal varices or even the development of new varices^[2,12,13]. One study concluded that, to avoid the consequences of a sudden increase in portal pressure, embolization should be indicated only in patients with absent or mild esophageal varices and with no signs of hepatic failure, such as ascites or jaundice^[14]. In addition, routine periprocedural endoscopy is recommended in this setting to minimize the incidence of embolization-related complications. In this report, we used an occlusion balloon catheter initially to occlude the surgical shunt. Further monitoring of the

patient over the next few days revealed no evidence of induction varices or ascites; thus we decided to use an AVP to enable closure of the shunt.

Our case raises several unique points not previously noted in the literature. Firstly, to our knowledge, this is the first report of a surgical shunt relate-HM successfully embolized with AVP resulting in an immediate improvement in intrahepatic portal perfusion, normalization of blood ammonia, and a gradual improvement of HM-related symptoms. Secondly, since we were able to document a temporary balloon occlusion of the surgical shunt prior to permanent embolization, it may be possible to predict clinical and laboratory improvement. Finally, in patients with normal function liver or Child-Pugh A grade cirrhosis, shunt occlusion may represent a suitable alternative therapy to LT that can help to relieve shunt-induced HM symptoms.

In conclusion, for the present case, we successfully occluded a large surgical splenorenal shunt using an AVP, which resulted in significant clinical improvement of the shunt-induced HM symptoms. This technique represents a viable alternative to surgery or coil embolization, although further study is necessary. In addition, trial balloon occlusion of the shunt prior to complete permanent embolization can be used to predict the clinical and laboratory improvement.

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Retrieval-balloon-assisted enterography in post-pancreaticoduodenectomy endoscopic retrograde cholangiopancreatography

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Abstract

This case reports an application of conventional duodenoscope in a post pancreaticoduodenectomy patient with the help of retrieval balloon assisted enterography. The 56-year-old woman had pancreaticoduodenectomy with Child reconstruction 9 mo ago because of pancreatic adenocarcinoma and now there are recurrent enlarged lymph nodes in the anastomotic stoma of hepaticojejunostomy. Considering the patient's late-stage cancer, a plastic stent was then successfully placed there to drainage. The main challenge in this case was the extremely long afferent loop and blind cannulation through the anastomotic stoma of hepaticojejunostomy. Retrieval balloon assisted enterography is very helpful for duodenoscope going through the reconstructed intestinal tract and for the cannulation. After two weeks, the patient remained free of painful symptoms and free of fever. Liver function improved well. Four months after the placement of stent, the patient died of cachexia without jaundice, fever and abdominal pain according to her daughter's statement.

INTRODUCTION

Endoscopic interventions are usually very challenging in patients with anatomic changes caused by previous gastrointestinal surgery. Methods using front view endoscopy, single-balloon enteroscopy (SBE) and double-balloon enteroscopy (DBE) systems may aid in cholangiopancreatography in postsurgical conditions. In this case, an endoscopic retrograde cholangiopancreatography (ERCP) procedure was successfully performed using a conventional duodenoscope on a post-pancreaticoduodenectomy patient with the help of retrieval-balloon-assisted enterography. The patient was a 56-year-old woman who had undergone a pancreaticoduodenectomy 9 mo previously in another hospital because of pancreatic adenocarcinoma. Recurrent enlarged lymph nodes were found 9 mo after surgery in the anastomotic stoma of the hepaticojejunostomy. A plastic stent was placed in the stenosis and resulted in improved liver function.

CASE REPORT

A 56-year-old woman presented complaining of abdominal pain, fever and jaundice for two weeks. She had pancreaticoduodenectomy 9 mo previously in another hospital, and the pathological diagnosis was pancreatic adenocarcinoma. Two weeks before this admission, she began to feel abdominal distension and intermittent pain, accompanied by jaundice and fever. The highest fever was 38.6 °C.

Magnetic resonance imaging (MRI) showed that the lymph nodes of the hepatoduodenal ligament were enlarged and causing compression of the anastomotic stoma of hepaticojejunostomy (Figure 1). The intrahepatic bile duct was markedly expanded. The lymph nodes near the aorta were also enlarged.

Laboratory tests revealed: total white cells $5.9 \times 10^9/L$, neutrophilic granulocyte (%) 71.9%, total bilirubin 104 $\mu\text{mol/L}$, direct bilirubin 81.6 $\mu\text{mol/L}$, glutamate pyruvate transaminase 34 U/L, glutamic-oxal(o) acetic transaminase 25 U/L.

We performed ERCP using a conventional duodenoscope on this patient, which showed compressed stenosis of the anastomotic stoma of hepaticojejunostomy consistent with the MRI. We then successfully placed a plastic stent in the anastomotic stoma.

Within a few days after placement of the biliary stent, the fever resolved and there was no complaint of abdominal pain. Serum bilirubin levels also went down. Five days later, X-ray of the biliary system showed that no contrast remained in the bile duct and the patient was discharged. Two weeks after discharge, there was still no complaint of abdominal illness or fever. Laboratory tests showed: total white cells $5.9 \times 10^9/L$, neutrophilic granulocyte (%) 63.1%, total bilirubin 49.7 $\mu\text{mol/L}$, direct bilirubin 33.0 $\mu\text{mol/L}$, glutamate pyruvate transaminase 31 U/L, glutamic-oxal(o) acetic transaminase 55 U/L.

The procedure was performed with the patient under pharyngeal anesthesia and sedation (intramuscular 10 mg diazepam injection). The patient was placed in the left lateral decubitus position. ERCP was performed under fluoroscopic control using a therapeutic duodenoscope with a total length of 120 cm, and a working channel of 3.7-mm diameter (Olympus V260). The duodenoscope was advanced into the stomach and then the afferent jejunal loop through the gastrojejunal anastomosis. The afferent loop after pancreaticoduodenectomy is always longer compared to that after a Billroth II gastrectomy. In this case, after arriving at the afferent loop and pushing the whole duodenoscope into the patient's stomach and afferent loop by retrieval-balloon-assisted enterography, we realized we were a long way (approximately 30 cm) from the anastomotic stoma of the hepaticojejunostomy (Figure 2A).

The position of the anastomotic stoma of hepaticojejunostomy after pancreaticoduodenectomy was actually invariably near the porta hepatis opening downward to the afferent limb. After using contrast to see the whole picture of the afferent loop, we inserted the wire-guided

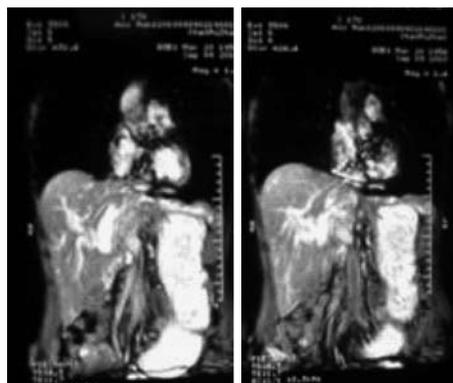


Figure 1 Magnetic resonance imaging showed compressed anastomotic stoma.

retrieval-balloon catheter to the extremity of the afferent limb, then inflated the balloon and drew back the catheter slowly. We focused on the possible position of the anastomotic stoma of the hepaticojejunostomy and tried to cannulate the intrahepatic bile duct using a guide wire and finally succeeded. It took only 9 min from when we saw the whole picture of afferent loop to successful cannulation (Figure 2B).

Consistent with the MRI, cholangiography showed the compressed anastomotic stoma of hepaticojejunostomy and expanded intrahepatic duct. We left the guide wire in the intrahepatic duct and removed the retrieval-balloon catheter. Because of the patient's late-stage cancer, we put a plastic stent through the stenosis of the anastomotic stoma. The stent went along the guide wire and was pushed by a long catheter to the proper site. Contrast came out of the intrahepatic duct into the afferent loop (Figure 2C). Five days later, X-ray showed that the stent remained in the correct position (Figure 3).

Four months later, the patient died of cachexia. According to a statement from her daughter, there was no jaundice, fever or abdominal pain during that time.

DISCUSSION

ERCP on patients with postsurgical anatomy changes, such as subtotal gastrectomy of Billroth II, Roux-en-Y hepaticojejunostomy and pancreaticoduodenectomy, is difficult to perform. Some case reports describe successful cannulation through front-viewing endoscopes^[1], SBE or DBE^[2-7]. However, rare cases of use of a conventional duodenoscope have been reported^[8-10].

One difficulty in ERCP with postsurgical anatomy changes is how to gain access to the right afferent loop. In our experience, for post-pancreaticoduodenectomy ERCP, the afferent loop was usually at the lesser curvature of stomach, so when we saw the gastrojejunal anastomosis after walking the duodenoscope along the greater curvature of stomach, the "upper tunnel" was the right afferent loop (Figure 4). Sometimes, it was difficult to get into the "upper tunnel" because of the relatively sharp angle. If it was hard to distinguish which was the

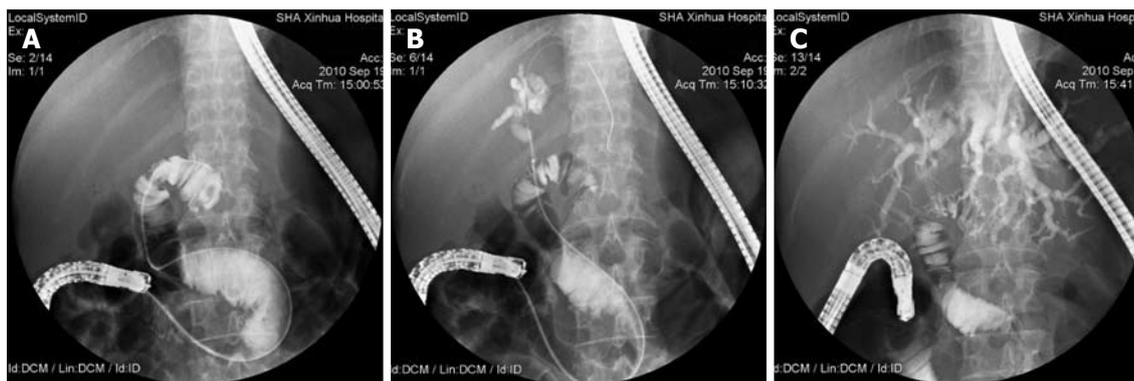


Figure 2 The process of blind cannulation through the anastomotic stoma of hepaticojejunostomy followed by placement of a plastic stent. A: The afferent loop was too long for the duodenoscope to reach the anastomotic stoma of hepaticojejunostomy; B: Successful cannulation into the intra hepatic bile duct with the help of the wire guided retrieval balloon catheter; C: A plastic stent was placed through the stenosis of the anastomotic stoma.



Figure 3 Five days after placement of biliary stent, X-ray of the biliary system showed the right position of the stent, and no contrast remained in the bile duct.

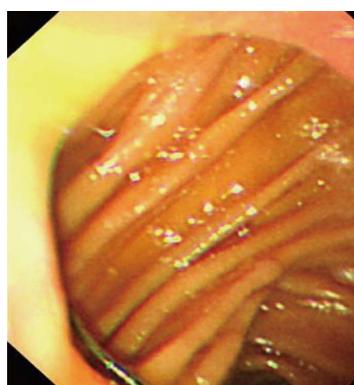


Figure 4 The "upper tunnel" was usually the exactly right afferent loop in post pancreaticoduodenectomy with child reconstruction.

upper, we would draw back the duodenoscope a little to "relax" the gastrojejunal anastomosis, making it easier to see distinguish the upper. Sometimes, we would also use a catheter (usually the wire-guided retrieval balloon that was used to remove the common bile duct stone) to explore into each limb, injecting contrast into the loop, followed by the scope advancing, and then found the afferent loop. It should be emphasized that in the ERCP with postsurgical anatomy changes, we could rely not only on what was visible through the endoscope, but also the X-ray images from enterology.

The main challenge in this case was the extremely long afferent loop. In an ordinary pancreaticoduodenectomy, the distance from the gastrojejunal anastomosis and the anastomotic stoma of hepaticojejunostomy is at least 60 cm. In theory, the duodenoscope could reach the anastomotic stoma, however, in this case the afferent loop was too long to reach. By using the retrieval balloon catheter and injecting contrast through it, we were able to see how much further we needed to go. The other key point to success in this case was that the surgeons knew the post-pancreaticoduodenectomy anatomy well and, as a result, knew where to cannulate. The retrieval-balloon catheter aided in approaching the anastomotic stoma of the hepaticojejunostomy and served as a sustainer to

achieve cannulation successfully.

The retrieval balloon could be used to explore the intestinal tract by injecting contrast through it under X-ray, especially in patients with postsurgical anatomy changes. We termed it retrieval-balloon-assisted enterography. It not only showed us the direction of the tract in order to guide the endoscope forward, but also facilitated forward movement of the endoscope with fewer injuries to the intestinal wall. In a subtotal gastrectomy of Billroth II with side-to-side jejunojunctionostomy of afferent and efferent loops, the retrieval-balloon catheter could also be used, by lying in the right limb, as a guide to prevent the duodenoscope from sliding out of the right limb into another upon forward motion. Upon successful accessing of the right limb, the retrieval balloon is visible in the tract ahead, instead of jumping out of it.

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Primary $\Delta 4$ -3-oxosteroid 5β -reductase deficiency: Two cases in China

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while the other was heterozygous for the mutation c.797G>A. Based on these mutations, a diagnosis of primary $\Delta 4$ -3-oxosteroid 5β -reductase deficiency could be confirmed. With ursodeoxycholic acid treatment and fat-soluble vitamin supplements, liver function tests normalized rapidly, and the degree of hepatomegaly was markedly reduced in both patients.

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Key words: Primary $\Delta 4$ -3-oxosteroid 5β -reductase gene; Cholestasis; Bile acid therapy; Aldo-keto reductase 1D1; Bile acid synthetic defects

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Abstract

Aldo-keto reductase 1D1 (*AKR1D1*) deficiency, a rare but life-threatening form of bile acid deficiency, has not been previously described in China. Here, we describe the first two primary $\Delta 4$ -3-oxosteroid 5β -reductase deficiency patients in Mainland China diagnosed by fast atom bombardment-mass spectroscopy of urinary bile acids and confirmed by genetic analysis. A high proportion of atypical 3-oxo- $\Delta 4$ -bile acids in the urine indicated a deficiency in $\Delta 4$ -3-oxosteroid 5β -reductase. All of the coding exons and adjacent intronic sequence of the *AKR1D1* gene were sequenced using peripheral lymphocyte genomic DNA of two patients and one of the patient's parents. One patient exhibited compound heterozygous mutations: c.396C>A and c.722A>T,

INTRODUCTION

Primary $\Delta 4$ -3-oxosteroid 5β -reductase deficiency (OMIM 235555, also called congenital bile acid synthesis defect type 2, CBAS 2) is a rare but potentially life-threatening form of bile acid deficiency that presents with neonatal progressive intrahepatic cholestasis and normal or slightly elevated γ -glutamyltransferase (GGT)^[1]. In the patients described to date, it is noted that early diagnosis and primary bile acid treatment lead to progressive normalization of liver function and avoidance of liver transplantation^[2].

The aldo-keto reductase 1D1 (*AKR1D1*) gene encodes the only known human $\Delta 4$ -3-oxosteroid 5β -reductase, which catalyzes the reactions responsible for changes to the steroid nucleus of cholesterol in both major bile acid synthesis pathways^[3]. Mutations in *AKR1D1* lead to a

deficiency of the bile acids that normally facilitate the detachment of γ -glutamyl-transpeptidase from the canalicular membrane. So, unlike other causes of cholestasis that present with markedly elevated GGT, CBAS 2 presents with normal or slightly elevated GGT^[1].

To our knowledge, an accurate diagnosis of CBAS 2 requires *AKR1D1* gene analysis, but only 8 reported cases of CBAS 2 worldwide have been diagnosed both biochemically and genetically^[1,4]. To further the knowledge of the clinical features and therapeutic treatment of CBAS 2, here we describe two patients in Mainland China with progressive intrahepatic cholestasis in early infancy diagnosed by fast atom bombardment-mass spectrometry (FAB-MS) analysis of urinary bile acids and genetic analysis.

CASE REPORT

Patients

A male patient (patient 1) was delivered at term after an uneventful pregnancy by a caesarean delivery with a birth weight of 3100 g. The infant represented his mother's third pregnancy; the first two pregnancies were terminated by abortion for social reasons. The patient's parents were both healthy and non-consanguineous. He developed progressive jaundice from 10 d of life with dark urine. At the age of 4 mo, recurrent pruritus and white stool were noticed, and exploratory laparotomy was performed. Hepatic pathology showed marked giant transformation of hepatocytes, lobular disarray, hepatocellular bile stasis, mildly interlobular fibrosis, proliferation and inflammation. After treatment with traditional Chinese medicine for 2 mo, the pruritus was relieved, but the jaundice persisted. At the age of 11 mo, he was referred to the hospital. On physical examination, moderate jaundice was present. The abdomen was soft, and the liver was detected 2 cm below the xiphoid process and 4.5 cm below the right costal margin, while the spleen was detected 2 cm below the left costal margin. There were no dysmorphic features or cardiac murmur. Laboratory studies at 11 mo showed total bilirubin (TBil) 146 $\mu\text{mol/L}$ (normal range: 5.1-17.1 $\mu\text{mol/L}$), direct bilirubin (DBil) 113 $\mu\text{mol/L}$ (normal range: 0-6 $\mu\text{mol/L}$), alanine aminotransferase (ALT) 210 IU/L (normal range: 0-40 IU/L), aspartate aminotransferase (AST) 207 IU/L (normal range: 0-40 IU/L), alkaline phosphatase 162 IU/L (normal range: 42-383 IU/L), GGT 65 IU/L (normal range: 7-50 IU/L), total bile acids (TBA) 6.1 $\mu\text{mol/L}$ (normal range: 0-10 $\mu\text{mol/L}$). Abdominal ultrasound showed hepatomegaly 4.5 cm below the right costal margin and splenomegaly 5 cm below the left costal margin.

Another male patient (patient 2) born at term after an uneventful pregnancy by a caesarean delivery with a birth weight of 3950 g was the first child of a non-consanguineous couple. The mother had hepatitis B and the father was healthy. He developed mild jaundice from 3 d of life and did not receive any medicine. At the age of 11 wk, he was referred to our hospital. On physical examination,

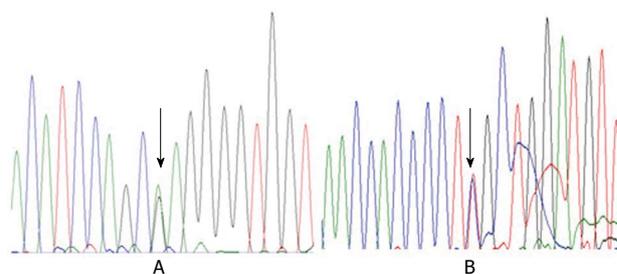


Figure 1 Genomic DNA sequences in exon 7 of the *aldo-keto reductase 1D1* gene in patient 1. A: The arrow identified a heterozygote mutation for c.797G>A (p.R266Q) in this patient; B: The reverse strand sequence shows the same result.

mild jaundice was present. The abdomen was soft, and the liver was 4 cm below the right costal margin and 3 cm below the xiphoid process. The spleen was not felt. There were no dysmorphic features or cardiac murmur. Laboratory studies showed TBil 96.1 $\mu\text{mol/L}$ (normal range: 5.1-17.1 $\mu\text{mol/L}$), DBil 64.4 $\mu\text{mol/L}$ (normal range: 0-6 $\mu\text{mol/L}$), ALT 176 IU/L (normal range: 0-40 IU/L), AST 183 IU/L (normal range: 0-40 IU/L), GGT 33 IU/L (normal range: 7-50 IU/L), TBA 3 $\mu\text{mol/L}$ (normal range: 0-10 $\mu\text{mol/L}$). Abdominal ultrasound showed hepatomegaly 4.5 cm below the right costal margin and splenomegaly 2 cm below the left costal margin.

Urine bile acid analysis

For patient 1, the first urine sample was taken at 11 mo of age when ursodeoxycholic acid (UDCA) (150 mg/d) was discontinued for 7 d. The FAB-MS analysis showed that the largest bile acid peaks were consistent with the presence of residual amounts of ursodeoxycholic acid metabolite (m/z 528), and the atypical bile acids of dihydroxy-oxo-cholenoic acids present in the glycine conjugate (m/z 460) and taurine conjugate (m/z 510) forms and monohydroxy-oxo-cholenic acids presents in the glycine conjugate (m/z 444) and the taurine conjugate (m/z 494) forms. Peaks attributable to the glycine and taurine conjugates of chenodeoxycholic acid and cholic acid (m/z 448, 464, 498, and 514) were present in traces or undetectable above background^[5]. A second urine sample was taken at 17 mo of age, when the patient was treated with UDCA (250 mg/d). Liver function test (LFT) was normal, with slightly elevated aminotransferase. The FAB-MS analysis showed that the largest bile acid peaks were consistent with ursodeoxycholic acid metabolite (m/z 528), a dihydroxy-oxo-cholenoic acid present as the glycine conjugate (m/z 460) and a monohydroxy-oxo-cholenic acid present as the glycine conjugate (m/z 444).

For the second patient, a urine sample was taken at 3 mo of age, when he was treated with UDCA (100 mg/d). LFT had improved but was not completely normal. The FAB-MS analysis was similar to that for patient 1.

Genetic analysis

The study protocol conforms to the ethical guidelines of the Declaration of Helsinki of 1975. With the approval

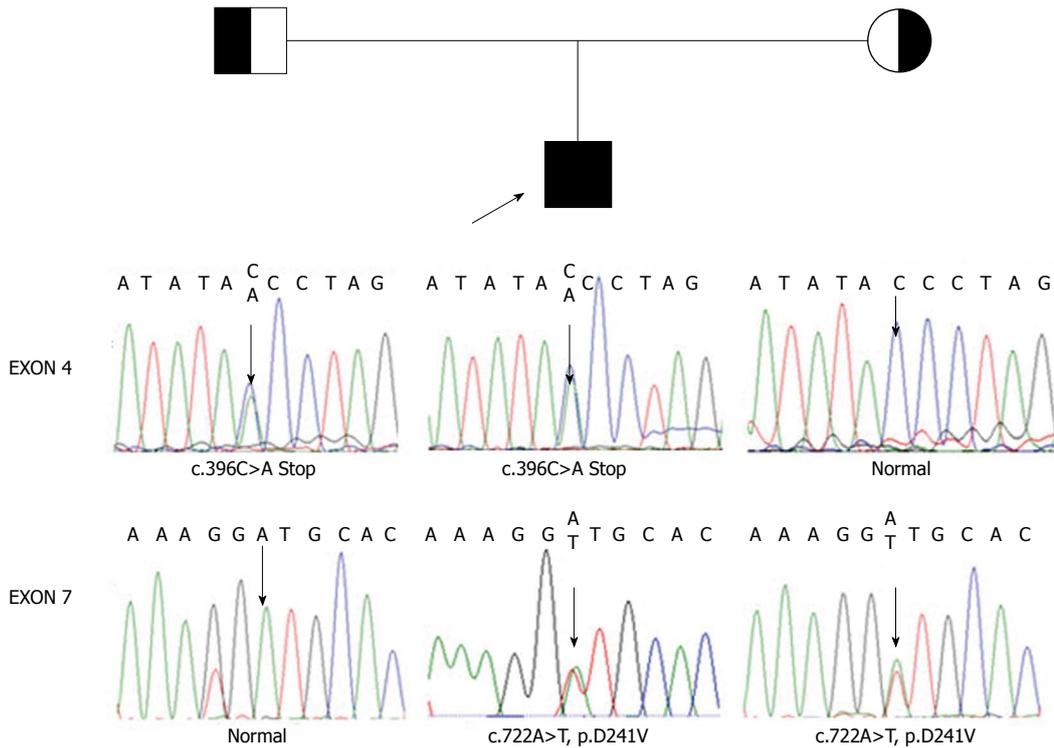


Figure 2 Pedigree for patient 2 shown with genomic DNA sequences in exons 4 and 7 of the *aldo-keto reductase 1D1* gene in patient 2 and his parents. The arrow in exon 4 identified C/A in patient 2s and his father, but C in his mother. The arrow in exon 7 identified A/T in patient 2 and his mother, but T in his father. These represent compound heterozygote with c.396C>A (nonsense mutation) from his father and c.722A>T (p.D241V) from his mother.

by the Ethics Committee on human research of the Children’s Hospital of Fudan University and the informed consent of the parents, 1 mL of whole blood was drawn from the patient and his parents. To confirm the diagnosis and establish the molecular basis of the disorder, genomic DNA was isolated from the white blood cells. Then, we sequenced all of the coding exons and adjacent intronic sequence of the *AKR1D1* gene. Patient 1 was found to be heterozygous for c.797G>A in exon 7, which leads to an amino acid substitution of arginine by glutamine at amino acid position 266 (p.R266Q) (Figure 1). Patient 2 was revealed to be compound heterozygous for c.396C>A (nonsense mutation) and c.722A>T (p.D241V), the former from his father and the latter from his mother (Figure 2).

Management and treatment

Patient 1 was treated with UDCA (40 mg/kg per day) for 5 mo. Liver transaminase concentration was normalized (Figure 3A), and the degree of hepatomegaly was markedly reduced. Jaundice has improved and disappeared. Recently, liver function tests showed TBil 6.7 μmol/L, DBil 2.9 μmol/L, ALT 8 IU/L, AST 16 IU/L, and GGT 10 IU/L.

Patient 2 was treated with UDCA (40 mg/kg per day) for 4 mo. The liver transaminase concentration was normalized, and the degree of hepatomegaly was markedly reduced. Jaundice has improved and disappeared. After FAB-MS analysis of urine bile acid, the patient was given chenodeoxycholic acid (CDCA, 25 mg/kg per day)

instead of UDCA for 4 mo. After the CDCA was discontinued, the patient’s total bilirubin and alanine aminotransferase was normal (Figure 3). However, the patient was not normally responsive to both visual stimuli and auditory stimuli. Magnetic resonance imaging at the age of 13 mo showed cerebral dysplasia with abnormally low levels of white matter.

DISCUSSION

Primary Δ4-3-oxosteroid 5β-reductase deficiency, first reported by Setchell *et al*^[6], is an extremely rare condition. We diagnosed two Chinese infants, both of whom presented with neonatal progressive intrahepatic cholestasis with markedly elevated serum conjugated bilirubin and aminotransferase but normal serum TBA and normal or slightly elevated GGT. Diagnosis of CBAS 2 was established by FAB-MS analysis of urinary bile acids and genetic analysis. According to clinical features and biochemical findings, bile acid synthesis deficiency was highly suspected^[1,7,8]. However, analyses of the bile acid profile and *AKR1D1* gene sequence were not available in our center at that time. Our patients were given a large dose of UDCA combined with a fat-soluble vitamin supplement, which was inadvertently found to produce a good response in our patients with CBAS 1.

The first urine sample of patient 1 was collected when UDCA (150 mg/d) was stopped 7 d, although there are several ions that may represent bile acid metabolites that are usually observed with UDCA therapy for metabolism

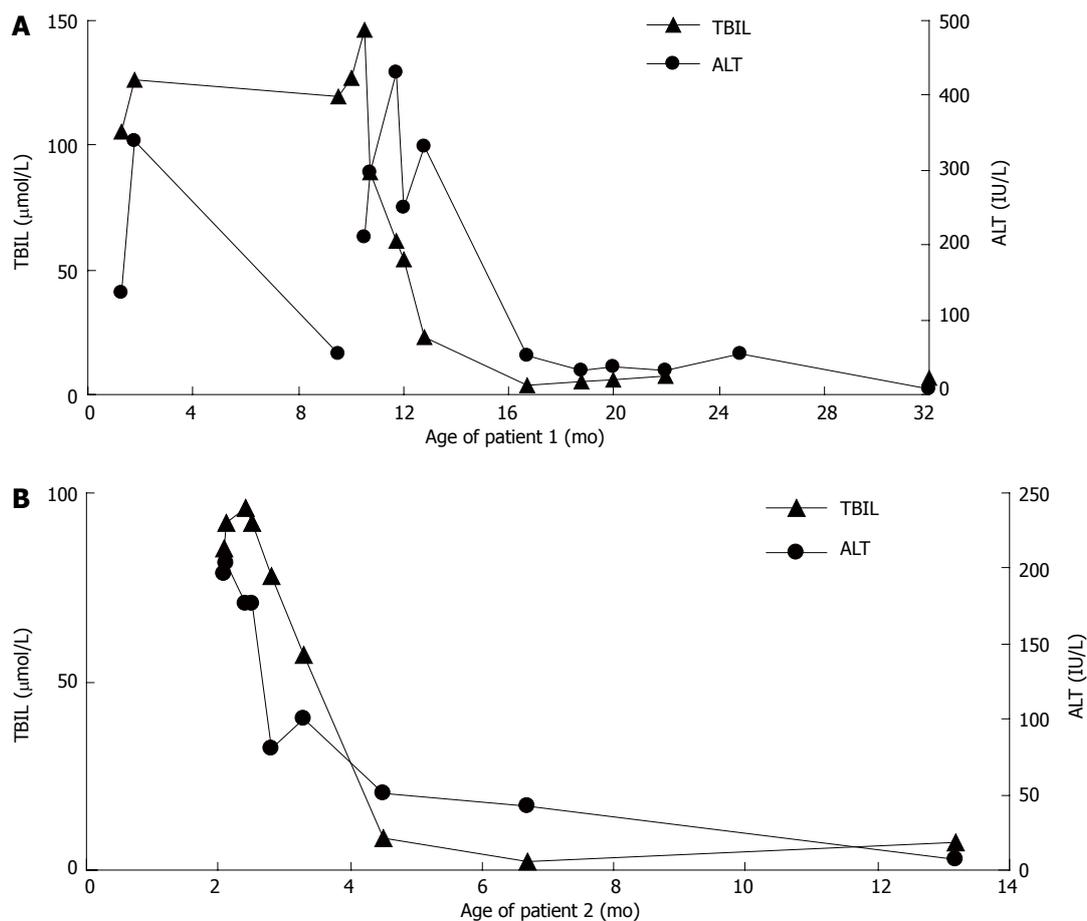


Figure 3 The responds of total bilirubin and alanine aminotransferase to treatment with ursodeoxycholic acid and/or chenodeoxycholic acid. A: Patient 1; B: Patient 2. TBIL: Total bilirubin; ALT: Alanine aminotransaminase.

of UDCA may be present, the profile was conspicuous by lack of normal primary bile acid conjugates that are characteristic of a bile acid synthetic defect involving a deficiency in the activity of the Δ 4-3-oxosteroid 5β -reductase deficiency. The second urine sample of patient 1 and the urine sample of patient 2 were collected when UDCA therapies were administered. The profiles were complicated by several ions that represent bile acid metabolites usually observed with UDCA therapy. Both were highly probable that these were primary defects in bile acid synthesis^[6]. To definitively confirm a primary defect^[4], we sequenced all the coding exons and adjacent intronic sequence of the *AKR1D1* gene.

Three new mutations in *AKR1D1* exons were detected in our patients, all of which altered the sequence and function of protein and were predicted to be disease causing by Mutation Taster (<http://www.mutationtaster.org/>). All of the altered amino acid loci are highly conserved among orthologous proteins in different species. There were two patients reported who were found with just one heterozygote mutation^[4]. We sequenced all the coding exons and adjacent intronic sequence of the *AKR1D1* gene, another heterozygous mutation might be outside the coding and splicing sequence or there might be a large DNA genomic aberration which couldn't be

found in Sanger sequencing^[4,8]. Based on biochemical changes in urine bile acids and mutations in *AKR1D1* gene, the diagnosis of CBAS 2 in both patients was confirmed.

UDCA is not recommended to be used in treatment of patients with the *AKR1D1* deficiency, because it has no ability to downregulate endogenous bile acid synthesis and prevent continued synthesis of atypical and potentially hepatotoxic bile acid intermediates and their metabolites^[9]. However, an analysis of the urinary bile acids of patient 1 showed that, even after liver function had normalized, large amounts of abnormal bile acids and UDCA remained. In addition, patient 2, who was treated by UDCA followed by CDCA, showed cerebral dysplasia with low levels of white matter. However, it would be difficult to determine whether this injury was due to the deficiency of delta 4-3-oxosteroid 5β -reductase and accumulation of abnormal bile acids in lipids in the cerebrum or intracranial space or to other metabolic complications.

In conclusion, we report the first two documented cases of CBAS 2 in Mainland China. Although rare, primary 3-oxo-delta 4-steroid 5β -reductase deficiency should be considered in patients with neonatal cholestatic jaundice with normal TBA and normal or slightly elevated GGT.

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Esophageal granular cell tumors: Report of 9 cases and a literature review

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Abstract

Esophageal granular cell tumors (GCTs) are rare tumors of the esophagus. We evaluated the clinical and pathologic features of 9 esophageal GCT patients (5 men and 4 women) from our institute and reviewed the related disease literature. Patient age ranged from 25 to 53 years (mean: 41 years). All the patients were asymptomatic or presented with non-specific symptoms. Most GCTs occurred in the distal esophagus and were less than 6 mm in diameter. Computational analysis showed that the average gray-scale endoscopic ultrasound images of esophageal GCTs were greater than that of esophageal leiomyomas. Eight patients were treated by endoscopic resection, and 1 patient underwent surgical excision. No post-therapy recurrence or metastasis developed during follow-up (mean: 36.4 mo, range: 1-72 mo).

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Key words: Esophageal granular cell tumor; Endoscopic

INTRODUCTION

Granular cell tumors (GCTs) are nerve sheath tumors composed of Schwann cells with granular cytoplasm^[1]. GCTs were first described in the 1920s by Abrikossoff in a series of 5 cases with benign tumors removed from the tongue. These tumors were once regarded as specific to skeletal muscle. However, subsequent studies identified GCTs in various other tissues, including skin, breast, respiratory tract, and biliary system^[1].

GCTs are relatively rare in the gastrointestinal tract, accounting for approximately 8% of all GCTs^[2,3]. Of these, only about 2% occur in the esophagus^[3]. Abrikossoff described the first case of an esophageal GCT in the early 1930s. To date, about 200 cases of esophageal GCTs have been described in the literature, mostly representing single case reports or small case series. Several aspects of esophageal GCTs remain to be elucidated, such as whether endoscopic ultrasound (EUS) can effectively diagnose the disease, what the appropriate management is, and whether GCTs undergo malignant degeneration. Therefore, we studied the clinical and pathologic features of esophageal GCTs by retrospectively analyzing all

esophageal GCT cases treated at our institution to address these unresolved issues.

CASE REPORT

Subjects

All the patients evaluated in this study were in- or outpatients treated at the First Affiliated Hospital, College of Medicine, Zhejiang University, between January 2001 and December 2011. All the patients were initially diagnosed with suspected esophageal submucosal protruding lesions (as revealed by conventional endoscopy), and were subsequently confirmed as GCT by histology. The study was approved by the hospital's ethics committee, and all the patients agreed to study participation.

EUS examinations

Patient preparation for EUS examinations was the same as those for conventional gastroscopy. The EUS equipment was composed of double cavity mirrors (Olympus GIF-2T 240), a mini-ultrasound probe with 12 MHz frequency, and the ultrasound device (Olympus EU M2000). Endoscopic specialists performed all operations, diagnoses, and therapeutic interventions.

Esophageal GCTs may be easily misdiagnosed as leiomyomas using EUS examination. To explore whether EUS can distinguish the two diseases by computer-aided image analysis, we compared the average gray-scale and gray-scale standard deviation of the EUS images for the two diseases. We selected the lesions with a range of interest for each of the cases, then determined the average gray-scale and gray standard deviation of the range of interest and compared the results for the two disease groups. The image analysis software was Photoshop CS2 9.0. The parameter indices of average gray-scale and gray standard deviation referred to the nature of echo and echogenicity, respectively.

Histological analysis

Eight cases underwent endoscopic resection, and the remaining case underwent surgical excision. The procedures for endoscopic resection of GCTs were the same as those used for other mucosal-based and submucosal esophageal lesions. In a case with esophageal GCT originating from the submucosa and having a diameter larger than 10 mm, the trans-thoracic operation was performed.

The tissue specimens obtained by endoscopic resection or surgical excision were fixed in 10% formalin and processed for paraffin embedding using standard methods. Sections of 4 mm thickness were stained with hematoxylin and eosin, and observed by light microscopy. Subsequently, specimens from all cases were stained for CD68, CD34, Ki-67, smooth muscle actin (SMA), S-100, GFAP, and p53 using standard immunohistochemistry techniques.

The criteria for histological diagnoses of GCT were: the tumor being mainly composed of larger tumor cells with cytoplasm rich in eosinophilic granules; the tumor cell morphology showing densely nested or stringy struc-

ture; the granules in the cytoplasm showing positive periodic acid-Schiff (PAS) staining; the tumor cells expressing S-100.

Follow-up examination and data analysis

To study the progression of GCTs, all the patients were followed-up with periodic endoscopic examination. Statistical analyses were performed using the SPSS 13.0 software for Windows. *P* values less than 0.05 (by a 2-tailed test) were considered statistically significant.

Results

The clinical characteristics of the patients are listed in Table 1. Our case series included 9 histologically confirmed esophageal GCT patients (5 men and 4 women). Patient age ranged from 25 to 53 years, with a mean age of 41 years. Of the 9 patients, 7 complained of nonspecific symptoms, including upper abdominal distension, poor appetite, belching, and acid reflux.

All 9 cases were initially diagnosed with suspected esophageal submucosal protruding lesions, as indicated by conventional endoscopy. The typical endoscopic appearance was isolated, tawny or grayish white submucosal nodules with the overlying mucosa being normal or slightly granular, without ulceration or mucosal depression (Figure 1). Seven cases involved the distal esophagus, and 2 cases involved the middle esophagus. Eight cases received computed tomography chest examination; all of which revealed a soft-tissue mass in the esophagus and partial narrowing of the esophageal lumen. The enhanced scan technique showed that all masses were moderately enhanced.

EUS was performed in 8 patients and showed that the tumors were slightly low echo and were circular or quasi-circular in shape. The surrounding esophageal structure appeared normal for all the lesions. Three cases originated from the mucosa, 2 from the submucosa, and 3 from the muscularis mucosa. The size of the tumors ranged from 4 to 11 mm in diameter. In comparison to esophageal leiomyomas, the esophageal GCTs showed slightly stronger echo. Quantitative gray-scale analysis confirmed this observation. Photoshop CS2 9.0 analysis of the EUS images of esophageal GCTs, compared to age-, sex-, size- and location-matched esophageal leiomyomas, indicated that the average gray-scale EUS images of esophageal GCTs were slightly and significantly greater than that of esophageal leiomyomas. Accordingly, we propose that the echo of GCTs may be stronger than that of esophageal leiomyomas.

Histological analysis showed that all tumors were composed of proliferating cells of slightly ovoid shape and with abundant eosinophilic granular cytoplasm. The tumors were PAS-positive. Diastase-resistant granules, representing lysosomes, were present within the cytoplasm of some cells. Immunohistological study showed that all the tumors were positive for S-100 and CD68, but negative for SMA, CD34, p53, GFAP, Ki-67, and CD117 (Figure 2).

No recurrence was observed after endoscopic or sur-

Table 1 Clinical characteristics of the patients with esophageal granular cell tumors

Case	Gender	Age	Symptoms	Location	Size (mm × mm)	Originating layer
1	Female	45	Upper abdominal distension	Distal	5 × 4	Undetermined
2	Female	25	Upper abdominal distension	Distal	5 × 4	Submucosa
3	Male	41	No	Distal	4 × 3	Muscularis mucosa
4	Female	41	Recurrent belching and acid reflux	Distal	5 × 4	Mucosa
5	Male	47	No	Distal	4 × 2	Muscularis mucosa
6	Male	50	Upper abdominal distension, poor appetite	Distal	6 × 4	Mucosa
7	Female	44	Abdominal distension and belching	Middle	5 × 3	Muscularis mucosa
8	Male	53	Substernal distension	Distal	11 × 10	Submucosa
9	Male	32	Upper abdominal distension	Middle	4 × 3	Mucosa

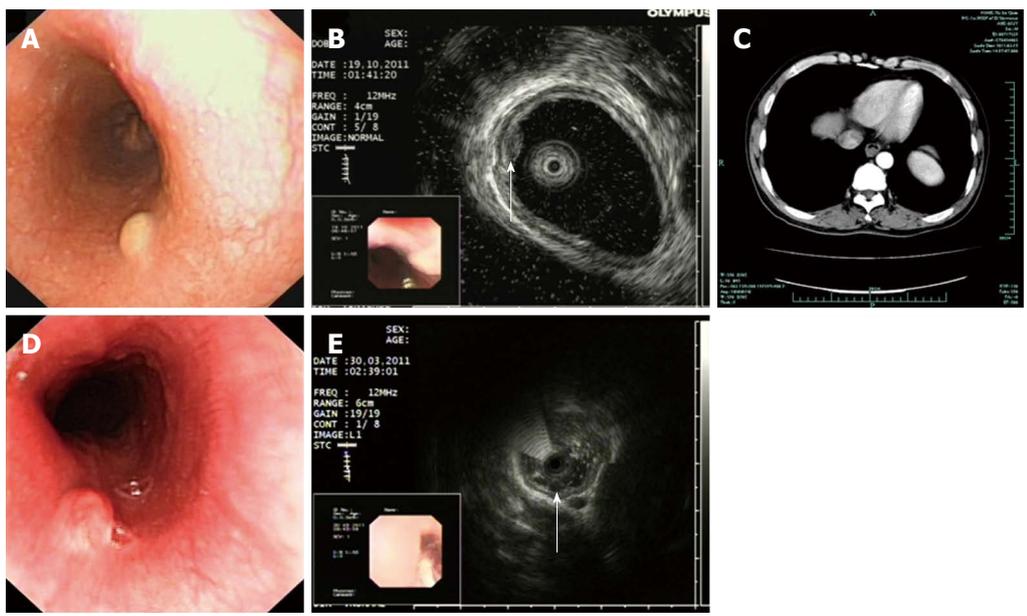


Figure 1 Clinical examination of esophageal granular cell tumors and esophageal leiomyomas. A: Conventional gastroscopy showing a representative granular cell tumor (GCT) with white-to-yellow surface color and smooth surface; B: Endoscopic ultrasound (EUS) showing a hypoechoic lesion originating from the mucosa layer; C: Computed tomography scan showing a soft-tissue mass in the esophagus and partial narrowing of the esophageal lumen; D: Conventional gastroscopy showed that the leiomyoma had a light pink surface color with smooth surface; E: EUS showing the leiomyoma with a slightly lower echo than the esophageal GCT.

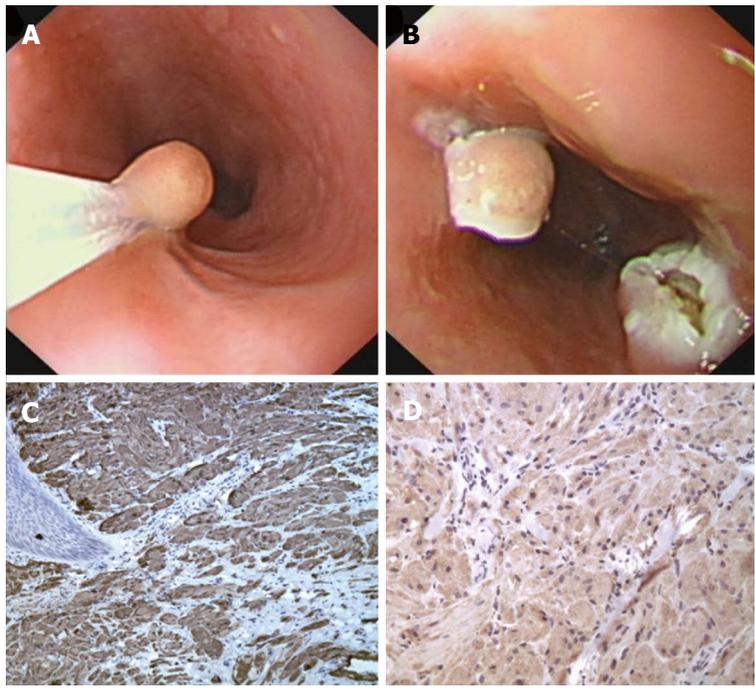


Figure 2 Clinical management and histological characteristics of esophageal granular cell tumors. A, B: A typical case of esophageal granular cell tumor treated by endoscopic resection; C: Histological analysis showing the lesion stained positively for S-100; D: Histological analysis showing the lesion stained positively for CD68.

gical resection or during the follow-up period (mean: 36.4 mo, range: 1-72 mo).

DISCUSSION

GCT is a rare type of esophageal submucosal tumor. Its character of dormant onset lends to the disease being overlooked or misdiagnosed. GCTs are female predominant^[4], and mainly occur in middle-age^[5,6]. In the current series of esophageal GCTs patients, the mean age was 41 years, but the sex distribution was more equal than in the collected literature. Since ethnicity may play a role in the development of GCTs^[7], the observed difference in sex distribution may reflect our patients' Chinese background. According to the collected literature, esophageal GCTs primarily occur in the distal esophagus. Orlowska *et al*^[9] reported that two-thirds of esophageal GCTs were found in the distal esophagus, with only 20% and 15% in the middle and proximal esophagus, respectively. Another case series reported by Goldblum *et al*^[5] showed that 12 of 13 patients had distal esophageal GCT. Similarly, our case series included 7 cases with GCTs in the distal esophagus, and only 2 cases of GCT in the middle esophagus.

In general, esophageal GCTs are initially observed by conventional gastroscopy and confirmed by histological analysis. The ability of EUS to show the size, origin, borders, and echo structure of the submucosal lesion, has dramatically improved the diagnostic accuracy for submucosal disorders^[8]. However, EUS may still misdiagnose some lesions with similar imaging features, such as esophageal GCTs and leiomyomas. In the current study, we applied computer-aided analysis techniques to comparatively analyze the ultrasound images of GCTs and leiomyomas. Our results showed that the gray value of esophageal GCTs was slightly, but significantly, greater than that of esophageal leiomyomas. This suggests that the EUS echo of esophageal GCTs is higher than that of leiomyomas, which possibly represents a discriminating feature of esophageal GCTs and leiomyomas. This feature may aid in the differential analysis of gray values on EUS images and help to improve the preoperative diagnostic accuracy of esophageal GCTs and possibly stimulate ideas for new strategies for earlier diagnosis and more effective treatment.

GCTs are potential malignant tumors. Fanburg-Smith *et al*^[9] proposed the first histological criteria for prospectively diagnosing malignancy in GCTs, which include tumor cell necrosis, tumor cell spindling, increased nuclear size, large nucleoli, mitotic activity, and nuclear pleomorphism. Due to the potential for malignancy, endoscopic resection or surgical excision is recommended for patients with esophageal GCTs. In the current study, 8 cases were treated by endoscopic resection and one

case was treated by surgical excision. All the patients were followed-up with periodic endoscopic examinations, during which no recurrence was detected. Our experience, as well as that of other treating physicians reported in the literature, defines endoscopic resection as a safe and effective therapeutic choice for esophageal GCTs^[10-12].

In conclusion, GCTs are uncommon esophageal tumors, which are mainly asymptomatic and located in the distal esophagus of middle-age subjects. Histology remains the gold standard for diagnosis of esophageal GCTs, while EUS may also provide important diagnostic information. Endoscopic resection or surgical excision should be performed due to the potential for malignancy.

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Successful disintegration, dissolution and drainage of intracholedochal hematoma by percutaneous transhepatic intervention

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shown that percutaneous transhepatic manipulation is a major cause of hemobilia after liver transplantation, but in our case, percutaneous transhepatic intervention was used to relieve the biliary obstruction and dissolve the biliary clot, with a good outcome.

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Key words: Hemobilia; Biliary clot; Fulminant hepatic failure; Percutaneous transhepatic biliary drainage

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Abstract

Hemobilia is a rare biliary complication of liver transplantation. The predominant cause of hemobilia is iatrogenic, and it is often associated with traumatic operations, such as percutaneous liver intervention, endoscopic retrograde cholangiopancreatography, cholecystectomy, biliary tract surgery, and liver transplantation. Percutaneous transhepatic cholangiography and liver biopsy are two major causes of hemobilia in liver transplant recipients. Hemobilia may also be caused by coagulation defects. It can form intracholedochal hematomas, causing obstructive jaundice. Herein we describe a patient with an intracholedochal hematoma resulting in significant obstructive jaundice after liver transplantation for fulminant hepatic failure. Previous studies have

INTRODUCTION

Fulminant hepatic failure is a syndrome characterized by impaired liver function in an acute setting. It is defined as the onset of hepatic encephalopathy and liver failure within 8 wk of jaundice^[1]. Liver transplantation remains the exclusive choice of treatment to improve the survival in the majority of patients with fulminant hepatic failure^[2]. Biliary complications following liver transplantation are a major cause of morbidity, but hemobilia is infrequent^[3]. Hemobilia may result in intracholedochal hematoma, causing obstructive jaundice. The current report describes a patient with intracholedochal hematoma leading to significant obstructive jaundice after liver transplantation for fulminant hepatic failure.

CASE REPORT

A 23-year-old female was admitted on July 15, 2010 with complaints of abdominal pain and distension, accompanied by fever, dark yellow urine, and xanthochromia for the past three days. The patient had been treated for psoriasis with traditional Chinese medicine for three months prior to admission. In addition, she had a 16-year history of hepatitis B virus infection. Despite being a carrier of the hepatitis B virus, the patient had no history of hepatitis and liver function tests had been normal during the follow-up.

On physical examination, the patient was conscious, and her orientation and calculations were normal. Jaundice was evident. The abdomen was slightly distended and soft, with tenderness and rebound tenderness, and a shifting dullness test was positive. The serum transaminase and total bilirubin levels were markedly elevated on admission. The laboratory test results were as follows: alanine transaminase (ALT), 5190 U/L; aspartate transaminase (AST), 7230 U/L; total bilirubin (TBIL), 142.8 $\mu\text{mol/L}$; and direct bilirubin (DBIL), 83.3 $\mu\text{mol/L}$ (Figure 1). The prothrombin time (PT) and activated partial thromboplastin time (APTT) were 26.5 s and 41.5 s, respectively. The international normalized ratio (INR) was 2.39. These results suggested significant impairment of coagulation function. In addition, serum hepatitis B virus DNA was present at 4.6×10^7 copies/mL.

The patient was treated with artificial liver support (ALS)-plasma exchange due to diminished liver function and impaired coagulation. Although the laboratory indicators improved (Figure 1), hepatic encephalopathy occurred and worsened progressively. Therefore, fulminant hepatic failure was diagnosed. On July 21, 2010 (post-admission day 7), the patient suffered a coma without pupillary light reflex, although the vital signs were stable. The patient underwent liver transplantation on July 23, 2010. A modified piggyback liver transplantation was carried out, and an end-to-end duct-to-duct anastomosis without a T-tube was performed to reconstruct the biliary tract. A continuous 6-0 polydioxanone suture was used for the posterior wall while interrupted sutures were applied for the anterior wall. She was maintained on a tacrolimus-based immunosuppressive regimen that included mycophenolate mofetil and glucocorticoid.

The patient regained consciousness on July 25, 2010 [post-transplantation day (PTD) 2]. The liver and coagulation functions were significantly improved. On PTD 4, the laboratory data were as follows: ALT, 553.8 U/L; AST, 187.4 U/L; TBIL, 58.7 $\mu\text{mol/L}$; DBIL, 28.5 $\mu\text{mol/L}$ (Figure 1); albumin, 37.2 g/L; PT, 9.2 s; APTT, 21.9 s; INR, 0.83; serum tacrolimus, 12.2 ng/mL; hemoglobin, 100 g/L; and platelet count, $61 \times 10^9/\text{L}$. Because red blood cells, plasma, and cryoprecipitate were transfused without platelets following a massive intra-operative hemorrhage (approximately 2500 mL), dilutional thrombocytopenia was diagnosed. Platelets were then transfused to treat the thrombocytopenia; however, the right leg became edema-

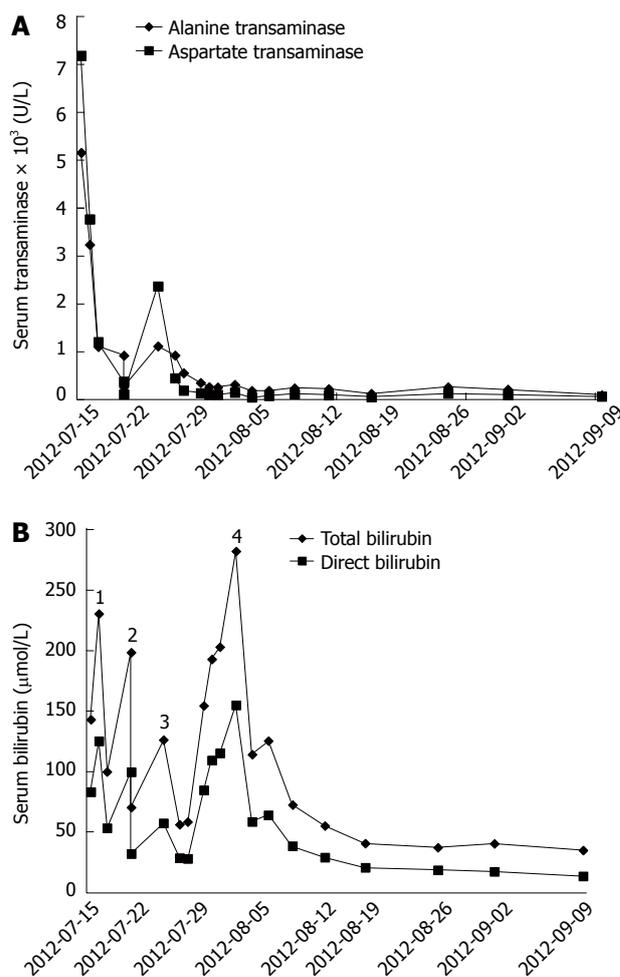


Figure 1 Laboratory test results of physical examination. A: Blood concentration of transaminases markedly decreased after artificial liver support (ALS)-plasma exchange, increased after liver transplantation due to ischemia reperfusion injury, and reached a peak on post-transplantation day 2, then decreased gradually to normal; B: Blood concentration of bilirubin. There are four marked peaks: bilirubin increased due to fulminant liver failure and decreased after two ALS-plasma exchanges, forming peaks 1 and 2; bilirubin increased after liver transplantation due to ischemia reperfusion injury and reached a peak, forming peak 3; bilirubin increased markedly because of the intrahepatic biliary hematoma and decreased after percutaneous transhepatic intervention, forming peak 4.

tous on PTD 3. Doppler ultrasound revealed a thrombus in the femoral vein (Figure 2), which was punctured due to the ALS-plasma exchange. Subsequently, low molecular weight heparin (LMWH) was used to treat the deep venous thrombosis which relieved the edema, but the volume of bloody ascites gradually increased. On PTD 5, the hemoglobin decreased to 77 g/L. Anticoagulation was therefore discontinued and blood was transfused. However, the jaundice became severe again. On PTD 10, the TBIL was 281.2 $\mu\text{mol/L}$, and the DBIL was 154.7 $\mu\text{mol/L}$ (Figure 1). A computed tomography (CT) scan showed dilation of the intrahepatic biliary ducts and a huge hematoma below the right lobe of liver (Figure 3A). Therefore, obstructive jaundice was suspected.

To confirm the diagnosis of obstructive jaundice and relieve the biliary obstruction, endoscopic retrograde

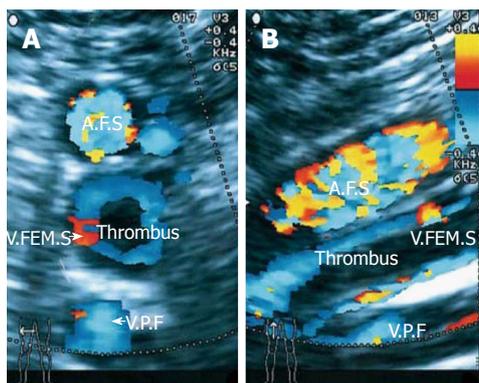


Figure 2 Doppler ultrasound shows a thrombus in the right femoral vein. A: Axial plane; B: Sagittal plane. A.F.S.: Superficial femoral artery; V.FEM.S.: Superficial femoral vein; V.P.F.: Profund femoral vein.

cholangiopancreatography was performed, but failed and caused pancreatitis. So as an alternative, percutaneous transhepatic intervention was administered on PTD 10. Cholangiography showed a long intrabiliary filling defect crossing the strictured biliary anastomosis (Figure 3B). As fecal occult blood testing was positive, hemobilia leading to biliary hematoma was suspected. A 0.018-inch diameter micro-guidewire, a 6F sheath, a 0.038-inch diameter micro-guidewire, and an 8F catheter were manipulated through the hematoma sequentially, making the hematoma break into pieces, enlarging the contact surface with flowing bile and making it easy to dissolve. Then the 8F biliary drainage catheter was placed across the anastomosis with one end opening into the duodenum and the other end exiting the body (Figure 3C). The amount of biliary drainage was approximately 400 mL per day, and the jaundice was markedly relieved 2 d later (TBIL, 114.1 $\mu\text{mol/L}$; DBIL, 58.9 $\mu\text{mol/L}$) (Figure 1). The patient recovered well and the jaundice gradually diminished 10 d after percutaneous transhepatic biliary drainage (PTBD); when the TBIL was 55.5 $\mu\text{mol/L}$, the external end of the biliary drainage catheter was closed. The glucocorticoid was gradually tapered and discontinued on PTD 30. On September 14, 2010 (PTD 54), she experienced no more discomfort and the TBIL was 35.3 $\mu\text{mol/L}$ (Figure 1). The patient was discharged the next day with the biliary drainage catheter *in situ* and the external port closed.

At the first follow-up evaluation on January 29, 2011, magnetic resonance cholangiopancreatography showed the intrahepatic biliary ducts were slightly dilated (Figure 3D). A CT scan revealed that the hematoma below the liver was almost absorbed and the intrahepatic biliary ducts were slightly dilated (Figure 3E). The liver function tests were within normal ranges: TBIL, 11.3 $\mu\text{mol/L}$; DBIL, 3.5 $\mu\text{mol/L}$; ALT, 15.0 U/L; AST, 22.1 U/L (Figure 1). The serum tacrolimus was 8.4 ng/mL. Cholangiography was also performed through the biliary drainage catheter, showing that the intrahepatic biliary ducts were slightly dilated and that there was a stricture at the site of the anastomosis (Figure 4A). To prevent a more severe stricture and obstructive jaundice, a balloon dilatation

of the anastomotic stricture was performed (Figure 4B) to relieve the stricture (Figure 4C). To avoid the risk of infection and stenosis of the lumen, the biliary drainage catheter was replaced with a new catheter (Figure 4D). In addition, the mycophenolate mofetil was discontinued 6 mo after liver transplantation. The patient was then maintained on immunosuppressive therapy with tacrolimus (3 mg/d). Two weeks later at the second follow-up evaluation, the biliary drainage catheter was removed. During the one-year follow-up, the patient had no discomfort and her liver functions remained normal.

DISCUSSION

Biliary complications are a major cause of morbidity in liver transplant recipients, with an incidence of 10%-30% and a mortality rate of up to 10%. Biliary leaks and strictures are the most common biliary complications, followed by sphincter of Oddi dysfunction, hemobilia, and biliary obstruction from stones, sludge, casts, or cystic duct mucocoeles^[3]. Hemobilia is defined as bleeding into the biliary tree. Hemobilia arises when there is communication between a vascular structure and the biliary tree. The predominant cause of hemobilia is iatrogenic, such as percutaneous liver intervention, endoscopic retrograde cholangiopancreatography, cholecystectomy, biliary tract surgery, and liver transplantation^[4]. Percutaneous transhepatic cholangiography and liver biopsy are two major causes of hemobilia in liver transplant recipients^[3]. In addition, coagulation defects may cause hemobilia^[5]. Jolobe^[6] reported that hemobilia is a possible cause of jaundice in patients receiving anticoagulants, although hemobilia after liver transplantation is infrequent^[3,4]. The LMWH used to treat the thrombosis within the right femoral vein may be the cause of hemobilia in the current case.

The clinical manifestations of hemobilia are determined by the amount and speed of hemorrhage in the biliary tract. Profuse hemobilia often causes severe symptoms with colicky pain and gastrointestinal hemorrhage. In contrast, occult bleeding, which is more frequent, often lacks clinical significance as the blood inconspicuously flows into the intestine. Even if the blood does coagulate within the ducts, the clots are often promptly dissolved by the fibrinolytic activity of bile. Sometimes, the clots may escape dissolution, causing obstructive jaundice^[5]. Sandblom *et al*^[7] constructed a model of the biliary tract into which blood could be injected. When hemorrhage is severe and rapid, the blood mixes with the bile and forms mushy clots; when hemobilia is minor and slow, it does not mix with the bile, but flows into the bottom of the system where it forms a solid, pure clot that is a cast of the lumen. These pure clots remain stable, whereas the mixed clots dissolve rapidly. Sandblom *et al*^[5,7] also reported that the fate of clots is related to the bile flow, especially noting that when the clots are exposed to flowing bile, the dissolution is rapid. Our case is very interesting because hemobilia occurred with an

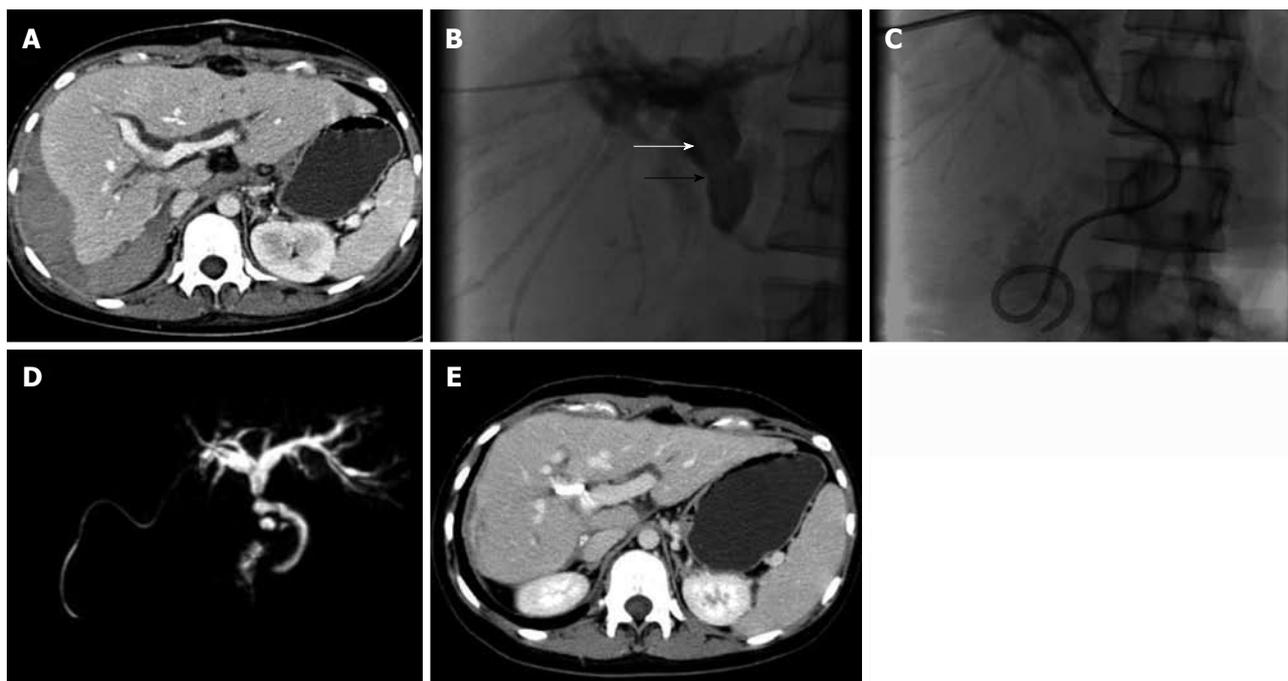


Figure 3 Diagnosis of obstructive jaundice. A: Before percutaneous transhepatic intervention, on computed tomography (CT) scan, intrahepatic biliary ducts are dilated and a huge hematoma is present below the right lobe of liver; B: Percutaneous transhepatic cholangiography shows a long intrabiliary filling defect (white arrow) crossing the strictured biliary anastomosis (black arrow); C: An 8F biliary drainage catheter was placed across the anastomosis with one end opening into the duodenum and the other end exiting the body; D: On magnetic resonance cholangiopancreatography, the intrahepatic biliary ducts were slightly dilated at the first follow-up; E: CT reveals that the hematoma below the liver was almost absorbed and the intrahepatic biliary ducts were slightly dilated at the first follow-up.

abdominal hemorrhage after administration of LMWH, and the biliary clot and the huge subhepatic hematoma formed simultaneously after discontinuation of LMWH. The abdominal hemorrhage was massive, causing the hemoglobin to decrease sharply. The hemobilia, however, was minor because there was no evidence of significant gastrointestinal bleeding.

The principles of managing hemobilia are to resuscitate the patient, control hemorrhage, and maintain biliary patency. Resuscitation with transfusion must be administered to obtain hemodynamic stability when the hemorrhage is severe and rapid. Additionally, coagulopathies should be corrected appropriately. When hemorrhage is active and prolonged, hemostatic interventions should be considered, including transcatheter arterial embolization (TAE), percutaneous thrombin injection, and surgery. TAE is the therapy of choice to achieve hemostasis, with a success rate of 75%-100%^[6]; however, TAE may not be appropriate in liver transplant patients because it can cause liver graft dysfunction and biliary ischemia. These complications are devastating, especially in the early post-transplantation period. Croutch *et al*^[8] reported that superselective arterial embolization is a safe treatment for hemobilia caused by PTBD in liver transplant recipients, and emphasized that it is important to use a microcatheter to achieve precise subselective localization and embolization, minimize spasm, and avoid occlusion of noninvolved arterial branches. In spite of this, the cases were all due to PTBD, which often causes small branches to bleed into bile ducts. For cases of hemobilia in anasto-

mosis or due to coagulopathy, TAE may not be appropriate.

If bleeding ceases and a biliary hematoma forms leading to biliary obstruction, it is important to maintain biliary patency. If a T-tube or PTBD is *in situ*, irrigation may be adequate to relieve obstruction; however, in recent years there has been a shift toward abandoning the use of T-tubes for biliary tract reconstruction because some studies have shown that biliary complications are positively related to T-tubes, such as biliary leakage after T-tube removal, anastomotic strictures, cholangitis, and biliary infections^[9]. Endoscopic nasobiliary drainage can also be used to decompress the biliary tract. It has been reported that endoscopic sphincterotomy with balloon extraction of clots could be an effective method to treat biliary clots^[4,10]. In addition, thrombolytic agents could be infused using a nasobiliary catheter to dissolve the biliary clots^[11]. Although the role of surgery is declining in the management of hemobilia and biliary clots, surgery is still indicated when non-surgical methods are not effective. The principles of surgery for hemobilia are to control the bleeding and clear the biliary tract. Methods involving ligation of the bleeding vessels, extraction of the biliary clots, lavage and biliary tract drainage are often used^[4,12].

In our case, percutaneous transhepatic intervention was used to break up the biliary hematoma and relieve bile stasis. During this intervention, a 0.018-inch diameter micro-guidewire, a 6F sheath, a 0.038-inch diameter micro-guidewire, and an 8F catheter were manipulated through the hematoma sequentially, breaking the hematoma up

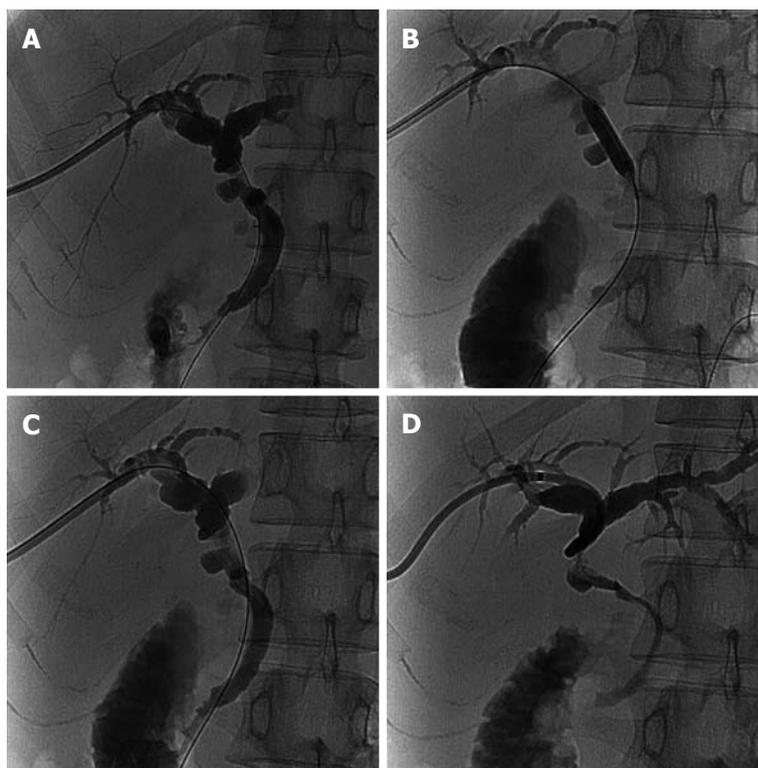


Figure 4 Cholangiography was performed through the biliary drainage catheter. A: At the first follow-up, percutaneous transhepatic cholangiography shows the slightly dilated intrahepatic biliary ducts and a stricture at the site of anastomosis; B: Balloon dilatation of the anastomotic stricture; C: Anastomotic stricture was relieved after balloon dilatation; D: A new biliary drainage catheter was placed.

into pieces, enlarging the contact surface with flowing bile and making it easy to dissolve. Our case provides good proof of the principles underlying Sandblom's clinical and experimental study. Previous studies suggested that percutaneous transhepatic manipulations were major causes of hemobilia after liver transplantation^[3], but in our case, percutaneous transhepatic intervention was used to relieve the biliary obstruction and dissolve the biliary clot with a good outcome.

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Gastric intramural hematoma accompanied by severe epigastric pain and hematemesis after endoscopic mucosal resection

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Abstract

Gastric intramural hematoma is a rare injury of the stomach, and is most often seen in patients with underlying disease. Such injury following endoscopic therapy is even rarer, and there are no universally accepted guidelines for its treatment. In this case report, we describe a gastric intramural hematoma which occurred within 6 h of endoscopic mucosal resection (EMR). Past medical history of this patient was negative, and laboratory examinations revealed normal coagulation profiles and platelet count. Following EMR, the patient experienced severe epigastric pain and vomited 150 mL of gastric contents which were bright red in color. Subsequent emergency endoscopy showed a 4 cm × 5 cm diverticulum-like defect in the anterior gastric antrum wall and a 4 cm × 8 cm intramural hematoma adjacent to the endoscopic submucosal dissection lesion. Following unsatisfactory temporary conservative management, the patient was treated surgically and made a complete recovery. Retrospectively, one possible reason for the patient's condition is that the arterioles in the submucosa or muscularis may have been damaged during deep and massive submucosal injection. Thus, endoscopists should be aware of this potential compli-

cation and improve the level of surgery, especially the skills required for submucosal injection.

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Key words: Gastric intramural hematoma; Endoscopic mucosal resection; Complication

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INTRODUCTION

Gastric intramural hematoma is an uncommon disorder. Previously reported cases were caused by coagulopathy, trauma, aneurysm, peptic ulcer disease, pancreatitis, endoscopic therapy or spontaneous hematomas^[1-3]. However, gastric intramural hematoma after endoscopic intervention is even rarer. Here, we describe a gastric intramural hematoma accompanied by severe epigastric pain and hematemesis which occurred within 6 h of endoscopic mucosal resection (EMR).

CASE REPORT

A 33-year-old man had dull epigastric pain for 3 mo, and endoscopy showed a 0.4 cm × 0.5 cm polypoid protrusive lesion in the stomach antrum (Figure 1A). He was admitted for endoscopic treatment. He had no notewor-

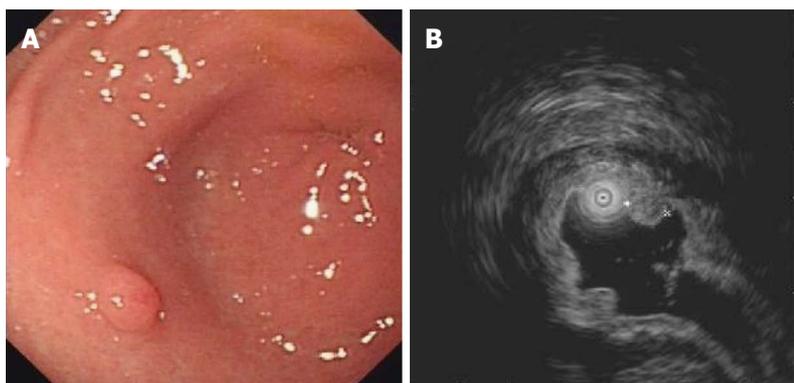


Figure 1 Subepithelial tumor in the stomach antrum. A: Endoscopic examination showed a subepithelial tumor in the antrum; B: Endoscopic ultrasonography revealed that the tumor was localized in the mucosal layer.



Figure 2 Endoscopic mucosal resection. A: After injection of 15 mL saline, the whole nidus was lifted satisfactorily. However, an area of the uplifted mucosal layer was slightly blue; B: The lesion was successfully removed by high-frequency electrocoagulation, and a white wound was observed.

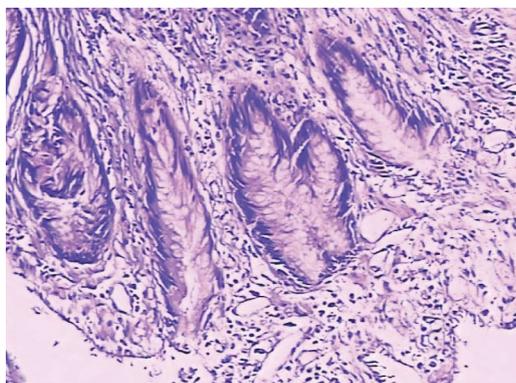


Figure 3 Pathologic examination revealed that the removed lesion from the gastric antrum was a tubular adenoma and *Helicobacter pylori* was not found.

thy medical history, and laboratory examinations showed the following: white blood cell count $8.14 \times 10^9/L$, hemoglobin 151 g/L, platelet count $206 \times 10^9/L$, prothrombin time 9.8 s, and activated partial thromboplastin time 26.7 s. Endoscopic ultrasonography showed that the lesion was localized in the mucosal layer (Figure 1B). Following discussion with the patient and his wife, EMR was carried out. He was given a submucosal 5-mL saline injection, and the nidus was not uplifted satisfactorily.

The operator then slowly injected another 10 mL saline into the distal lesion until the result was satisfactory (Figure 2A). The lesion was successfully removed by high-frequency electrocoagulation, and a white wound was observed (Figure 2B). The removed lesion was sent for pathologic examination which later revealed tubular adenoma and negative *Helicobacter pylori* infection (Figure 3). The patient's condition was stable after surgery. He received 40 mg omeprazole intravenously, and walked into the ward.

Half an hour after surgery, the patient experienced sudden persistent epigastric pain and paroxysmal colic without nausea or vomiting. He received an injection of anisodamine. Bedside ultrasonography of the abdomen did not reveal any apparent abnormalities, and the levels of serum lipase and amylase were normal. Although his pain was slightly reduced, he continued to have intermittent upper abdominal pain. Two hours later, he vomited 150 mL of gastric contents which were bright red in color. After vomiting, his epigastric pain was slightly reduced, and his vital signs were stable. Emergency plain film of the abdomen (KUB) did not reveal radiological evidence of a perforated viscus, and emergency endoscopy showed a 4 cm \times 5 cm diverticulum-like defect (Figure 4A) on the anterior gastric antrum wall, there was also a 4 cm \times 8 cm intramural hematoma on the greater curvature side of the lower gastric body adjacent to the EMR

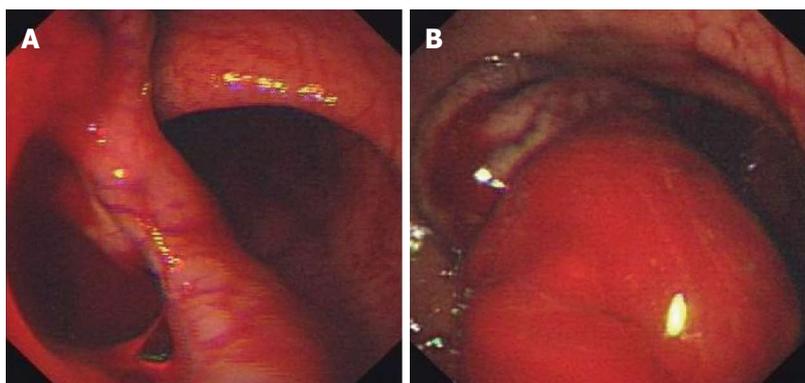


Figure 4 Emergency endoscopy. A: A 4 cm × 5 cm diverticulum-like defect in anterior gastric antrum wall; B: A 4 cm × 8 cm intramural hematoma adjacent to the endoscopic submucosal dissection lesion and active bleeding.

wound, and active bleeding was observed (Figure 4B). Gastrointestinal decompression was performed. After 4 h observation, the patient still had intense paroxysmal pain, and 100 mL of blood was drained from the stomach. He underwent emergency laparotomy under endoscopic guidance, and approximately 300 mL intragastric blood was found. There was a 4 cm × 5 cm × 8 cm intramural hematoma on the distal anterior gastric wall, and the gastric wall was intact with no evidence of perforation. An incision was made in the gastric wall, and the hematoma in the stomach and gastric wall was evacuated manually. The area of suspicious bleeding in the gastric wall was sutured, and no source of active bleeding was identified. The operators sutured the EMR wound, and a subhepatic drain was inserted.

Recovery was uneventful and the patient was allowed oral intake three days after surgery. There was no evidence of gastric leak upon commencing diet, and the subhepatic drain was removed one week later. The patient was well at discharge. At the one-year follow-up, he was well and without complications.

DISCUSSION

Recently, EMR and endoscopic submucosal dissection (ESD) have been widely used. The most common complications of these endoscopic techniques are bleeding and perforation. Intraoperative and postoperative gastric intramural hematomas are clinically rare. EMR and ESD have been used in our hospital for 5 years, and this is the first case of postoperative gastric intramural hematoma. To date, there is only one reported case of intraoperative gastric intramural hematoma, which occurred during ESD^[4].

The pathological changes related to hematoma are damaged vessels and blood is confined to the space around the vessels. In previously reported cases, gastric intramural hematomas can result from coagulopathy, trauma, aneurysm, peptic ulcer disease, pancreatitis, repeated vomiting or spontaneous hematomas^[1,2]. However, the past medical history of our patient was entirely negative, and laboratory examinations showed normal

coagulation profiles and platelet count.

It is well known that there are few blood vessels in the gastric mucosal layer, while the submucosa and muscularis contain many blood vessels. Before lesion excision, ESD or EMR operators should inject hypertonic saline mixed with methylene blue around the lesion, to lift the lesion and completely separate the mucosal layer and muscularis. Thus, the wound would be confined to the submucosa, the muscularis would not be damaged, and complications would be very infrequent. In our case, the probable reasons for gastric intramural hematoma were that the very deep submucosal injection (retrospectively, lifting of the mucosal layer was slightly unsatisfactory, and the muscularis may have been injected), massive submucosal injection (15 mL), and arterioles in the submucosa or muscularis may have been damaged during submucosal injection. The blood from the damaged arterioles was confined between the submucosa and muscularis and formed a hematoma. As the hematoma grew, the mucosal layer was torn and the patient experienced epigastric pain. As the pressure increased, blood entered the gastric cavity from the EMR wound, and the patient vomited bright red gastric contents.

There is no established standard therapy for gastric intramural hematoma. In previous reports, most patients recovered following conservative management; however, surgery or arterial embolization may be necessary, depending on the patient's status^[1,3]. Although there was no radiographic evidence of perforation in our patient, he had severe epigastric pain and vomited blood. In order to avoid serious complications, a laparotomy was selected instead of conservative treatment in this patient.

Reported cases of gastrointestinal intramural hematoma following endoscopic procedures are very few and mostly confined to the esophagus^[5]. This type of gastric intramural hematoma is extremely rare, however, several authors have reported the incidence of gastric intramural hematoma following endoscopic therapy^[3,4,6]. Gastric intramural hematoma is dangerous, and can cause misery for the sufferer. When carrying out endoscopic procedures, endoscopists should be aware of this potential complication. In addition, endoscopists should improve

the level of surgery, especially the skills required for submucosal injection.

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 Oncology 2012 Gastrointestinal
 Cancers Symposium
 San Francisco, CA 3000,
 United States

January 19-21, 2012
 2012 Gastrointestinal Cancers
 Symposium
 San Francisco, CA 94103,
 United States

January 20-21, 2012
 American Gastroenterological
 Association Clinical Congress of
 Gastroenterology and Hepatology
 Miami Beach, FL 33141,
 United States

February 3, 2012
 The Future of Obesity Treatment
 London, United Kingdom

February 16-17, 2012
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 Research Group Conference
 London, United Kingdom

February 23, 2012
 Management of Barretts
 Oesophagus: Everything you need
 to know
 Cambridge, United Kingdom

February 24-27, 2012
 Canadian Digestive Diseases Week
 2012
 Montreal, Canada

March 1-3, 2012
 International Conference on
 Nutrition and Growth 2012
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 Orlando, FL 32808, United States

March 26-27, 2012
 26th Annual New Treatments in
 Chronic Liver Disease
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March 30-April 2, 2012
 Mayo Clinic Gastroenterology and
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 San Antonio, TX 78249,
 United States

March 31-April 1, 2012
 27th Annual New Treatments in
 Chronic Liver Disease
 San Diego, CA 92121, United States

April 8-10, 2012
 9th International Symposium on
 Functional GI Disorders
 Milwaukee, WI 53202, United States

April 13-15, 2012
 Asian Oncology Summit 2012
 Singapore, Singapore

April 15-17, 2012
 European Multidisciplinary
 Colorectal Cancer Congress 2012
 Prague, Czech

April 18-20, 2012
 The International Liver Congress
 2012
 Barcelona, Spain

April 19-21, 2012
 Internal Medicine 2012
 New Orleans, LA 70166,
 United States

April 20-22, 2012
 Diffuse Small Bowel and Liver
 Diseases
 Melbourne, Australia

April 22-24, 2012
 EUROSON 2012 EFSUMB Annual

Meeting
 Madrid, Spain

April 28, 2012
 Issues in Pediatric Oncology
 Kiev, Ukraine

May 3-5, 2012
 9th Congress of The Jordanian
 Society of Gastroenterology
 Amman, Jordan

May 7-10, 2012
 Digestive Diseases Week
 Chicago, IL 60601, United States

May 17-21, 2012
 2012 ASCRS Annual Meeting-
 American Society of Colon and
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 Hollywood, FL 1300, United States

May 18-19, 2012
 Pancreas Club Meeting
 San Diego, CA 92101, United States

May 18-23, 2012
 SGNA: Society of Gastroenterology
 Nurses and Associates Annual
 Course
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 2012-Digestive Disease Week
 San Diego, CA 92121, United States

June 2-6, 2012
 American Society of Colon and
 Rectal Surgeons Annual Meeting
 San Antonio, TX 78249,
 United States

June 18-21, 2012
 Pancreatic Cancer: Progress and
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 Lake Tahoe, NV 89101, United States

July 25-26, 2012
 PancreasFest 2012
 Pittsburgh, PA 15260, United States

September 1-4, 2012
 OESO 11th World Conference
 Como, Italy

September 6-8, 2012
 2012 Joint International

Neurogastroenterology and Motility
 Meeting
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 The Viral Hepatitis Congress
 Frankfurt, Germany

September 8-9, 2012
 New Advances in Inflammatory
 Bowel Disease
 La Jolla, CA 92093, United States

September 8-9, 2012
 Florida Gastroenterologic Society
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 Current Problems of
 Gastroenterology and Abdominal
 Surgery
 Kiev, Ukraine

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 1st World Congress on Controversies
 in the Management of Viral Hepatitis
 Prague, Czech

October 19-24, 2012
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 Gastroenterology 77th Annual
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 Course
 Las Vegas, NV 89085, United States

November 3-4, 2012
 Modern Technologies in
 Diagnosis and Treatment of
 Gastroenterological Patients
 Dnepropetrovsk, Ukraine

November 4-8, 2012
 The Liver Meeting
 San Francisco, CA 94101,
 United States

November 9-13, 2012
 American Association for the Study
 of Liver Diseases
 Boston, MA 02298, United States

December 1-4, 2012
 Advances in Inflammatory Bowel
 Diseases
 Hollywood, FL 33028, United States

GENERAL INFORMATION

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Acknowledgments

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Chinese journal article (list all authors and include the PMID where applicable)

- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

In press

- 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

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

- 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]

No author given

- 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]

Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

- 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]

No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS A Careaction* 2002; 1-6 [PMID: 12154804]

Books*Personal author(s)*

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 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

Author(s) and editor(s)

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

- 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

Electronic journal (list all authors)

- 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>

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean \pm SD or mean \pm SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as ν (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 $24.5 \mu\text{g/L}$; CO₂ volume fraction, 50 mL/L CO₂, not 5% CO₂; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

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Restriction enzymes: *EcoRI*, *HindI*, *BamHI*, *Kbo I*, *Kpn I*, *etc.*

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Matrix metalloproteinase-9: A deleterious link between hepatic ischemia-reperfusion and colorectal cancer

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Abstract

Despite the advent of improved surgical techniques and the development of cytotoxic chemotherapeutic agents useful for the treatment of colorectal cancer, the primary clinical challenge remains that of preventing and combating metastatic spread. Surgical resection is the best treatment for colorectal metastases isolated to the liver. However, in rodent models, the hepatic ischemia-reperfusion (I/R) applied during the surgery accelerates the outgrowth of implanted tumors. Among the adverse effects of I/R on cellular function, several studies have demonstrated an over expression of the matrix metalloproteinase-9 (MMP-9) in the ischemic liver. Since several studies showed high local levels of expression and activity of this proteolytic enzyme in the primary colorectal adenocarcinoma, the role of MMP-9 might be considered as a potential common mediator, favoring both growth of local tumor and the dissemination of colorectal carcinoma metastases.

INTRODUCTION

Colorectal cancer is one of the most commonly diagnosed cancers in developed countries and it is ranked as the second leading cause of death among cancer patients^[1]. The majority of these cases is sporadic and develops from a precursor lesion, the adenomatous polyp. At the diagnosis, colorectal cancer is localized to the colon (Dukes A and B stages) in 54% of cases, while it is metastatic to lymph nodes and distant organs (Dukes C and D stages) in the remaining 46%. The survival data demonstrate that the prognosis depends on the severity of the disease: around 80% of patients have long term survival with node negative disease (Dukes A and B stages), this percentage drop to 4% for 5-year survival in patients with metastases (Dukes D stage)^[2]. The liver is the most frequent metastatic site of colorectal cancer, and the complete surgical resection of isolated metastases in this organ has been shown to improve disease outcomes^[3]. Unfortunately, this therapeutic procedure does not avoid a high rate of metastasis recurrence within the

liver. Experimental evidence accumulated over the last two decades has suggested the association between the increase risk of metastatic relapse and their surgical resection in the liver. Indeed, besides the presence of residual or dormant malignant cells, the blood loss incurred during the operation disseminates colon cancer cells into the peripheral blood^[4]. In order to limit the blood loss during the parenchymal resection, surgeons occlude the inflow of blood to the liver.

HEPATIC ISCHEMIA-REPERFUSION INJURY

However, hepatic ischemia-reperfusion (I/R) is an additional and strong stimulus that promotes the outgrowth of micrometastases in the liver^[5]. Local response to I/R is quite complex, but it can be classified into two distinct phases. The acute hepatocellular injury is caused by reactive oxygen species, intracellular and mitochondrial Ca²⁺ overload, complement activation and release of cytotoxic cytokines, and is reflected by a rise in plasma liver enzymes. The late phase is characterized by neutrophil infiltration causing further damage to the parenchyma, mainly through a protease-dependent pathway^[6,7].

In a mouse model of colorectal liver metastases, van der Bilt and colleagues have demonstrated that intermittent clamping prevents both early and late hepatocellular damage and I/R-accelerated tumor growth^[8]. These results might be associated with the reduction of neutrophil infiltration in the parenchyma, which contributes to tumor growth by producing proliferation and angiogenesis-stimulating factors and cytokines^[8].

METALLOPROTEINASE 9 AND COLORECTAL CANCER

Numerous publications have demonstrated that the disease progression in animal models of tissue invasion and metastasis correlate with enhanced secretion of MMPs by tumor and/or stromal cells. Indeed, the metastatic cascade is characterized by the stromal invasion, “intravasation” of the circulatory system at the primary site, “extravasation” at the secondary site and outgrowth of new tumors. These progressive steps require degradation of the extracellular matrix components by proteolytic enzymes, such as matrix metalloproteinases (MMPs)^[9]. Increased expression of various isoforms (MMP-1, -2, -3, -7, -9, -12, -13) has been also related with the pathophysiology of the transformation of human neoplastic colorectal mucosa to adenomatous polyps, invasive colorectal cancers, and metastases^[10]. Among different MMPs, MMP-9 is of particular interest. Indeed, MMP-9 (also known as gelatinase B) is the main enzyme responsible for the degradation of type IV collagen (a major component of basement membranes) and the denatured collagens (gelatins). Importantly, this capacity of MMP-9 has been previously associated with colorectal cancer progression and dissemi-

nation of metastasis^[11]. Likewise, this gelatinase might also degrade different matrix substrates including collagen type I, V, VII, X and XI, elastin, fibronectin and laminin. In addition to these extracellular membrane components, MMP-9 cleaves different bioactive molecules, such as growth factors, cytokines, chemokines and also pro-MMPs (pro-MMP-2, pro-MMP-9 and pro-MMP13) and contributes to transform these molecules into their active forms^[12]. Various leukocyte subsets (including neutrophils, monocytes/macrophages and T lymphocytes) produce and release MMP-9.

Although MMP-9 is virtually absent in native livers, it is highly expressed in damaged livers after I/R injury^[13]. Its role has been investigated by Hamada and coworkers^[14], using MMP-9 deficient mice and mice treated with a specific neutralizing anti-MMP9 antibody. The authors demonstrated that, when MMP-9 is inhibited, mice were characterized by significant improvement in liver preservation outcomes. This beneficial effect (in both MMP-9 knockout and neutralizing antibody-treated mice) was also associated with a reduction of leukocyte recruitment and cytokine expression within this organ^[14]. Finally, in a next important step, Nicoud and coworkers demonstrated a direct relationship between the hepatic I/R-induced elevations in MMP-9 and the growth of metastatic colorectal carcinoma^[15]. To achieve this goal, they have subjected mice to hepatic ischemia after tumor cell injection and treated them with or without doxycycline (a broad-spectrum MMP inhibitor). Mice subjected to 30 min of 70% liver ischemia at the time of the tumor inoculation developed significantly larger tumor (expressed as number and volume), and, concomitantly, they were characterized by elevated MMP-9 levels in the systemic circulation and within liver. Accordingly, treatment with the MMP inhibitor strongly reduced the number and volume of the colorectal carcinoma metastasis in the liver. These results indicate that the inhibition of MMP-9 after the hepatic surgical resection of colorectal carcinoma metastases might be clinically relevant to prevent tumor relapse.

CONCLUSION

In summary, these studies have provided important and novel insights on the pivotal role of MMP-9 in the hepatic growth of colorectal carcinoma metastases. Furthermore, a therapeutic strategy targeting the early inhibition of MMP-9 might be a very promising approach to reduce the hepatic metastasis relapse following surgical manipulation of the liver. These preliminary results from basic research also represent a relevant input for developing more selective pharmacological inhibitors of MMP-9 to be tested *in vivo* in animal models of hepatic cancer dissemination. Although clinical studies investigating the role of MMP-9 in human beings are still lacking, these animal studies might suggest the scientific rationale for future applications targeting MMP-9 activity and expression in cancer care and prevention.

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Carcinoma of the gastroesophageal junction in Chinese patients

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Abstract

Carcinoma of the gastroesophageal junction (GEJ) is defined as carcinoma that crosses the GEJ line, irrespective of where the tumor epicenter is located. This group of cancer is rare but controversial. Based on study results from the majority of epidemiologic and clinicopathologic investigations carried out in Western countries, this cancer is believed to arise from Barrett's esophagus (BE) and includes both distal esophageal and proximal gastric carcinomas because of similar characteristics in epidemiology, clinicopathology, and molecular pathobiology in relation to BE. As such, the most recent American Joint Committee on Cancer staging manual requires staging all GEJ carcinomas with the rule for esophageal adenocarcinoma (EA). This mandate has been challenged recently by the data from several studies carried out mainly in Chinese patients. The emerging evidence derived

from those studies suggests: (1) both BE and EA are uncommon in the Chinese population; (2) almost all GEJ cancers in Chinese arise in the proximal stomach and show the features of proximal gastric cancer, not those of EA; (3) application of the new cancer staging rule to GEJ cancer of Chinese patients cannot stratify patients' prognosis effectively; and (4) prognostic factors of GEJ cancer in Chinese are similar, but not identical, to those of EA. In conclusion, the recent evidence suggests that GEJ cancer in Chinese shows distinct clinicopathologic characteristics that are different from EA. Further investigations in molecular pathology may help illustrate the underlying pathogenesis mechanisms of this cancer in Chinese patients and better manage patients with this fatal disease.

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Key words: Esophagus; Stomach; Cancer; Gastroesophageal junction; Staging; Barrett's esophagus

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INTRODUCTION

Carcinomas of the gastroesophageal junction (GEJ) is defined by the World Health Organization (WHO) as tu-

mors “that cross the oesophagogastric junction... regardless of where the bulk of the tumours lies”^[1]. Therefore, this cancer may arise from the distal esophagus, grow downward, cross the GEJ line, and invade the proximal stomach, or originate from the proximal stomach, grow upward, and invade the distal esophagus. For better surgical management of patients, Siewert and Stein classify this cancer into 3 types: Type I cancer shows epicenter in the distal esophagus 1-5 cm above the GEJ; Type III cancer centers within the proximal stomach 2-5 cm below the GEJ; and Type II tumor straddles the GEJ within a 3-cm longitudinal spread of 1 cm above and 2 cm below the GEJ^[2-4].

At present, the underlying mechanisms of tumorigenesis for this uncommon cancer are poorly understood^[5-7]. Several investigators from Western countries believe adenocarcinoma of the proximal stomach, i.e., gastric cardiac carcinoma, to be similar, or even identical, to Barrett’s esophagus (BE)-associated distal esophageal adenocarcinoma (EA) on the basis of comparable characteristics in epidemiology^[8-14], clinical presentations^[15-23], molecular pathobiology^[24], and histopathology^[17,25-27]. This notion has been adopted by the American Joint Committee on Cancer that published the 7th edition of the cancer staging manual (AJCC 7) in 2009, requiring staging all GEJ cancers with the rule for EA^[28].

Over the past decade, a growing body of evidence has been published on epidemiology^[29-31] and clinicopathology^[32-36] of GEJ carcinoma in Chinese patients with the standardized criteria. The emerging data suggest that GEJ carcinoma in Chinese is heterogeneous in histology and shows clinicopathologic features different from those of EA. This article critically reviews the most recent evidence on this fatal cancer in Chinese patients with the intention to promote clinical research and to better manage Chinese patients with this cancer.

BE AND DISTAL EA REMAIN SCARCE IN CHINESE

Several recent population-based studies using the BE diagnostic criteria of the American Gastroenterology Association show a very low frequency of BE in the Chinese population^[29,37-43]. In 2008, Tseng *et al*^[29] reported a frequency of 0.28% of patients with columnar-lined esophagus out of 19 810 consecutive subjects at annual health check-up with upper endoscopy. By histology, among those with columnar-lined esophagus only 12 subjects had intestinal metaplasia and were qualified as BE patients, rendering a prevalence rate of 0.06% in the general population^[29]. In referral patients for upper endoscopy, Kuo *et al*^[30] reported only 1.8% of BE cases from 735 consecutive subjects. Similar results were also described in another upper endoscopy study of 5179 patients with a prevalence rate of BE at 1% and 0.35% for referral and screening cases at annual health check-up. These results have been repeatedly confirmed by several other endoscopy studies^[40-42,44,45]. In addition to this very

low prevalence rate, BE in most Chinese subjects is in the short- or ultra-short segment, i.e., shorter than 3 cm in the longitudinal length^[40,46-49]. Chen *et al*^[49] studied 4120 qualified BE cases in a meta analysis of 308 original research articles published over the period from 1997 to 2007. They reported overwhelming BE cases (78%) in the short-segment and most in tongue- and island-like endoscopic mucosal lesion patterns (78%). The long segment BE is infrequent and has not been reported in Chinese women. In a histopathology study of distal esophageal mucosa in patients with proximal gastric carcinoma, Sun *et al*^[50] reported the finding of columnar-lined esophagus in up to 65% of the cases, 97% of which was confined within 1 cm above the GEJ line.

Similarly, EA remains rare in the Chinese population^[51-57], unlike that in patients from the West where the incidence of EA has been rapidly rising in the most recent years and EA has outnumbered esophageal squamous cell carcinoma^[58,59]. A population-based epidemiology study carried out in Taiwan over a 25-year period from 1979 to 2003 showed a steadily increasing trend for esophageal squamous cell carcinoma but not for EA^[60]. In another study in Hong Kong, investigators even reported a decreasing trend for EA over a 20-year period from 1984 to 2003^[31]. Among 10 751 new esophageal cancer cases reported to the Hong Kong Cancer Registry, the number of EA cases decreased from 224 in 1984 to 131 in 1998 to 2003, a dramatic drop of over 40% in incidence^[31].

By histopathology, investigators from a major tertiary medical center in Taiwan did not find a single case of EA over a 20-year period from 1987 to 2007^[35]. Most recently, armed with the WHO diagnostic criteria^[1] and the recently defined histological definition of the GEJ line^[61,62], pathologists in Nanjing studied histopathologic features of consecutive 206 radical resections of tumors in the distal esophagus in a homogenous Chinese population and identified only 2 (1%) cases of true EA^[34]. In that study, esophageal squamous cell carcinoma stays predominant^[34].

These clinical study data suggest that BE-related diseases including EA remain uncommon in the Chinese population in the most recent years and may not be the source of their GEJ cancer^[63].

CLINICOPATHOLOGIC FEATURES ARE NOT THOSE OF EA

To answer the question as to where GEJ cancer in Chinese patients arises and what clinicopathologic differences in this cancer between Chinese and Westerners could be, a recent comparison study on clinicopathologic features of GEJ cancer with the WHO diagnostic criteria was conducted between Chinese patients treated in Nanjing, China, and American patients treated in Boston, the United States^[33]. The researchers reported remarkable differences in almost all clinicopathologic characteristics of GEJ cancer between these two different ethnic

patient populations. In general, Chinese patients were 6-year younger, more in the female gender, and presented with tumors 1.5 cm larger in size. Their tumors were all centered in the proximal stomach and heterogeneous in histology with substantial proportions of the cases showing uncommon types such as adenosquamous cell carcinoma, neuroendocrine carcinoma, and pancreatic acinar-like adenocarcinoma^[32,33,64]. In contrast in American patients, almost all tumors were centered in the distal esophagus and homogeneous as EA in histology^[33]. As to the peri-tumor mucosal diseases in Chinese patients, although distal esophageal columnar metaplasia (14%) and dysplasia (0) were uncommon or absent, chronic gastritis (81%) and *Helicobacter pylori* (*H. pylori*) infection (35%) were widespread. Again, in a sharp contrast, distal esophageal columnar metaplasia (87%) and dysplasia (67%) in the Americans were overwhelming; but chronic gastritis (24%) and *H. pylori* infection (19%) were uncommon in the uninvolved proximal gastric mucosa^[33]. The results suggest that GEJ cancer in American patients is indeed associated with BE and shows the clinicopathologic features of EA^[25,65-68]. In contrast, GEJ cancer in Chinese is in fact primary proximal gastric cancer and different from EA.

Despite the fact that the results of this single comparison study confirm the rationale on the AJCC 7 classification of this cancer as EA in American patients, the new AJCC 7 mandate for classification of all GEJ cancers as EA may be questionable and ineffective in Chinese patients.

APPLICATION OF STAGING RULES ON EA CANNOT EFFECTIVELY PREDICT SURVIVAL IN CHINESE PATIENTS

The updated AJCC 7 staging guideline classifies all GEJ cancers as EA and requires staging these tumors as esophageal cancer^[28]. The validity and effectiveness of this new mandate has been found problematic in Chinese patients. Researchers in Nanjing, China, investigated 142 cases of GEJ cancer and reported inferior stratification of survival prediction to survival stratification with the staging rule for gastric cancer, especially for pN and summary pIII C stages, when these cases were staged with the scheme for EA based on the AJCC 7 new guideline^[69]. They reported that the pN stage was more predictive in survival than the pT, which is consistent with the features of gastric cancer. In addition, they described a useful survival predictive value for celiac nodal disease and the lymph node ratio in patients with this cancer. In contrast, using the staging guideline for EA, they discovered illogical patient survival characteristics. For example, the Kaplan-Meier curves for patients staged at pIII A predicted erroneously better survival than those staged at pIA and pIIB. Moreover, the survival curves also crossed in the cases staged at pII B and pIII B, indicating the existence of intra-group hetero-

geneity. Importantly, even with the staging scheme for gastric cancer, the survival curves for patients with this cancer were not distinctive and showed incorrectly better survival prediction for patients staged at p I B and p II B than those at p I A and p II A. Interestingly enough, patients staged at pN3b had the 5-year survival rate worse than those with pM1 and pIV diseases^[69]. These observations, taken along with the group clustering in p II A, p II B, p III A, and p III B, illustrate a poor discriminatory ability of the new AJCC 7 staging rule for this cancer in Chinese^[70,71].

One of intriguing facts in Chinese patients with this cancer is that despite the larger tumor size and higher overall pathologic stage with stage pIII-IV in 70% of cases, the 5-year survival rate for patients with stage pIII tumors is significantly better in Chinese than in American patients^[69]. A similar result for patients with proximal gastric cancer staged at pIII has been reported previously in an epidemiology study on the data derived from the Surveillance, Epidemiology, and End Results database^[11]. In that study, although the overall patient survival curves are almost identical between EA and proximal gastric cancer groups, a distinct separation in survival curves between these two groups is demonstrated for patients with pIII diseases; importantly, the patients with proximal gastric cancer and staged at pIII show a much better survival trend than those with EA^[11]. These results demonstrate a unique characteristic for proximal gastric cancer, which is distinctly different from that of EA^[69].

The aforementioned preliminary data suggest that GEJ cancer in Chinese cannot be staged predicatively as EA with the new AJCC 7 guideline, as confirmed in a recent South Korean study^[72]. Even staged with the rules for gastric cancer, these cases cannot be monotonically stratified for prognosis prediction^[69], suggesting the existence of discrete pathobiological characteristics that set this cancer apart from EA and less characteristically from conventional gastric cancer^[73]. Regardless, the study of GEJ cancer in Chinese patients treated in Nanjing is limited by a relatively small sample size, advanced pT3 disease in the majority of the cases, and a lack of consistent surgical lymphadenectomy procedure carried out in all cases^[69]. Further investigation with defined criteria and a larger sample size is needed to validate those interesting results.

PROGNOSTIC FACTORS AND SIRT1 GENE EXPRESSION

In the most recent reports on prognostic factors of EA with a large sample size, well-defined clinicopathologic characteristics, and robust follow-up from Western countries, the worse 5-year disease-specific prognostic factors are found to be associated with higher pT, pN stages, advanced age over 76 years, signet-ring cell histology, poor tumor differentiation, and extra-nodal diseases^[74-77]. These prognostic factors for EA have been reported to

be similar, but not identical, to those in Chinese patients with GEJ cancer in recent publications^[78-80]. However, because of the limited number of reports on this issue, the results are a bit inconsistent. For instance, in one report with 514 surgical resection cases of GEJ cancer at a major medical center in China, tumor gross and histology type, stage, vascular invasion, and extent of surgical resection were found to be significant prognostic factors^[78]. In another detailed clinicopathology study report^[79], patient age over 70 years, tumor size larger than 8 cm, poor differentiation, the number of positive lymph nodes over 16, and advanced summary pathology stage were shown to be associated with worse outcomes. In contrast, lymphovascular invasion, which is associated with worse survival in EA^[80], is not shown to be a significant prognostic factor. Interestingly, celiac nodal metastasis and the lymph node ratio for the number of lymph node retrieved and nodal disease are reported to be significant prognostic factors^[81-83]. The investigators further reported that the ratio of the number of positive nodes identified *v*s the number of total lymph nodes evaluated was related to significantly worse overall survival^[79]. Furthermore, the patient survival rate becomes significantly worse for the lymph node ratio over 0.2, compared to the cases with negative nodal disease. The relative risk of worse prognosis for the ratio over 0.4 or 0.5 is 37-fold or 75-fold^[79]. This powerful prognostic prediction by the lymph node ratio is very practical in clinical settings where nodal dissection by individual surgeons and nodal retrieval by pathologists vary and a unified nodal dissection protocol is not universally executed. This simple, easy-to-use, and objective prognostic indicator in gastric cancer has been repeatedly confirmed by many other investigators around the world^[84-91].

Advanced age has been proven to be one of independent worse prognostic factors in GEJ cancer^[74,75,79]. This may result from genetic abnormalities in aging associated genes such as Sirt1, which is a recently discovered, aging-related histone deacetylase involved in regulation of multiple critical steps of stress responses, nutrient metabolism, and aging through deacetylation of a variety of subcellular molecules such as p53, forkhead transcription factors, PGC-1 α , NF- κ B, Ku70, and histones^[92-97]. An increasing body of evidence in molecular biology suggests a complex role for Sirt1 to play in tumorigenesis^[96-101]. Feng *et al*^[102] are the first to use tissue microarray and immunohistochemical methods to investigate the Sirt1 gene expression in proximal gastric cancer including GEJ cancer in Chinese patients. They reported that compared to normal controls, Sirt1 gene expression was significantly higher in a subgroup of GEJ cancer cases, which was significantly associated lymph node metastasis, higher pathologic stages, and worse survival prognosis with significantly lower 1- and 3-year survival rates (80% and 49%), compared to the Sirt1-negative cancer patient groups (89% and 71%), suggesting a prognostic predictive value for this molecule in patients with this cancer. It should be interesting to know how Sirt1 plays

in the initiation and progression of GEJ cancer in Chinese patients.

CONCLUSION

GEJ cancer is uncommon but poorly understood for its natural history, pathogenesis mechanisms, and prognosis. An increasing body of evidence accumulated in recent years suggests that GEJ cancer in Chinese patients arises mainly in the proximal stomach associated with chronic gastritis and shows a heterogeneous histology pattern. This cancer is distinctly different from EA and cannot be accurately stratified with the scheme for EA, as required by the updated AJCC 7 cancer staging guideline for patient survival prognosis prediction. Although the new AJCC 7 staging rule for gastric cancer may be used for this cancer, there exists considerable heterogeneity and indistinctive survival characteristics, suggesting the possibility of a distinct disease entity for this cancer.

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Role of autoimmunity in primary biliary cirrhosis

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Abstract

Primary biliary cirrhosis (PBC) is an autoimmune liver disease characterized by the presence of serum autoantibodies and chronic nonsuppurative destructive cholangitis. The pathogenesis of PBC involves environmental factors, genetic predisposition and loss of immune tolerance. In recent years, it has become univocally accepted that an inappropriately activated immune response is one of the most important factors in PBC. In this study, the role of autoimmunity in PBC is summarized and a feasible research orientation is recommended.

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Key words: Primary biliary cirrhosis; Autoimmunity; Humoral immunity; Cellular immunity; Nonspecific immunity

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INTRODUCTION

Primary biliary cirrhosis (PBC) is an organ-specific autoimmune disease of the liver characterized by the presence of serum antimitochondrial antibodies (AMAs) and the destruction of small and medium-sized bile ducts. The histological manifestations are damaged biliary epithelial cells (BECs) and infiltration of T cells, B cells, macrophages, eosinophils and natural killer (NK) cells in the portal area, which eventually leads to cirrhosis and liver failure^[1]. PBC affects middle-aged women, and the natural disease history is 10 to 20 year. The annual incidence rates range between 0.7 and 49 cases per million persons, while the prevalence rates range between 6.7 and 402 cases per million persons^[2,3]. Advanced biochemical assays and improved acquisition of disease will lead to increased detection worldwide. Currently, the only recommended first-line therapy, early treatment with ursodeoxycholic acid (UDCA) at a dose of 13-15 mg/kg per day, can delay progression of histology and ameliorate long-term prognosis^[4]. However, approximately 25% of patients have no response to UDCA, and in these cases liver transplantation is needed^[5]. Exploration of pathogenesis is needed to discover novel target treatments. Deficiencies in autoimmune tolerance are critical factors of disease initiation and perpetuation. Therefore, the aim of this study was to demonstrate the function of autoimmunity in PBC.

HUMORAL IMMUNITY

Autoantibody-AMA

High titer of AMAs is the serological hallmark of PBC. It can be detected even before clinical symptoms or bio-

chemical anomalies. The proportion of AMA-negative patients has been minimized due to the development of sensitive detection technology^[6,7]. The highly disease-specific autoantibody, AMA-M₂, recognizes components of the oxo-acid dehydrogenase complex (OADC) that are ubiquitously expressed on the inner mitochondrial membrane, including the E₂ subset of the pyruvate dehydrogenase complex (PDC-E₂). The antigens are released from apoptotic blebs of the BEC, or come from molecular mimicry of infectious agents, or from alteration of xenobiotics^[8]. However, transgenic mice aberrantly expressing PDC-E₂ components on BECs do not show serological and histological features of PBC^[9], indicating that aberrant PDC-E₂ expression is not sufficient for disease development.

Although AMA is found in most PBC patients, it may have no pathogenic role due to the following observations: (1) the titer of AMA is not associated with biochemical or histological manifestations, and there is no indication for a significant change in AMA levels after drug therapy^[10,11]; (2) approximately 5% of patients are AMA-negative with typical histological injury and exhibit the same treatment response as AMA-positive patients^[12,13]; and (3) although the antigens are ubiquitous, the disease is organ-specific.

However, some studies have identified novel features of AMAs in recent years. Lleo and colleagues^[14,15] demonstrated that PDC-E₂ with antigenic reactivity was only detectable in apoptotic blebs of human intra-hepatic BECs; Moreover, in the presence of AMA, macrophages increased the expression of TNF-related apoptosis-inducing ligand (TRAIL) and produced intense inflammatory cytokines, such as tumor necrosis factor alpha (TNF- α) and interleukin (IL)-6. These results provide a mechanism for the biliary specificity and a physiopathologic role of AMA in PBC. Rigopoulou *et al.*^[16] found that AMA-IgG₃ was associated with a more severe disease. The presence of IgA-anti-PDC-E₂ in sera or saliva might be associated with the progression of PBC^[17]. The mechanism responsible is likely that a greater concentration of IgA in bile ducts can make cells more susceptible to apoptosis through constant transcytosis, resulting in subsequent bile duct damage^[18]. In addition, the production of AMA-IgM from peripheral blood mononuclear cells (PBMCs) from PBC patients was reduced after exposure to UDCA^[19].

Autoantibody-antinuclear antibodies

In addition to AMA, serum antinuclear antibodies (ANAs) are positive in approximately 1/3 patients with PBC. These antibodies provide important evidence for AMA-negative PBC. The typical staining pattern and clinical significance^[20-28] of ANAs are shown in Table 1.

B cells and plasma cells

Plasma cells that originate from B cells are sources of antibodies. As antigen presentation cells, B cells can secrete many kinds of cytokines and present costimulatory signals

to active antigen-specific T lymphocytes. Migita *et al.*^[29] found that serum B-cell-activating factor (BAFF) levels were significantly higher in PBC patients than in healthy controls and HCV-infected patients, and were positively correlated with aspartate amino-transferase (AST) and total bilirubin levels. In liver, CD5⁺ and CD20⁺ cells were associated with BEC damage, suggesting that B cells have a role in regulating the portal destruction in PBC^[30]. Therefore, B cell depletion therapy might be an alternative to UDCA. In murine experiments, I μ (-/-)NOD.c3c4 mice demonstrated a decreased number of activated NK cells in the liver. The degree of granuloma formation, bile duct destruction, and salivary gland histology were also shown to be significantly attenuated^[31]. Moreover, anti-CD20 therapy every 2 wk in transforming growth factor-beta receptor II dominant negative (dnTGF- β R II) mice at age of 4-6 wk could reduce the number of B cells and CD8⁺ T cells in liver^[32]. In clinical therapy, two doses of 1000 mg rituximab separated by 2 wk were safe and effective in patients with an incomplete UDCA response. After treatment, not only did serum levels of total IgG, IgM, and IgA decrease significantly, but T regulatory (Treg) cells also increased, which was associated with increased mRNA levels of forkhead box 3 (Foxp3) and TGF- β in CD4⁺ T cells^[33].

In contrast, in 2-octynoic acid-bovine serum albumin (2OA-BSA)-induced mice, treatment with anti-CD20 and anti-CD79 antibodies increased the number of CD4⁺ and CD8⁺ T cells infiltrating around damaged bile ducts in portal areas, leading to more severe cholangitis^[34]. A similar phenomenon occurred in I μ (-/-)dnTGF- β R II mice. Adoptive transfer of CD19⁺ cells from dnTGF- β R II mice into recombination activating gene-1 (Rag-1)(-/-) mice resulted in decreased liver inflammation and bile duct damage^[35]. However, anti-CD20 therapy in dnTGF- β R II mice at age of 20-22 wk had little effect on liver lesions^[32]. The efficacies of different B cell depletion approaches in diverse murine models are shown in Table 2. Together, these findings suggest that there is a subclass of B cells that have a regulatory role by producing IL-10^[36]. However, further exploration of the function of B cells in PBC is needed before B cell depletion therapy can be applied to routine clinical work.

Elevated IgM

In addition to high titers of circulating AMAs, PBC patients have high levels of serum IgM that are not related to titers of AMAs. Compared with AMA-positive PBC, the level of serum IgM was lower in AMA-negative patients^[37,38]. Plasma cells in the portal tracts of PBC patients are found to be predominantly IgM-positive, while those cells predominantly express IgG in other forms of liver disease, such as autoimmune hepatitis and chronic hepatitis C^[39,40]. It has been shown that after treatment with UDCA, the level of IgM decreases at both short-term and long-term follow-up^[41], which is possibly due to a reduction in naïve B cell and IgM-memory B cell activation through down-regulation of the NF- κ B signal-

Table 1 The characteristics of antinuclear antibodies in primary biliary cirrhosis patients

Staining pattern	Autoantigen	Prevalence (%)	Clinical significance
Nuclear dot	SP100 ^[20,28]	25	Highly specific for PBC; Urinary tract infections
	PML ^[21,28]	19	Highly specific for PBC; Coexistence with anti-sp100
	SP140 ^[22]	15	Coexistence with anti-sp100 and anti-PML
Nuclear periphery (Nuclear pore complex)	gp210 ^[23]	25	Association with disease severity and poor prognosis
	p62 ^[24]	30-55	Association with disease severity and poor prognosis
Nuclear periphery (Nuclear envelope)	Lamin ^[25]	6-8	Not highly specific for PBC
	LBR ^[24]	2-6	Highly specific for PBC
Anticentromere	Centromere ^[26]	30	Association with portal hypertension
?	AchR M3 ^[27]	83	Unknown

PBC: Primary biliary cirrhosis; PML: Promyelocytic leukemia; LBR: Lamin B receptor.

Table 2 Differences in B cell depletion therapies in primary biliary cirrhosis murine models

Results	Model	Therapy (time)
Amelioration	NOD.c3c4 mice	Igμ knockout
	dnTGF-βR II mice	Anti-CD20 (4-6 wk)
Exacerbation	2OA-BSA-induced mice	Anti-CD20/Anti-CD79 (6 wk)
	dnTGF-βR II mice	Igμ knockout
No effect	dnTGF-βR II mice	Anti-CD20 (20-22 wk)

ing pathway^[19]. The mechanism of IgM elevation is still unclear in PBC, but abnormal Ig class switching may be involved. Lleo *et al.*^[42] showed that IgM was inversely associated with levels of CD40L promoter methylation in CD4⁺ T cells, suggesting that the CD40-CD40L interaction is involved in the production of high amounts of IgM. Moreover, CD40L is an essential molecule involved in Ig class switching.

CELLULAR IMMUNITY

It is thought that activated CD4⁺ T cells can recognize peptide PDC-E₂163-176, while activated CD8⁺ T cells can recognize peptide PDC-E₂159-167 and PDC-E₂165-174 in PBC. Moreover, a large number of autoreactive T lymphocytes infiltrate the portal area.

CD4⁺ T cells

Effector CD4⁺ T cells: In patients with PBC, it is well accepted that an enhanced ratio of Th1 to Th2 cells is one of the most important factors^[43,44] in the onset of disease. After treatment with UDCA, serum interferon-gamma (IFN-γ)^[45] and liver IL-2^[45,46], which are Th1 cell-related cytokines, are decreased. However, the level of serum IFN-γ rebounded after 6 mo of therapy^[45]. Recently, besides Th1 cells, studies have shown that IL-17⁺ cells accumulate around the damaged bile ducts^[47,48], and BECs produce Th17-inducible cytokines (IL-6, IL-1β and IL-23) when stimulated with pathogen-associated molecular patterns (PAMPs)^[47]. In addition, the ratio of Th17 to Tregs is enhanced in PBMCs^[49].

Several experiments using murine models have indicated a central role of CD4⁺ T cells in the pathogenesis of PBC. NOD.c3c4-SCID mice can develop autoim-

mune cholangitis after adoptive transfer of splenocytes or CD4⁺ T cells^[50]. In IL-2Rα(-/-) mice, marked aggregation of IL-17⁺ cells within portal tracts compared to the periphery has been demonstrated. Interestingly, CD4⁺ T cells from the livers of normal C57BL/6J mice can secrete higher levels of IL-17 compared to those from spleens, indicating the role of the liver microenvironment in Th17 induction^[48].

It is currently unknown whether Th1 or Th17 cells are more important in the pathogenesis of the disease. IL-12p40(-/-)dnTGF-βR II mice have a dramatic reduction in histological autoimmune cholangitis and a significant decrease in the levels of intra-hepatic proinflammatory cytokines^[51], while worsening hepatic histology and elevated inflammatory cytokine production have been observed in IL-6(-/-)dnTGF-βR II mice^[52]. These findings might suggest that IL-12-inducible Th1 cells are more important than IL-6-inducible Th17 cells. However, the definitive conclusions on these mechanisms will require further investigation.

Treg cells: CD4⁺CD25^{high} regulatory T cells play a critical role in self-tolerance and the prevention of autoimmune disease. Patients with PBC display a relative reduction of circulating CD4⁺CD25^{high} Tregs compared to controls^[53,54]. The frequency of circulating Tregs can increase after 1 year of treatment with UDCA^[13]. However, the number of Foxp3⁺ cells is higher in the liver in PBC^[53], which is most likely due to the localization of CD8⁺ T cell blasts in the liver portal area^[55].

In addition to CD4⁺ Tregs, Bernuzzi *et al.*^[56] found that the CD8⁺ Treg (CD8⁺CD28⁻) population has striking phenotypic alterations, including decreased CD39 and increased CD127. Although CD8⁺ Tregs were not significantly different quantitatively between patients and healthy subjects, the *in vitro* induction of CD8⁺ Tregs by incubation with IL-10 was significantly reduced in PBC patients.

Murine models constructed by altering the signaling pathway of Treg cells can imitate PBC-like manifestations, which underscores the vital function of Tregs in disease. Zhang *et al.*^[57] demonstrated that Scurfy mice, which have complete ablation of Foxp3⁺ Tregs, exhibited a high titer of AMAs and elevated serum cytokines

Table 3 The characteristics of primary biliary cirrhosis murine models and primary biliary cirrhosis patients

	NOD.c3c4 ^[50]	dnTGF-βR II ^[59]	IL-2Rα(-/-) ^[58,85]	Ae2a,b(-/-) ^[86]	2OA-BSA induced ^[87]	Human
Disease onset	8-20 wk	4 wk	4 wk	6 mo?	4-12 wk	40-60 yr
AMA	56%	100%	100%	40-80%	100%	95%
ANA	80%-90%	(-)	80%	(-)	?	30%
Cytokines	Th1 ↑	Th1 ↑	Th1, Th17 ↑	Th1 ↑	Th1 ↑	Th1, Th17 ↑
Treg cells	↓	↓	↓	↓	↓	↓
Ig	IgG, IgM ↑	IgA ↑	IgG, IgA ↑	IgG, IgM ↑	?	IgM ↑
Liver inflammation						
CD8+/CD4+T	↑	↑	↑	↑	↑	↑
Treg	?	↓	↓	↓	?	↑
Fibrosis	?	Slight	?	Slight	Slight	Evident in late stage
Complication	Bile cyst and Extrahepatic damage	Colitis	Colitis Anemia	?	?	Thyroid disorder Sjogren syndrome

ANA: Antinuclear antibodies; AMA: Antimitochondrial antibodies.

(TNF-α, IFN-γ, IL-6, IL-12p40, and IL-23). In addition, these mice also had a substantial number of lymphocytes infiltrated around portal areas with bile duct damage. Both IL-2Rα(-/-)^[58] and dnTGF-βR II murine models^[59] show PBC-like characteristics.

CD8⁺ T cells

Compared to CD4⁺ T cells, CD8⁺ T cells play a more significant role in mediating the destruction of the bile duct. In PBC patients, CD45RO^{high}CD57⁺CD8^{high} T cells expressing elevated α4β7 and IL-5 accumulate around the portal area^[60]. Moreover, the ratio of CD8⁺ T cells to FoxP3⁺ cells is significantly higher in the liver at late stages of the disease^[53]. Biliary ductule damage by CD8⁺ T cells is also observed in non-obese diabetic autoimmune biliary disease (NOD.ABD) mice^[61]. CD8⁺ T cells from NOD.ABD mice can transfer liver inflammation into NOD.c3c4-SCID recipients. The liver of dnTGF-βR II mice has an increased number of CD8⁺ T cells and a higher ratio of CD8 to CD3 or CD4 cells^[59]. Furthermore, adoptive transfer of CD8⁺ T cells from dnTGF-βR II mice to Rag-1(-/-) recipients results in PBC-like liver lesions and AMA-positive sera, while CD4⁺ T cells predominantly cause bowel inflammation^[62]. Relative to IL-2Rα(-/-) mice, IL-2Rα(-/-) CD4(-/-) mice have increased biliary ductular damage. These findings also suggest that CD8⁺ T cells may mediate bile ductular injury under the background of Treg function loss^[63].

INNATE IMMUNITY

The innate immune system is the first line of defense against infection. Emerging evidence suggests that the liver is one component of the body's immune system^[64]. Traditional immunosuppressive drugs are not effective for PBC compared to other autoimmune diseases. Thus, the role of the innate immune system in PBC has garnered a lot of focus in recent years.

Toll-like receptor

The liver can encounter a number of pathogenic microorganisms and their by-products from the gut by acting

as a traffic hub. The expression of toll-like receptor (TLR) is normally regulated by a negative signaling pathway in the liver, which prevents inappropriate activation of inflammation.

After *in vitro* challenge with TLR ligands, circulating monocytes in PBC patients produce higher pro-inflammatory cytokines, including IL-1β, IL-6, IL-8, and TNF-α^[65]. The expressions of TLR3^[66] and TLR4^[67,68] are increased in BECs. Transfection of poly(I:C) into BECs induces a marked increase in mRNAs encoding a variety of chemokines/cytokines^[66]. In addition, the expressions of TLR4 in BECs^[67] and circulating monocytes^[69] both increase significantly after stimulation with lipopolysaccharide (LPS). Furthermore, the level of RP105, which is involved in the negative regulation of TLR4 signaling, is decreased in PBC monocytes^[69]. The interplay between LPS and TLR4 results in myeloid differentiation factor 88 (MyD88) recruitment, NF-κB activation and coding gene expression^[67,69]. After exposure to CpG, PBMCs from PBC patients produce higher levels of IgM and AMAs^[70,71]. Importantly, the effect can be inhibited by K⁺ channel blockers^[70] and UDCA^[19].

These results indicate that patients with PBC exhibit hypersensitivity of TLR signaling, which leads to a breakdown of self-tolerance. However, activation of the TLR signal pathway alone may not explain the entire course of the disease^[72]. Mice immunized with 2OA-BSA coupled with poly(I:C) showed alterations in the disease process and acceleration of fibrosis of liver.

NKT cells

NKT cells are a subset of lymphocytes possessing both T cell receptors (TCRs) and NK-specific receptors, and they play an important part in the modulation of the innate immune response and cytotoxicity.

Alpha-galactosylceramide (α-GalCer) is an activator of NKT cells, and the frequency of CD1d-α-GalCer-restricted NKT cells is similar between the peripheral blood and liver of healthy people. In contrast, the frequency of these cells in liver is significantly higher than in peripheral blood in PBC patients, and also higher than in healthy individuals^[73].

To define the function of CD1d-restricted NKT cells in the pathogenesis of PBC, Chuang *et al*^[74] generated CD1d(-/-)dnTGF- β R II mice and found that they developed decreased hepatic lymphoid cell infiltration and mild cholangitis. After immunization of 2-OA-BSA-induced PBC mice with α -GalCer, Wu *et al*^[75] found that the disease was exacerbated, including signs of portal inflammation, bile duct destruction and liver fibrosis. However, *in vivo* depletion of NK and NKT cells in the same murine model only suppressed AMAs and cytokine production, but did not change the portal cholangitis^[76]. Therefore, these data support the role of NK and NKT cells in the loss of autoimmune tolerance in PBC; however, the development of PBC also requires other pathological factors.

NK cells

NK cells are another component of the innate immune system, and function by secreting cytokines and lysing target cells. NK cells account for 30% of the total resident lymphocytes in the liver. Chuang *et al*^[77] reported an obvious higher frequency and absolute number of NK cells in both the blood and liver of PBC patients. Moreover, the cytotoxic activity and perforin expression of isolated NK cells were increased. Recently, Shimoda *et al*^[78] demonstrated that TLR4 ligand-stimulated NK cells destroyed autologous BECs in the presence of IFN- α synthesized by TLR 3 ligand-stimulated monocytes. In addition, there was an increased number of CD56⁺ cells scattered around the destroyed small bile ducts.

CHEMOKINES

Chemokines, which are 8-12 kDa heparin-binding cytokines, direct lymphocyte trafficking and positioning in tissues and play roles in modulating immune responses and shaping the severity of disease^[79]. In PBC, infiltrating memory T cells are culprits regarding the destruction of bile ducts.

Chemokines, interferon-gamma-inducible protein-10 (IP-10) and monokine-induced by gamma interferon (MIG), are increased in plasma and the portal area in PBC patients compared to controls. Moreover, the frequency of CXCR3⁺ cells is higher in both PBMCs and injured bile ducts. Intriguingly, daughters and sisters of PBC patients demonstrate increased plasma levels of IP-10 and MIG, but the frequency of circulating CXCR3⁺ cells is normal^[80]. Knockout of CXCR3 in Poly(I:C)-induced mice displayed a delayed and milder progression of cellular inflammation^[81].

Other chemokines may also be involved in recruiting inflammatory cells into the liver in PBC. BECs are a source of chemokine production that attracts monocytes into the liver^[82,83]. Stimulated by poly(I:C), BECs produce augmented CX3CL1 in the presence of monocytes pretreated with LPS through CD40-CD154 contact^[83]. In addition, BECs pretreated with LPS also secrete elevated CX3CL1^[83,84].

CONCLUSION

Substantial amounts of data to date have illustrated that autoimmunity plays a critical role in the pathogenesis of PBC. The adaptive immune response, the innate immune system and their interplay participate in the development of disease. However, there are clearly limitations and unanswered questions that still remain: (1) although some murine models have been established, they cannot imitate PBC in humans completely (Table 3); (2) *in vitro* experiments based on human samples may not completely reflect the endosomatic problems; and (3) the data to date are mainly based on PBMCs, which lack reliability without assessment in the local liver microenvironment. In order to address these problems, better murine models are needed. In the future, researchers must identify new mechanisms through the analysis of both *in vivo* and *in vitro* approaches for the development of effective treatment strategies for PBC.

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Diagnostic and therapeutic progress of multi-drug resistance with anti-HBV nucleos(t)ide analogues

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Abstract

Nucleos(t)ide analogues (NA) are a breakthrough in the treatment and management of chronic hepatitis B. NA could suppress the replication of hepatitis B virus (HBV) and control the progression of the disease. However, drug resistance caused by their long-term use becomes a practical problem, which influences the long-term outcomes in patients. Liver transplantation is the only choice for patients with HBV-related end-stage liver disease. But, the recurrence of HBV after transplantation often caused by the development of drug resistance leads to unfavorable outcomes for the recipients. Recently, the multi-drug resistance (MDR) has become a common issue raised due to the development and clinical application of a variety of NA. This may complicate the antiviral therapy and bring poorly prognostic outcomes. Although clinical evidence has suggested that combination therapy with different NA could effectively reduce the viral load in patients with MDR, the advent of new antiviral agents with high potency and high genetic barrier to resistance brings hope to antiviral therapy. The future of HBV researches relies on how to

prevent the MDR occurrence and develop reasonable and effective treatment strategies. This review focuses on the diagnostic and therapeutic progress in MDR caused by the anti-HBV NA and describes some new research progress in this field.

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Key words: Hepatitis B virus; Multi-drug resistance; Nucleos(t)ide analogues; Gene mutation; Liver transplantation

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INTRODUCTION

Globally, it is estimated that about 400 million are chronically infected with hepatitis B virus (HBV). The prevention and control of hepatitis B is an important public health issue. HBV infection may not only result in fulminant, subfulminant, and chronic hepatitis, but may also contribute to the development of liver cirrhosis and hepatocellular carcinoma in the long-term^[1]. Decades of clinical experiences have shown that the administration of nucleos(t)ide analogues (NA) could postpone the progression of the disease. NA mainly suppresses the

HBV replication and reduces the damage to hepatocytes by hindering the synthesis of reverse transcriptase (RT), which is the prerequisite for viral replication. The main disadvantage of NA is the recurrence of HBV after therapy interruption. Hence, the long-term treatment becomes a mandate. But, this paves the way for other challenges like HBV genome mutation and clinical drug resistance. The reports of multi-drug resistance (MDR) to NA in the recent years are increasing, and become an important issue for clinicians to modulate the treatment strategies during drug resistance.

MECHANISMS OF ANTIVIRAL RESISTANCE

HBV has a DNA genome. But, it replicates through an RNA intermediated reverse transcription. The lack of proofreading ability of the viral encoded, RNA-dependent DNA polymerase could result in potential mutations at each nucleotide position within the entire genome^[2]. This, together with the large number of virions produced (10^{12} - 10^{15} /d), indicates that every mutation of the 3.2 kb HBV genome can theoretically be produced daily^[3,4]. Under the selective pressure of antiviral agents, such diversity is likely to give rise to drug-resistant mutants^[5]. RT is the target of NA and also the chief place of gene mutations. Several factors are associated with the development of antiviral resistance, including viral fitness, potency, and genetic barrier to resistance of the antiviral agents^[6]. Studies have shown that there are five pathways leading to antiviral resistance^[7]. (1) The L-nucleoside pathway (rtM204V/I), leads to the resistance of lamivudine (LAM), emtricitabine, telbivudine (LdT), and clevudine. This pathway includes entecavir (ETV) resistance in LAM-experienced patients; (2) The acyclic phosphonate pathway (rtN236T), associates with the resistance to adefovir (ADV) and tenofovir disoproxil fumarate (TDF)^[8,9]; (3) A shared pathway (rtA181T/V), results in the selection of HBV quasispecies when treated with L-nucleosides or acyclic phosphonates; (4) Naïve entecavir resistance pathway (rtL180M + rtM204V with either rtT184, S202, or M250 codon changes). In this pathway, three mutations are required to appear simultaneously accounting for the very low resistance profile of entecavir^[10]; and (5) Multidrug resistance pathway, complex patterns and clusters of specific mutations in HBV polymerase are associated with it.

TDF is recommended since 2008, as first line drug in patients chronically-infected with HBV and led to high rates of virologic success^[11,12]. Its effect on HBV DNA suppression appears similar to ETV and LdT and superior to LAM and ADV^[13,14]. It remains largely active even against ADV-resistant or ETV-resistant mutations^[15-17]. However, the rtA194T polymerase mutation has been found in HBV/HIV co-infected patients during TDF treatment and may be associated with TDF resistance^[18-20]. Whether the rtA194T mutation truly confers resistance against TDF has remained controversial, as the

in vitro phenotypic assays showed variable results across laboratories^[21,22]. Thus, the potential impact of this mutation on TDF susceptibility deserves further study^[20]. The primary antiviral drug resistance mutations in the polymerase gene are listed in Table 1^[23].

HBV strains, resistant to at least two anti-HBV agents from different subclasses of NA without a cross-resistance profile, are defined as MDR^[24]. The main reasons for MDR are the sequential monotherapy to treat primary resistance and use of agents with similar cross-resistance profiles. The development of MDR is a major challenge for antiviral therapy, and the improper administration of NA may lead to serious outcomes. Thus, more researches on the choice of antiviral agents in treating patients with MDR have been carried out and some significant solutions have been achieved.

THE CURRENT SITUATION AND STRATEGIES OF DIFFERENT TYPES OF MDR

LAM + ADV resistance

LAM, the first oral antiviral agent against HBV, is safe and well tolerated even in patients with decompensated liver cirrhosis^[25]. Globally, it has been mostly used with a low genetic barrier to resistance and cumulative incidence of resistance as high as 70% after 5 years of treatment^[26,27]. Early studies had suggested that, ADV monotherapy had shown similar antiviral effects to combination therapy with LAM+ADV for LAM-resistant patients in the short-term, and a strategy of switching to ADV monotherapy had widely been adopted^[28]. However, recent studies have showed that ADV resistant mutations emerge more frequently during sequential ADV monotherapy in LAM resistance than in treatment-naïve patients^[29,30]. The rate of ADV resistance in LAM-resistant patients was shown to be as high as 18% at 1 year, compared with 0% in LAM-naïve patients^[31]. Another long-term study reported that the cumulative genotypic resistance and virologic breakthrough at 5 years of sequential ADV monotherapy in LAM-resistant patients were 65.6% and 61.8%, respectively^[32]. Fung *et al.*^[33] reported that the cumulative rate of ADV resistance in LAM-resistant patients at 2 years was 18% for patients who were switched to ADV and 7% for patients who had ADV added to their treatment regimen. In another study of 42 LAM-resistant patients (HBeAg-negative), the ADV resistance rates at 15-18 mo of treatment were 21% (3/14) for patients who were switched to ADV and 0% for patients who had ADV added^[34]. It can be assumed that the ADV resistance rate in LAM-resistant patients can be greatly reduced by adding rather than switching to ADV. There are more researches exploring the mechanisms of LAM + ADV dual-resistance, as these two agents were launched early. When the mutations causing resistance to LAM and ADV are not on the same viral genome, a combination therapy of these two agents will likely be effective in suppressing the mutants

Table 1 Primary antiviral drug resistance mutations in the polymerase gene^[23]

	Domain A	Domain B	Domain C	Domain D	Domain E	Numbers of mutations
Lamivudine and Telbivudine	--	rtV173L rtL180M rtA181T/V	rtM204V/I	--	--	1
Adefovir	--	rtA181T/V		rtN236T	--	1
Tenofovir	--	rtA181T/V		rtN236T	--	?
Entecavir	--	rtL180M rtT184	rtM204I/V rtS202	--	rtM250I/V	3

resistant to each of the drugs. In contrast, when the antiviral resistance mutations are on the same viral genome, the combination treatment may not be adequate^[30]. *In vitro* analysis have shown that most of MDR mutations collocate on the same viral genome^[35], but the *in vivo* confirmation on the same is lacking. There is no unified clinical treatment strategy for LAM + ADV dual-resistance, but different methods of mono or combination therapy have been carried out.

Due to the limited alternative of NA in the early stage, interferon (IFN) had been tried as a choice for dual-resistance to LAM and ADV. Phenotypic analysis have indicated that IFN- α suppresses equally the mutant strains and wild-type strains *in vitro*^[36]. Furthermore, IFN- α also suppresses the replication of LAM-resistant and ADV-resistant mutants *in vivo*^[37]. Besides that, IFN- α administration predictably have reduced the resistance to NA when combined with LAM^[38,39], as IFN- α exhibits at least two HBV-specific antiviral activities independent of the viral polymerase sequence with one reducing the levels of core protein and replicative intermediates, and the other leading to posttranscriptional degradation of HBV RNA^[40]. However, there are certain potential limitations with IFN therapy such as low probability of sustained response, parenteral administration, relatively poor tolerability, and frequent and potential serious adverse effects in patients with advanced liver disease^[41]. These deficiencies limited the clinical application of IFN, and more researches focused on the application of oral NA in MDR.

In vitro studies show that the majority of MDR mutations to LAM and ADV collocate on the same viral genome^[31]. Therefore, the combination therapy with LAM and ADV may not effectively deal with the patients, who are resistant to these two agents. The advent of ETV enabled a new choice for antiviral therapy. Since TDF is not available in many Asian countries, the 2008 updated guidelines by Asian Pacific Association for the Study of the Liver recommended ETV in LAM and ADV resistant patients^[42]. But satisfactory clinical results were not acquired in these patients treated with ETV. Heo *et al*^[30] compared the clinical efficacy of LAM + ADV combined therapy and ETV monotherapy in patients with dual-resistance to LAM and ADV. The mean reduction in serum HBV DNA concentration was significantly lower in the LAM + ADV than in the ETV group. But, the difference in mean decline in serum alanine aminotransferase (ALT) levels over 12 mo of treatment and the rate

of HBeAg seroconversion at 12 mo did not differ significantly between two groups. Park *et al*^[43] reported that ETV monotherapy could not reach an optimal clinical efficacy in ADV-refractory chronic hepatitis B patients with prior LAM resistance. In this long-term study (up to 4 years), the authors have suggested that an early virologic response is essential for a successful ETV monotherapy in this group of patients. Clinically, initial virologic response at 3 mo (IVR-3) is an independent predictor for virologic response, and it may help determine whether to maintain ETV monotherapy or not. Choe *et al*^[44] evaluated the antiviral efficacy of ETV in patients, who had failed to achieve viral response during LAM and ADV rescue therapy. The virologic response was achieved in 1 of 18 patients with pre-existing rt204 mutations, whereas it was achieved in all 4 patients without pre-existing rt204 mutations regardless of the presence of rt181 or rt236 mutations. The poor treatment efficacy of ETV in patients with LAM and ADV dual-resistance might have resulted from the pre-existing rt204 mutations, which could further lead to ETV resistance.

As the ideal clinical efficacy was not achieved by ETV monotherapy, researches continued to seek other treatment strategies with ETV combination therapies. A study tried combination of ETV and ADV in LAM-resistant chronic hepatitis B patients with suboptimal response to LAM + ADV^[45]. This strategy provided superior virologic response and favorable resistance profiles, when compared with combination therapy of LAM and ADV. But similar to ETV monotherapy, an optimal virologic response still could not be reached. This may likely due to the relatively low antiviral potency of ADV, which suggested to replace ADV with another drug with a similar resistance profile and higher potency against LAM-resistant mutants.

TDF shares the similar molecular structure with ADV. It has higher antiviral potency and lower rate of developing drug resistance. Despite its structural similarity to ADV, TDF partially suppresses ADV-resistant HBV, and it is also highly effective against LAM-resistant virus, suggesting that this drug may be an effective treatment for patients who have previously failed treatment with LAM and ADV^[46]. Van Bommel *et al*^[47] introduced TDF for LAM-Resistant patients with high HBV DNA level during ADV therapy. The administration of TDF (300 mg daily for all the patients except one) led to an undetectable HBV DNA level in 19 of 20 patients within

a median of 3.5 mo. The only patient who did not become HBV DNA negative during the observation period received a reduced TDF dose (300 mg every second day) because of renal insufficiency. Patterson *et al.*^[48] reported similar efficacy with TDF in another group of similar patients. But, the virologic response to TDF in this study appeared to be inferior to that observed in treatment-naïve patients. The development of antiviral resistance to TDF in the long-term is uncertain, but the combination therapy with high potency NA without cross-resistance is still a superior strategy for MDR. Recently, Petersen *et al.*^[49] reported a multicenter study with ETV + TDF as rescue therapy in pretreated chronic hepatitis B patients. HBV DNA was undetectable in 51 out of 57 patients with different types of resistance, and the ALT levels improved in most of them, suggesting a reduction in liver inflammation. Besides, this strategy was efficient, safe, and well tolerated in patients with and without advanced liver disease. The updated 2009 American Association for the Study of the Liver Diseases (AASLD) practice guideline recommended TDF + ETV in patients with sequential LAM and ADV treatment failure^[27].

LAM + ETV resistance

ETV is an effective antiviral agent with high potency and high genetic barrier to resistance. In NA-naïve patients, the 5-year cumulative probability of genotypic ETV resistance and genotypic ETV resistance associated with virologic breakthrough was only 1.2% and 0.8%, respectively^[50]. However, the emergence of resistance to ETV occurs more frequently in LAM-refractory population. Based on the previous reports, the resistance to ETV in LMV-refractory patients was detected in 8% of patients after 12 mo, 43% after 48 mo, and 51% after 60 mo of treatment^[51,52]. In a clinical study, the cumulative rates of ETV genotypic resistance in patients with LAM resistance are 6%, 15%, 36%, 46%, and 51% from years 1 to 5, respectively^[53]. The resistance to ETV in the sequential monotherapy in LAM-refractory patients shares the same pathway (mutation of rtM204V/I) with LAM resistance. Yatsuji *et al.*^[54] reported a patient with dual-resistance to LAM and ETV, but was effectively treated with ADV. Interestingly, the typical mutation strains of ETV (rtL180M + M204V + S202G) were observed in this patient. An *in vitro* study indicated that the rtL180M + M204V + S202G mutant had no resistance against ADV, and this case report confirmed this view clinically. Another study included 12 patients with dual-resistance to LAM and ETV, and half of them reached complete virological response with the combination therapy of ADV and ETV after 18 mo. In addition, no enrolled patients developed virologic breakthrough and had mutations resistant to ADV at the end of follow-up. However, not all the patients realized virologic response, which may due to the low potency of ADV and high pretreatment levels of HBV DNA^[55]. This combination therapy strategy may be helpful as ETV is effective to rtA181T related mutant and ADV is effective to rtM204V related mutant. The

optimal strategy for such dual-resistance patients has not been determined due to less availability of literature data.

LAM + ADV + ETV resistance

Recently, MDR to three NA has been described in case reports. Liu *et al.*^[56] reported LAM + ADV could be helpful in a patient, who was resistant to LAM, ADV, and ETV. It could be speculated that the HBV DNA replication had been suppressed due to LAM and ADV. The immune response against the MDR strains might also have contributed to the clearance of these strains. The exact effect of this strategy remains to be observed due to the short follow-up period. Sayan *et al.*^[24] reported a case of another patient, who was effectively treated with ETV and TDF. Three primer drug resistance mutations in different domains of HBV viral polymerase, such as rtA181V/T, rtL180M + rtM204V mutations and the rtN236T, were characterized in the same genome, which might explain the MDR profile. In another study of MDR, amino acid changes consisting of L80V, L91I, M204I, S219A, N238D, and Y245H were found on the same dominant viral genome strain. These newly discovered mutation types may also relate to MDR^[57]. This type of resistance, usually caused by sequential monotherapy, may be effectively treated by combination therapy. However, the mechanisms and preventive methods for MDR to three NA need to be studied.

Treatment strategy of MDR in liver transplant recipients

Liver transplant is an effective method for patients with HBV-related end-stage liver disease. In a liver transplant setting, three different clinical phases have to be considered: (1) treatment of HBV infection during waiting list; (2) prophylaxis of hepatitis B recurrence after liver transplant; and (3) treatment of recurrent hepatitis B when prophylactic measures have failed^[58]. The main factors associated with HBV recurrence were HBeAg status at transplant listing and serum HBV-DNA level at transplant^[59]. The goals of antiviral therapy in the pretransplant patients include the reduction of viral load to low or nondetectable serum HBV DNA levels^[60]. The development of drug resistance for patients in the waiting list for liver transplant is a common problem. Osborn *et al.*^[61] found that the antiviral therapy failure in patients with HBV in the waiting list did not impair clinical outcomes when recognized early and also when the salvage therapy was promptly initiated and neither the survival rate with transplant nor without transplant was negatively impacted by antiviral therapy failure.

However, the recurrence of HBV after transplant is still a troublesome problem and may influence the long-term outcomes. The combination of both LAM and hepatitis B immunoglobulin (HBIG) has emerged as the most effective prophylactic strategy in HBV transplant recipients with a 3-year recurrence rate of 0-10%^[58,62,63]. But, the drug resistance to this combination therapy has also emerged. In addition to the typical resistance to LAM, it was detected in 45% of the immunosuppressed

patients within the first year following liver transplant^[64]. The escape mutation from anti-HBs occurs in the common α determinant region of the surface gene, which is a highly conservative region of the HBsAg protein^[65]. IFN is not currently used for the treatment of hepatitis B after transplantation, given the risk of graft rejection^[66,67]. The availability of ADV has changed the clinical course of LAM-resistant patients, and it is effective and safe among liver transplant recipients^[68-70]. Researches showed that ADV could be an effective rescue therapy for patients with LAM-resistant hepatitis B post-liver transplant^[71,72]. In a retrospective review, viral recurrence was noted in 5 out of 23 liver transplant recipients, who are on combination prophylaxis of LAM and HBIg with 1 patient receiving TDF and 4 receiving ADV. Only 1 death related to HBV recurrence was reported in this population, who had been switched to ADV^[73].

Apart from ADV, ETV appears to be a more attractive candidate than ADV for use in the transplant setting^[74]. A study of 30 patients receiving a combination of ETV and low-dose HBIg confirmed the efficacy and safety of ETV in preventing recurrence of HBV after liver transplant^[75]. Another small study of 8 patients showed that ETV was safe and effective as prophylaxis after liver transplant with no interactions observed with the immunosuppressive medications^[76]. Fung *et al.*^[77] reported that although only 26% of patients had complete viral suppression at the time of transplant and 91% of patients underwent loss of HBsAg after 2 years of follow-up. Also, 98.8% of patients achieved undetectable HBV DNA levels with ETV as the sole antiviral agent after liver transplant. It can be inferred that ETV may play an effective role in recipients resistant to both LAM and HBIg.

ETV could be a choice in liver transplant recipients, and TDF may also be effective when used alone or in combination therapy. Villet *et al.*^[78] reported a recipient who was resistant to LAM, ADV, and HBIg. The combination therapy of LAM and TDF effectively suppressed the HBV DNA replication. Karlas *et al.*^[79] reported that the combination therapy with ETV and TDF may prevent post-liver transplant hepatitis B recurrence even without HBIg maintenance therapy. He illustrated that the combination oral antiviral therapy might substitute for HBIg as indefinite prophylactic regimen due to profound antiviral efficacy and low risk of viral resistance. The optimal combination method for MDR in liver transplant recipients is still uncertain, and the therapeutic experiences in non-transplant patients could be learned.

NEW PERSPECTIVES ON THE RESEARCH OF MDR

There are still some limitations on the current research on drug resistance. For instance, the genetic analysis of resistance to NA inhibitors is usually focused on the RT domain, and only infrequently takes into consideration of the whole genome of intra-host HBV variants^[80-82].

Thai *et al.*^[83] reported that the rtM204I/V substitution is insufficient for establishing resistance against LAM. The analysis of 639 HBV whole-genome sequences obtained from 11 patients showed that rtM204I/V was independently acquired by more than one intra-host HBV variant, indicating the convergent nature of LAM resistance. Currently, the most commonly used methods for detecting HBV drug-resistance mutations are direct sequencing and reverse hybridization^[84]. However, these methods do not enable haplotype analysis, and hence, they cannot be used to determine the collocation of mutations on the same viral genome. This limits the accurate identification of viral mutants that are resistant to drugs with high genetic barrier^[85]. Recently, several next-generation sequencing technologies, such as ultra-deep pyrosequencing, are available to generate more data^[85-88]. These methods may offer significant advantages in explaining and predicting the responses of HBV patients to antiviral therapy and enable quantification of HBV quasispecies variants.

Recently, some studies analyzed the mutation pattern in relation to the HBV genotypes and found that the rtL180M mutation is significantly connected to the rtM204V mutation in genotypes A, B, and C. Also, the HBV genotypes differ in their mutation pattern of LAM resistance^[89]. Another study indicated the association of genotype C with higher rates of hepatitis B recurrence after transplant due to LAM resistance^[90]. It can be inferred that HBV genotypes may play an important role in the progression of HBV-related liver disease and response to antiviral therapy. The assessment of HBV genotype prior to the treatment may help to individualize the antiviral therapy and reduce the incidence of treatment failures and complications^[91,92]. However, the relationship between HBV genotypes and antiviral resistance is unclear. Extensive researches may provide new perspectives for the prevention and optimal rescue therapy to patients with drug resistance.

PREVENTION OF MDR

Treatment failure in anti-HBV therapy could be regarded as an iatrogenic factor, and a judicious use of NA in chronic hepatitis B patients is the most effective prophylaxis against the development of MDR. Thus, proper strategy should be applied by clinicians at the beginning of therapy. An antiviral agent with the highest potency and a high genetic barrier to resistance should be selected^[93,94]. The pros and cons of initiating the treatment with combination therapy in minimizing the development of antiviral resistance are currently being investigated^[95,96]. Avoiding the sequential use of NA monotherapy is an effective preventive method for MDR^[24]. A roadmap suggested that HBV DNA concentration at week 12 after initial treatment should be checked to identify patients with primary treatment failure, which is defined by < 1 log copies/mL reduction of HBV DNA concentration. For patients with primary treatment failure with good drug compliance, addition of another NA is indicated. The second assessment of HBV DNA should then be

Table 2 Patterns and pathways of antiviral drug resistance in chronic hepatitis B in the context of cross-resistance^[99]

Pathway	Amino acid substitution in the rt domain	LAM	LdT	ETV	ADV	TDF
	WT	S	S	S	S	S
L-nucleoside (LAM/LdT)	M204I/V	R	R	I	S	S
Acyclic phosphonate (ADV)	N236T	S	S	S	R	I
Shared (LAM, LdT, ADV)	A181T/V	R	R	S	R	I
Double(ADV, TDF)	A181T/V + N236T	R	R	S	R	R
D-Cyclopentane (ETV)	L180M + M204V/I ± I169 ± T184 ± S202 ± M250	R	R	R	S	S

I: Intermediate sensitivity; R: Resistant; S: Sensitive based on cell culture and clinical; LAM: Lamivudine; LdT: Telbivudine; ETV: Entecavir; TDF: Tenofovir; ADV: Adefovir.

Table 3 Management of antiviral-resistant hepatitis B virus in updated guidelines

	AASLD 2009 ^[27]	EASL 2012 ^[101]
LAM-resistance	Add ADV or TDF In patients with no prior exposure to other NA: Add LAM or ETV	Switch to TDF or add ADV In patients with no prior exposure to other NA: Switch to ETV or TDF
ADV-resistance	In patients with prior LAM resistance: Switch to TDF plus LAM or ETV	In patients with prior LAM resistance: Switch to TDF and another NA
ETV-resistance	Switch to TDF	Switch to or add TDF
TDF-resistance	--	In patients with no prior exposure to LAM: Switch to ETV In patients with prior LAM resistance: Add ETV

LAM: Lamivudine; LdT: Telbivudine; ETV: Entecavir; TDF: Tenofovir; ADV: Adefovir; NA: Nucleos(t)ide analogues.

done at week 24^[97,98]. Treatment strategies in patients with partial virologic response are based on the potency and genetic barrier of the antiviral agent. Patients receiving NA with a high genetic barrier can remain the treatment beyond 48 wk. Patients receiving a less potent NA should continue treatment and be re-assessed at week 48, and those, who receive NA with a low genetic barrier, should add a more potent drug due to the high risk of resistance when the treatment is not adapted^[26]. To avoid MDR, the combination therapy of NA with cross-resistance should be avoided. The patterns and pathways of antiviral drug resistance in chronic hepatitis B in the context of cross-resistance are listed in Table 2^[99]. Patient's adherence to antiviral therapy is another important factor in avoiding resistance. Adherence may be monitored using patient reports, dispensed medication counts, or HBV DNA detection^[93]. If low response or virologic breakthrough is observed with the primary treatment, gene sequencing should be done to find out the type and location of mutations, in order to guide the optimal rescue therapy. Recently, An *et al.*^[100] reported that the family history, negative conversion time of HBV DNA, and different NA were independent risk factors of gene resistant mutation, which provided a theoretical basis for predicting drug resistance and salvage treatment.

There is still no consensus statement on the management of MDR in current HBV treatment guidelines. However, the guidelines for primary treatment failure could be used in order to prevent MDR. The management of antiviral-resistant HBV in guidelines is listed in Table 3^[27,101]. In these updated guidelines, the combination therapy was not recommended in all circumstances. The antiviral agents with high potency and high genetic

barrier to resistance, such as ETV and TDF, could also be used alone.

CONCLUSION

In summary, MDR to NA is a thorny issue in the anti-HBV therapy. The clinical efficacy in patients has been improved to some extent by the combination therapy. ETV and TDF can be regarded as the optimal choice for patients with MDR. Further investigations on the mechanisms and optimal treatment modalities are still lacking. The progress and emergence of new genetic testing technology will probably improve the anti-HBV therapy. The newly discovered forms of gene mutations to resistance may provide useful clues to solve the problem of MDR. The efficacy of IFN in patients with MDR needs further exploration. The development of new anti-HBV agents, which act not only on RT, but also on other targets of HBV, may be a new approach to prevent MDR in NA.

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***Gardenia jasminoides* attenuates hepatocellular injury and fibrosis in bile duct-ligated rats and human hepatic stellate cells**

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Abstract

AIM: To investigate the anti-hepatofibrotic effects of *Gardenia jasminoides* in liver fibrosis.

METHODS: Male Sprague-Dawley rats underwent common bile duct ligation (BDL) for 14 d and were treated with *Gardenia jasminoides* by gavage. The ef-

fects of *Gardenia jasminoides* on liver fibrosis and the detailed molecular mechanisms were also assessed in human hepatic stellate cells (LX-2) *in vitro*.

RESULTS: Treatment with *Gardenia jasminoides* decreased serum alanine aminotransferase (BDL vs BDL + 100 mg/kg *Gardenia jasminoides*, 146.6 ± 15 U/L vs 77 ± 6.5 U/L, $P = 0.0007$) and aspartate aminotransferase (BDL vs BDL + 100 mg/kg *Gardenia jasminoides*, 188 ± 35.2 U/L vs 128 ± 19 U/L, $P = 0.005$) as well as hydroxyproline (BDL vs BDL + 100 mg/kg *Gardenia jasminoides*, 438 ± 40.2 μ g/g vs 228 ± 10.3 μ g/g liver tissue, $P = 0.004$) after BDL. Furthermore, *Gardenia jasminoides* significantly reduced liver mRNA and/or protein expression of transforming growth factor β 1 (TGF- β 1), collagen type I (Col I) and α -smooth muscle actin (α -SMA). *Gardenia jasminoides* significantly suppressed the upregulation of TGF- β 1, Col I and α -SMA in LX-2 exposed to recombinant TGF- β 1. Moreover, *Gardenia jasminoides* inhibited TGF- β 1-induced Smad2 phosphorylation in LX-2 cells.

CONCLUSION: *Gardenia jasminoides* exerts antifibrotic effects in the liver fibrosis and may represent a novel antifibrotic agent.

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Key words: *Gardenia jasminoides*; Liver fibrosis; Collagen type I; Transforming growth factor- β 1/Smad2 pathway; α -smooth muscle actin

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INTRODUCTION

Chronic liver injury causes the accumulation of extracellular matrix (ECM) such as α -smooth muscle actin (α -SMA) in the liver and subsequently contributes to liver fibrosis and later cirrhosis^[1-6]. This eventually leads to hepatic dysfunction, portal hypertension, and hepatocellular carcinoma (HCC)^[7-9]. Hepatic stellate cells (HSC) are the principal liver cells that promote liver fibrosis^[3,9-12]. Upon activation by various stimuli such as transforming growth factor (TGF)- β 1, HSC transdifferentiate into myofibroblasts and then produce excessive ECM proteins, resulting in liver fibrosis^[13-15]. Strategies aimed at disrupting TGF- β 1 synthesis and/or signaling pathways markedly ameliorates liver fibrosis in an experimental model^[16].

Herbal medicines have been frequently investigated for their hepatoprotective and antifibrotic effects in both humans^[17] and animal models^[18]. A number of studies have shown that administration of Chinese herbs lead to a decrease in hepatic TGF- β 1 expression and severity of fibrosis in rats^[19,20]. Yin-Chen-Hao-Tang (YCHT) decoctions have long been used as antiinflammatory, antipyretic, choleric and diuretic agents for liver disorders and jaundice. Several studies provide clinical evidence of its effectiveness in the treatment of various liver disease. YCHT is an aqueous extract derived from three herbs: *Artemisia capillaries* Thunb (Herba *Artemisiae Capillaris*, Yin-Cen-Hao), *Gardenia jasminoides* Ellis (*Fructus Gardeniae*, Zhi-zi) and *Rheum officinale* Baill (Emodin, Da-huang) with a ratio of 4:3:1 in weight. YCHT was reported to suppress liver fibrosis in rats induced by a choline-deficient diet^[21]. Long-term administration of YCHT in rats ameliorated hydrophilic bile acids-induced hepatic injury presumably by reducing oxidative stress and the degree of hepatic fibrosis^[22]. These studies have indicated that YCHT as a promising therapeutic agent in chronic liver disease. Recent studies showed that *Artemisia capillaries* and Emodin (the main compound of *Rheum officinale*) are well-known herbal hepatotherapeutic drugs for the treatment of liver fibrosis^[23-25]. However, whether *Gardenia jasminoides* has an anti-fibrotic effect on liver fibrosis and the involved detailed mechanism has not been fully understood yet.

The aim of the current study is to investigate the beneficial effects of *Gardenia jasminoides* on liver fibrosis using the bile duct ligation (BDL) rat model *in vivo* and TGF- β 1-stimulated HSCs *in vitro*.

MATERIALS AND METHODS

Materials

Recombinant human TGF- β 1 was obtained from R

and D (Minneapolis, MN, United States). α -SMA and TGF- β 1 antibodies were purchased from Abcam (Cambridge, MA, United States). Phospho-Smad2 and Smad2 antibodies were obtained from Cell Signaling Technology (Boston, MA, United States). α -tubulin antibody was purchased from Sigma-Aldrich (St. Louis, MO, United States). DMEM and fetal bovine serum (FBS), penicillin/streptomycin and trypsin were obtained from Invitrogen (Carlsbad, CA, United States). *Gardenia jasminoides* standard (purity > 99%) was purchased from the Institute of Chinese Pharmaceutical and Biological Products.

Preparation of *Gardenia jasminoides*

Gardenia jasminoides was prepared as described previously^[22] by boiling the dried *Gardenia jasminoides* fruits with distilled water for 5 h. The extract was filtered, freeze-dried, and kept at 4 °C. The yield of water extract of *Gardenia jasminoides* was 8.33% (w/w). The dried extract was dissolved in distilled water before use.

Animal experiments

All animal experimental protocols were approved by the local animal care and use committee according to criteria outlined in Guide for the Care and Use of Laboratory Animals from the National Academy of Sciences (National Institutes of Health publication 86-23, 1985 revision). For experiments with BDL, rats were randomly divided into five groups ($n = 8$). Each day, four animals underwent BDL, and a sham-operated animal was used as a healthy control. Twenty-four hours after surgery, the four BDL animals were randomly assigned to receive 14 d of daily gavage consisting of ddH₂O (the treatment control and vehicle), while treatment groups received 25, 50 and 100 mg/kg *Gardenia jasminoides* (suspended in ddH₂O) by gavage. The sham controls also received ddH₂O by gavage. At the end of experiment, rats were anesthetized, serum was collected and livers were removed. Some portions of liver tissue were fixed in 10% formalin or embedded in paraffin specimen medium. Others were snap frozen in liquid nitrogen and stored at -80 °C until use.

Serum biochemistry and liver histology

Serum alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were analyzed with kits from Thermo Fisher Scientific (United States). Formalin-fixed tissue was embedded in paraffin, and sections of liver (4 μ m) were stained with hematoxylin and eosin (HE) to evaluate the morphological changes and stage of liver fibrosis according to the Ishak Stage Score. The liver hydroxyproline content was measured as described^[26].

Quantitative real-time polymerase chain reaction

Total RNA was extracted using TRIzolTM Reagent (Invitrogen, United States) according to the manufacturer's instructions. Total RNA (1 μ g) was reverse transcribed, followed by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) using the Bio-Rad

Table 1 Primer sequences for real-time reverse transcription polymerase chain reaction

Gene	Primer sequences	Size (bp)	Accession number
Rat	TGF-β1 Forward: 5'-TACAACAGCACCCGCGACCG-3' Reverse: 5'-TGCGTTGTGCGGTCCACCA-3'	117	NM_021578.2
	Col I Forward: 5'-ACCGGGCCGATGATGCCAAC-3' Reverse: 5'-ATGTGCGGGCGGGTTCCTTG-3'	129	NM_053304.1
	GAPDH Forward: 5'-GGGCCTCTGCTCCTCCCTGTTTC-3' Reverse: 5'-ACGGCCAAATCCGTTACACCC-3'	107	NM_017008.3
Human	TGF-β1 Forward: 5'-TGTTCCGCTCTCGGCAGTG-3' Reverse: 5'-GCCTCGATGCGCTTCCGCTT-3'	184	NM_000660.4
	Col I Forward: 5'-GAGCGGACGCTAACCCCTC-3' Reverse: 5'-AGGGCGGTGGCCGCTAAGAG-3'	110	NM_000088.3
	GAPDH Forward: 5'-CAGCTCCCGTTCGCTCTC-3' Reverse: 5'-ACCAGGCGCCCAATACGACC-3'	143	NM_002046.4

TGF-β1: Transforming growth factor-β1; Col I : Collagen type I ; GAPDH: Glyceraldehydes-3-phosphate dehydrogenase.

iCycler Iq system. The qRT-PCR reactions were carried out in a total volume of 25 μL containing 12.5 μL of SYBR Premix Ex Taq™ (2 ×) (TaKaRa Biotechnology Co., Ltd, Dalian, China), 2 μL of cDNA, 5 pmol of forward and reverse primers, and 9.5 μL of distilled H₂O. The sequences of the rat and human primers used are listed in Table 1, respectively. PCR was performed at 95 °C for 30 s, and then run for 45 cycles at 95 °C for 5 s and 60 °C for 20 s. The relative amount of mRNA was calculated using the comparative C_t (ΔΔC_t)^[27]. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as a reference for normalizing data. The derived normalized values were the mean of three runs.

Western blotting

Total proteins were extracted and subjected to SDS-PAGE and analyzed by immunoblotting as described previously^[28]. Primary antibodies against α-SMA, p-Smad2, Smad2 and α-tubulin were used. Horseradish peroxidase-coupled secondary antibodies were bought from Promega (Promega). The protein bands were detected employing ECL chemiluminescence (Thermo Scientific).

Cell culture

LX-2, a well-characterized cell line derived from human HSCs, was used in the *in vitro* studies. Recombinant TGF-β1 and *Gardenia jasminoides* (Standard) were used at doses shown in individual figure legends. Cells were cultured in DMEM supplemented with 10% fetal bovine serum, 1 mmol/L L-glutamine, and 100 IU/mL streptomycin/penicillin.

Statistical analysis

SPSS version 16.0 for Windows was used for all analysis. All values are expressed as mean ± SD. The statistical significance between experimental groups was determined by *t* test or analysis of variance. *P* values < 0.05 were considered statistically significant.

RESULTS

Treatment with *Gardenia jasminoides* attenuated liver fibrosis in BDL rats

Both BDL and sham-operated rats were orally administered continuously either with the vehicle or *Gardenia*

jasminoides (25, 50, 100 mg/kg body weight) for 2 wk. BDL in rats is associated with significant increases in liver fibrosis; this was confirmed by HE staining of liver tissues. In contrast, *Gardenia jasminoides* treatment groups had significantly less liver fibrosis (Figure 1), with the lowest scores in the 100 mg/kg *Gardenia jasminoides* treatment group.

Gardenia jasminoides treatment also reduced the elevated levels of serum ALT and AST, which are indicators of hepatocellular damage induced by BDL (Figure 2A and B). Hydroxyproline analysis revealed significantly lower levels of this fibrosis marker in the livers of the *Gardenia jasminoides* treatment groups (Figure 2C). This was also confirmed by hepatic histology. In particular, the hydroxyproline content in the 100 mg/kg group was reduced to levels similar to those in the control group.

To uncover the mechanisms underlying this beneficial phenomenon, we investigated the effects of *Gardenia jasminoides* on the expression of fibrotic gene. mRNA expression of TGF-β1 and collagen I (Col I) was significantly reduced in the livers of *Gardenia jasminoides* treatment groups (Figure 2D and E). Western blot analysis also revealed a significant reduction in α-SMA protein expression in the livers of groups treated by 50 and 100 mg/kg *Gardenia jasminoides*, respectively (Figure 2F). Taken together, these data suggest that the *Gardenia jasminoides* therapy greatly reduces fibrosis formation in BDL rats.

Treatment with *Gardenia jasminoides* attenuates TGF-β1-induced HSC activation

Because *Gardenia jasminoides* reduced markers of hepatic fibrosis in BDL rats, we next examined whether this therapy has similar antifibrotic effects in a human HSC line (LX-2 cells). The mRNA levels of TGF-β1 and Col I were significantly increased in response to recombinant TGF-β1 for 24 h. However, *Gardenia jasminoides* downregulated the mRNA levels of TGF-β1 and Col I in LX-2 exposed to TGF-β1 in a dose-dependent manner (Figure 3A and B). The protein expression of α-SMA as detected by Western blotting was also reduced in LX-2 cells after *Gardenia jasminoides* treatment (Figure 3C). Together, these results indicate that *Gardenia jasminoides* exerts its antifibrotic effects in the liver by repressing TGF-β1, Col I and α-SMA expression.

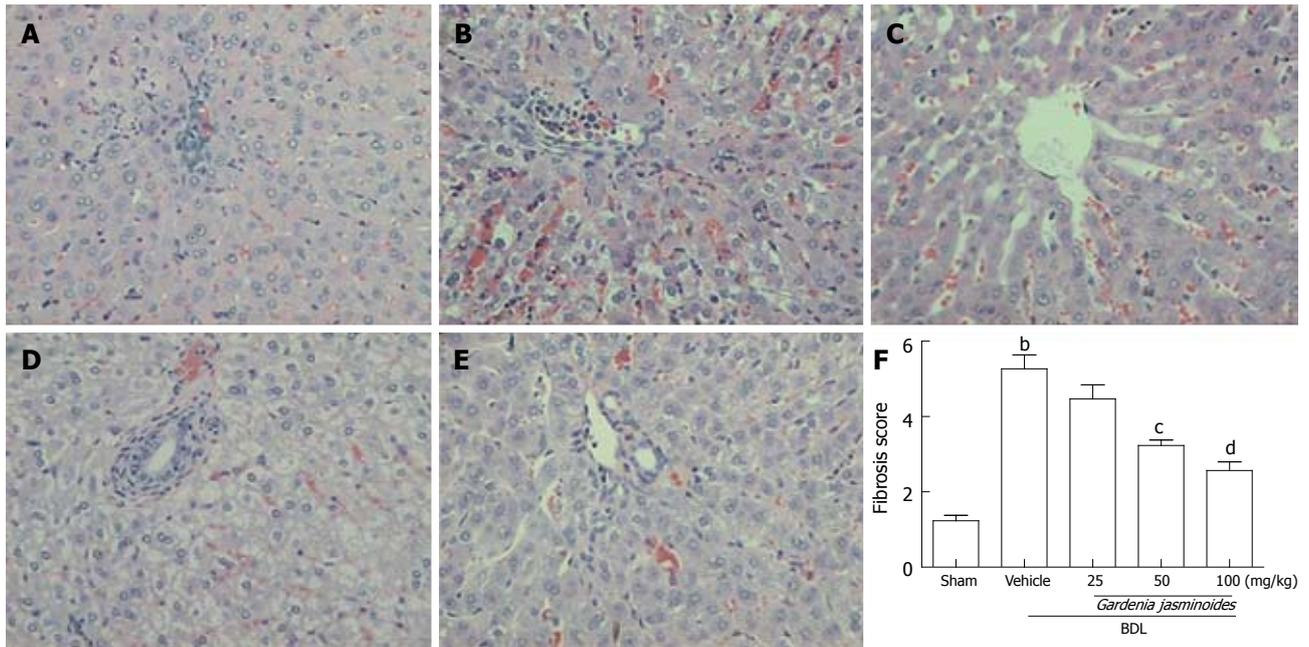


Figure 1 *Gardenia jasminoides* markedly improved the histology in bile duct ligation rats. Representative pictures of hematoxylin and eosin staining (magnification $\times 200$) from rats subjected to bile duct ligation (BDL) or sham-operated rats treated with vehicle or *Gardenia jasminoides*. A: Sham; B: BDL + vehicle; C: BDL + *Gardenia jasminoides* (25 mg/kg per day); D: BDL + *Gardenia jasminoides* (50 mg/kg per day); E: BDL + *Gardenia jasminoides* (100 mg/kg per day); F: Scores of double-blinded assessments of liver histology with respect to fibrosis. ^b $P < 0.01$ vs sham; ^c $P < 0.05$, ^d $P < 0.01$ vs BDL + vehicle ($n = 8$).

***Gardenia jasminoides* reduces Smad2 phosphorylation in TGF- β 1-stimulated LX-2 cells**

Phosphorylated Smad2 plays an important role in the activation of TGF- β 1-induced Col I and α -SMA expression^[29-31]. To determine whether *Gardenia jasminoides* attenuation of Col I and α -SMA expression is mediated through this pathway, we measured Smad2 expression and phosphorylation levels. As shown in Figure 4, *Gardenia jasminoides* significantly reduced Smad2 phosphorylation induced by TGF- β 1 in LX-2 cells without affecting the total amount of Smad2, thereby suggesting that the inhibitory effects of *Gardenia jasminoides* on the expression of Col I and α -SMA is mediated by the blocking of TGF- β 1-stimulated Smad2 phosphorylation.

DISCUSSION

Liver fibrosis is a key risk factor for the development of cirrhosis and chronic liver failure. Activation of HSCs is a crucial component of this process^[11,12]. In the current study, we evaluated the therapeutic effects of *Gardenia jasminoides* *in vivo* in a BDL cholestatic rat model and *in vitro* in human hepatic cells.

Chronic cholestasis leads to liver necrosis, fibrosis, and cirrhosis, partly due to an accumulation of toxic bile acids in the liver^[32]. Therapy with *Gardenia jasminoides* is based on the hepatoprotective properties of YCHT decoctions containing *Gardenia jasminoides*^[22]. Because previous studies have demonstrated that the other two ingredients, *Artemisia capillaris* Thunb and *Rheum officinale* Baill, have hepatoprotective properties, we hypothesized that *Gardenia jasminoides* might also improve hepatic function in patients

with cholestatic liver diseases. As seen in this report, this hypothesis is supported by our experimental results. Biochemical and gene expression analyses demonstrated that elevated markers of liver dysfunction such as ALT and AST were reduced by *Gardenia jasminoides*.

Continuous accumulation of the ECM causes hepatofibrosis. Collagen is the main component of the extracellular matrix in fibrotic tissue^[3]. Hydroxyproline, a major component of collagen, was used as an indicator for evaluation of the degree of liver fibrosis^[33]. *Gardenia jasminoides* treatment (50 and 100 mg/kg) significantly attenuated collagen accumulation as evidenced by the inhibition of BDL-elevated hydroxyproline concentrations and the proportion of fibrotic tissue.

It is well-known that HSCs activation plays a pivotal role in the process of liver fibrosis, and α -SMA is a marker of activated HSCs^[12,34,35]. In the current study, Western blotting indicated that *Gardenia jasminoides* (50 and 100 mg/kg) markedly suppressed the activation of HSCs. To determine whether human cells would show react similarly to *Gardenia jasminoides*, we treated human HSCs cell line, LX-2 cells, with different concentrations of *Gardenia jasminoides*. In contrast to vehicle, *Gardenia jasminoides* decreased the expression of TGF- β 1, Col I and α -SMA. These data are consistent with the results of our *in vivo* study. Together, these results provide compelling evidence supporting the beneficial effects of *Gardenia jasminoides* on the rat model of cholestasis and human liver cells.

Further analysis of this antifibrotic effect suggests that *Gardenia jasminoides* suppressed the expression of Col I and α -SMA *via* the TGF- β 1/Smad2 signaling

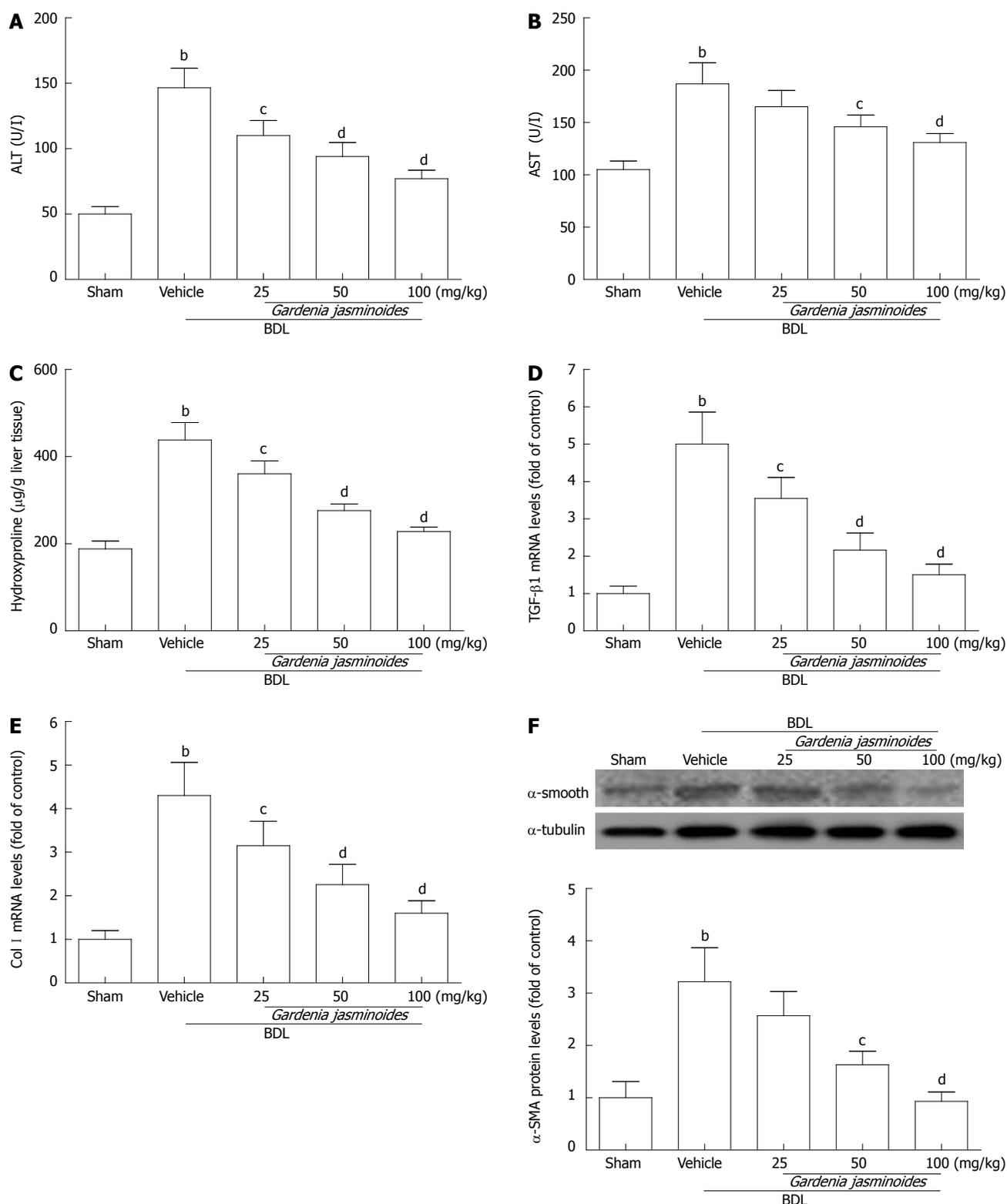


Figure 2 Biochemical and fibrotic gene expression in bile duct ligation rats. *Gardenia jasminoides* significantly improved liver function and reduced the expression of liver fibrosis marker genes from rats submitted to bile duct ligation (BDL) or sham-operated rats treated with vehicle or *Gardenia jasminoides*. A: Serum levels of alanine aminotransferase (ALT); B: Serum levels of aspartate aminotransferase (AST); C: Liver hydroxyproline content; D, E: Liver mRNA expression of transforming growth factor-β1 (TGF-β1) (D) and collagen type I (Col I) (E); F: Liver protein expression of α-smooth muscle actin (α-SMA) detected by Western blotting. ^bP < 0.01 vs sham; ^cP < 0.05, ^dP < 0.01 vs BDL + vehicle (n = 8).

pathway. HSCs are the major target of TGF-β1, which helps to stimulate the transdifferentiation of HSCs into fibrogenic myofibroblasts^[36]. The production of TGF-β1 is upregulated in myofibroblasts and proliferating bile

duct epithelia after BDL, which further contribute to the fibrogenic process in an autocrine/paracrine manner^[37]. Downstream signaling in HSCs involves signaling transcription factors such as Smad2. In addition, TGF-β1 is

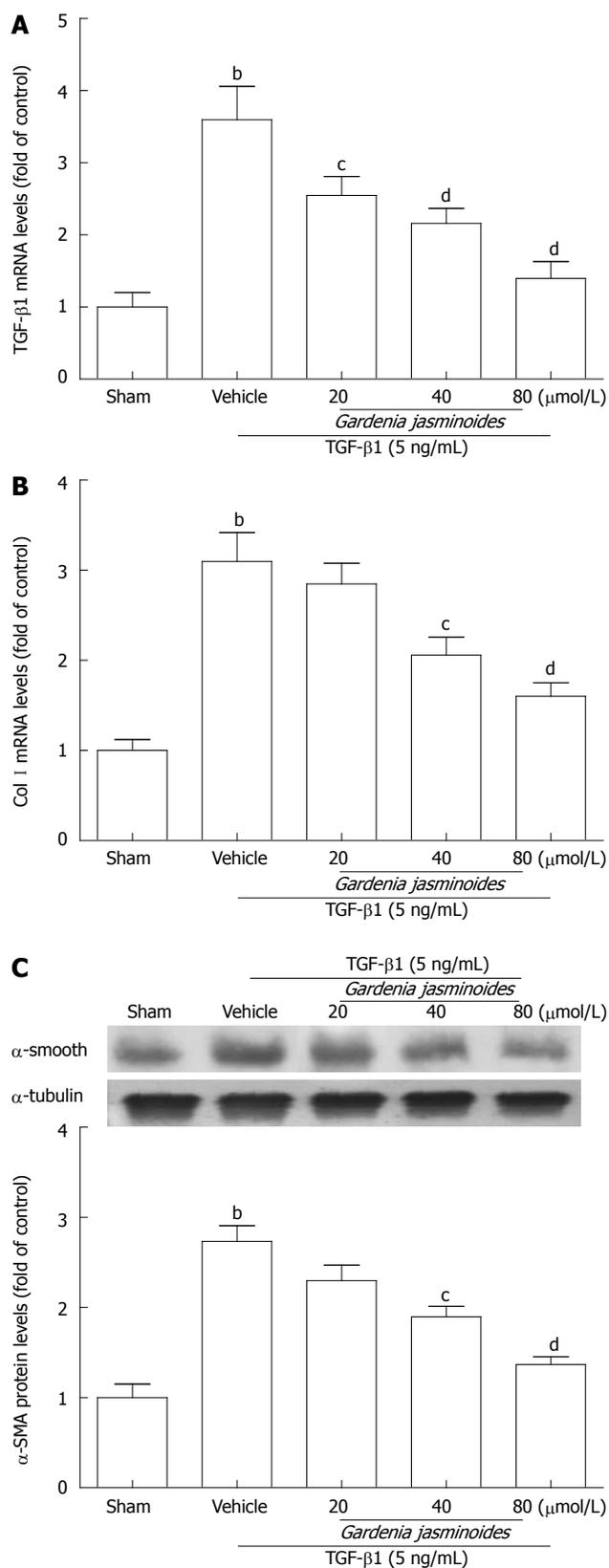


Figure 3 *Gardenia jasminoides* significantly reduced the expression of fibrotic marker genes in a human hepatic stellate cells line. Cells were exposed to transforming growth factor-β1 (TGF-β1) (5 ng/mL) in combination with the indicated concentrations of *Gardenia jasminoides* or vehicle for 24 h. A: mRNA expression of TGF-β1 in LX-2 cells; B: mRNA expression of collagen type I (Col I) in LX-2 cells; C: Western blotting analysis of α-smooth muscle actin (α-SMA) expression in LX-2 cells. ^b*P* < 0.01 vs sham; ^c*P* < 0.05, ^d*P* < 0.01 vs TGF-β1 + vehicle (*n* = 3).

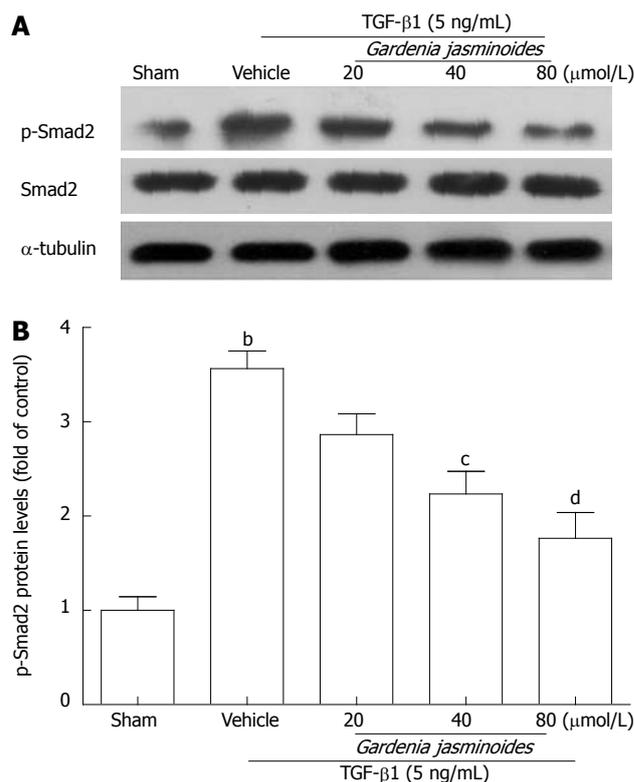


Figure 4 *Gardenia jasminoides* significantly reduced transforming growth factor-β1-induced Smad2 phosphorylation and Smad2 protein expression in LX-2 cells. Cells were exposed to transforming growth factor-β1 (TGF-β1) (5 ng/mL) in combination with the indicated concentrations of *Gardenia jasminoides* or vehicle for 24 h. The expression of p-Smad2 and Smad2 in LX-2 cells was evaluated by Western blotting. ^b*P* < 0.01 vs sham; ^c*P* < 0.05, ^d*P* < 0.01 vs TGF-β1 + vehicle (*n* = 3).

thought to mediate the activation of Smad2 through its phosphorylation^[38]. Here, we found that *Gardenia jasminoides* reduced Smad2 phosphorylation in a dose-dependent manner using LX-2 cells. These findings are consistent with a significant antifibrotic effect of *Gardenia jasminoides* mediated through the inhibition of the TGF-β1/Smad2 pathway. How *Gardenia jasminoides* represses Smad2 phosphorylation remains to be determined.

In summary, we demonstrate that *Gardenia jasminoides* improves the therapeutic response in a rat model of cholestasis and *in vitro* in human hepatic cells. These findings suggest that *Gardenia jasminoides* might be beneficial in patients with chronic cholestatic disorders. To further elucidate the detailed mechanisms, additional comparative studies will be needed to investigate the hepatoprotective of *Gardenia jasminoides* on other liver disease models as well as patients with liver fibrosis.

COMMENTS

Background

Liver fibrosis is a major cause of morbidity and mortality worldwide. However, there are only a few effective antifibrotic therapies for patients with liver fibrosis. Yin-Chen-Hao-Tang (YCHT) decoctions has long been used as antiinflammatory, antipyretic, choleric and diuretic agent for liver disorders and jaundice and several studies provide clinical evidence for its effectiveness in the treatment of various liver disease. However, whether *Gardenia jasminoides*, one of the

components of YCHT decoctions, has anti-fibrotic effect on liver fibrosis and the involved detailed mechanism has not been fully understood yet. In the present study, the anti-hepatofibrotic effects of *Gardenia jasminoides* were evaluated.

Research frontiers

Strategies aimed at disrupting transforming growth factor β 1 (TGF- β 1) synthesis and/or signaling pathways markedly ameliorates liver fibrosis in experimental model. Inhibition of TGF- β 1 signaling pathway may be related to the protective effects of *Gardenia jasminoides* on the bile duct ligation (BDL) rat model *in vivo* and TGF- β 1-stimulated HSCs *in vitro*.

Innovations and breakthroughs

Treatment with *Gardenia jasminoides* decreased serum alanine aminotransferase and aspartate aminotransferase as well as hydroxyproline after BDL. Protective mechanisms of *Gardenia jasminoides* are associated with reduced hepatic mRNA and/or protein expression of TGF- β 1, collagen type I (Col I) and α -smooth muscle actin (α -SMA). *Gardenia jasminoides* significantly suppressed the expression of TGF- β 1, Col I and α -SMA in hepatic stellate cells exposed to recombinant TGF- β 1. Moreover, *Gardenia jasminoides* inhibited TGF- β 1-induced Smad2 phosphorylation in hepatic stellate cells.

Applications

By understanding the effects and mechanism of *Gardenia jasminoides* on liver fibrosis, the present study may present a promising strategy in the treatment of patients with liver fibrosis.

Peer review

The present manuscript describes the effect of *Gardenia jasminoides* extract (YCHT) on the process of fibrosis that develops in rat liver after ligation of the common bile duct. This Chinese herbal medicine reduced the levels of serum transaminases, hydroxyproline, TGF- β 1 (and its mRNA), collagen type 1 and α -smooth muscle actin. The treatment also decreases the TGF- β 1-induced Smad2 phosphorylation in a human stellate cell line LX-2. This study clearly demonstrates that treatment of fibrosis, even by classical medical treatment has an effect.

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Oridonin induces apoptosis in gastric cancer through Apaf-1, cytochrome c and caspase-3 signaling pathway

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Abstract

AIM: To investigate the effect and mechanism of oridonin on the gastric cancer cell line HGC-27 *in vitro*.

METHODS: The inhibitory effect of oridonin on HGC-27 cells was detected using the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. After treatment with 10 $\mu\text{g}/\text{mL}$ oridonin for 24 h and 48 h, the cells were stained with acridine orange/ethidium bromide. The morphologic changes were observed under an inverted fluorescence microscope. DNA fragmen-

tation (a hallmark of apoptosis) and lactate dehydrogenase activity were examined using DNA ladder assay and lactate dehydrogenase-release assay. After treated with oridonin (0, 1.25, 2.5, 5 and 10 $\mu\text{g}/\text{mL}$), HGC-27 cells were collected for annexin V-phycoerythrin and 7-amino-actinomycin D double staining and tested by flow cytometric analysis, and oridonin-induced apoptosis in HGC-27 cells was detected. After treatment with oridonin for 24 h, the effects of oridonin on expression of Apaf-1, Bcl-2, Bax, caspase-3 and cytochrome c were also analyzed using reverse-transcript polymerase chain reaction (RT-PCR) and Western blotting.

RESULTS: Oridonin significantly inhibited the proliferation of HGC-27 cells in a dose- and time-dependent manner. The inhibition rates of HGC-27 treated with four different concentrations of oridonin for 24 h (1.25, 2.5, 5 and 10 $\mu\text{g}/\text{mL}$) were $1.78\% \pm 0.36\%$, $4.96\% \pm 1.59\%$, $10.35\% \pm 2.76\%$ and $41.6\% \pm 4.29\%$, respectively, which showed a significant difference ($P < 0.05$). The inhibition rates of HGC-27 treated with oridonin at the four concentrations for 48 h were $14.77\% \pm 4.21\%$, $21.57\% \pm 3.75\%$, $30.31\% \pm 4.91\%$ and $61.19\% \pm 5.81\%$, with a significant difference ($P < 0.05$). The inhibition rates of HGC-27 treated with oridonin for 72 h at the four concentrations were $25.77\% \pm 4.85\%$, $31.86\% \pm 3.86\%$, $48.30\% \pm 4.16\%$ and $81.80\% \pm 6.72\%$, with a significant difference ($P < 0.05$). Cells treated with oridonin showed typical apoptotic features with acridine orange/ethidium bromide staining. After treatment with oridonin, the cells became round, shrank, and developed small buds around the nuclear membrane while forming apoptotic bodies. Lactate dehydrogenase (LDH) release assay showed that after treated with 1.25 $\mu\text{g}/\text{mL}$ and 20 $\mu\text{g}/\text{mL}$ oridonin for 24 h, LDH release of HGC-27 caused by apoptosis increased from $22.94\% \pm 3.8\%$ to $52.68\% \pm 2.4\%$ ($P < 0.001$). However, the change in the release of LDH caused by necrosis was insignificant, suggesting that

the major cause of oridonin-induced HGC-27 cell death was apoptosis. Flow cytometric analysis also revealed that oridonin induced significant apoptosis compared with the controls ($P < 0.05$). And the apoptosis rates of HGC-27 induced by the four different concentrations of oridonin were $5.3\% \pm 1.02\%$, $12.8\% \pm 2.53\%$, $28.5\% \pm 4.23\%$ and $49.6\% \pm 3.76\%$, which were in a dose-dependent manner ($P < 0.05$). After treatment for 24 h, DNA ladder showed that oridonin induced a significant increase in DNA fragmentation in a dose-dependent manner. RT-PCR revealed that mRNA expression levels were up-regulated compared with the controls in caspase-3 (0.917 ± 0.103 vs 0.357 ± 0.019 , $P < 0.05$), cytochrome c (1.429 ± 0.111 vs 1.002 ± 0.014 , $P < 0.05$), Apaf-1 (0.688 ± 0.101 vs 0.242 ± 0.037 , $P < 0.05$) and Bax (0.856 ± 0.101 vs 0.278 ± 0.027 , $P < 0.05$) ($P < 0.05$), whereas down-regulated in Bcl-2 (0.085 ± 0.012 vs 0.175 ± 0.030 , $P < 0.05$). Western blotting analysis also confirmed this result.

CONCLUSION: Apoptosis of HGC-27 induced by oridonin may be associated with differential expression of Apaf-1, caspase-3 and cytochrome c, which are highly dependent upon the mitochondrial pathway.

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Key words: Oridonin; Gastric cancer; Proliferation; Apoptosis; Apaf-1/caspase-3/cytochrome C

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INTRODUCTION

Gastric cancer is a common cancer of the digestive system and the second most common type of cancer worldwide. Its incidence varies among different regions and countries. There are approximately 934 000 new cases of gastric cancer worldwide each year, of which 56% occur in East Asia. Among these new cases in East Asia, 41% come from China and 11% from Japan^[1,2]. Most patients

present with advanced stage. Surgery plus chemotherapy remains the first-line therapy. Despite rapid advances in surgical procedures and radiotherapy/chemotherapy, few chemotherapeutic drugs for gastric cancer have shown promising results. Treatment efficacies vary from person to person, with a prevalence of cure of $< 30\%$ ^[3]. Therefore, it is important to search and develop new and more effective anti-gastric cancer drugs. In recent years, traditional Chinese medicine (TCM) has played an increasingly important role in the prevention and treatment of tumors. In particular, the integration of TCM with Western medicine has appreciably improved the efficacy of drug combinations and prolonged patient survival.

Rabdosia rubescens, a medicinal herb in TCM, has therapeutic actions (e.g., heat-clearing, detoxifying, anti-inflammation, antinociceptive, anti-tumor). Oridonin (molecular formula: $C_{20}H_{20}O_6$; relative molecular weight: 364.42) is a tetracyclic diterpenoid compound. It is a monomer component extracted from *Rabdosia rubescens*. In China, structural and pharmacological studies on *Rabdosia rubescens* started in the mid 1980s. Results indicated that oridonin is one of the most important anti-tumor components of *Rabdosia rubescens*^[4,5]. Studies have suggested that oridonin has certain anti-tumor effects on cervical cancer, human epidermal squamous cell carcinoma, leukemia, liver cancer, malignant melanoma, colon cancer, breast cancer, and other tumors^[6-12]. A study on colon cancer found that the mechanism of oridonin-induced apoptosis and aging of colon cancer cells might lie in increased histone acetylation and changes in the expressions of p16, p21, p27 and c-myc^[13]. A study on laryngeal cancer indicated that oridonin can induce apoptosis through inhibiting the epidermal growth factor receptor signaling pathway and by increasing oxidative stress^[14]. Oridonin can induce the apoptosis of hepatoma cells through the reactive oxygen species-mitogen-activated protein kinase-p53 pathway^[15]. In another study on cervical cancer, oridonin induced the apoptosis of cervical cancer cells through the phosphatidylinositol 30-kinase-Akt pathway^[16]. In addition, some recent studies suggested that oridonin can also inhibit the proliferation of tumor cells by increasing the autophagy of tumor cells^[17-19]. All of these findings suggest that oridonin has good anti-tumor effects.

Our previous studies on gastric cancer also suggested that oridonin has a notable inhibitory effect on the proliferation of gastric cancer cells^[20]. However, the exact mechanism by which oridonin induces the apoptosis of gastric cancer cells remains undefined. In this study, the effect of oridonin on gastric cancer cells and its possible mechanism of action were explored.

MATERIALS AND METHODS

Experimental reagents

The human gastric cancer cell line HGC-27 as well as the instruments and equipments needed for this study were provided by the Zhejiang Provincial Key Laboratory of Gastroenterology (Zhejiang, China). Roswell Park Memo-

rial Institute (RPMI) 1640 medium was purchased from the Shanghai Pufei Biotech (Shanghai, China). Fetal bovine serum (FBS), 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, acridine orange/ethidium bromide (AO/EB) fluorescent dye and lactate dehydrogenase (LDH) test reagents were purchased from YK Biotech (Hangzhou, China). Bcl-2, Bax, Apaf-1, caspase-3 and β -actin primers were synthesized by Invitrogen (Shanghai, China). Trizol RNA extraction reagent was purchased from Invitrogen (Carlsbad, CA, United States). Taq DNA polymerase and PrimeScript™ reverse-transcript polymerase chain reaction (RT-PCR) Kit and other PCR reagents were purchased from TaKaRa Corporation (Osaka, Japan).

Cell culture

HGC-27 cells were routinely cultivated in RPMI 1640 medium containing 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin at 37 °C with 5% CO₂. Cells were passaged at 80% confluency using 1 mmol/L ethylene diamine tetraacetic acid (EDTA)-0.025% trypsin for 3-5 min, and subcultured at a ratio of 1:3-1:5. Cells at the logarithmic growth phase were collected for experiments.

Determination of inhibition of cell growth

HGC-27 cells at the logarithmic growth phase were obtained. The cell concentration was adjusted to 4×10^4 /mL. Then, 200 μ L of the above-mentioned cell suspension was added into each well of several 96-well plates. When cells adhered to the wall, the stock solution of oridonin (50 mg/mL) was added into each well to obtain final concentrations of oridonin of 0, 1.25, 2.5, 5 and 10 μ g/mL. Five parallel wells were established for each drug concentration. After each well was removed from the incubator after different time periods of cultivation (based on experimental design), 20 μ L MTT [prepared using 5 mg/mL phosphate-buffered saline (PBS) at pH 7.4] was added. The well was removed again after 4 h of cultivation. The supernatant was aspirated carefully; 150 μ L dimethyl sulfoxide was added to each well and homogenized. A Multi-Mode Microplate Reader (Infinite M200; Tecan, Geneva, Switzerland) was used to determine the absorbance (A), with a detection wavelength of 570 nm and a reference wavelength of 630 nm. The inhibition of cell growth was calculated using the following formula:

$$\text{Inhibition (\%)} = [1 - (A_{\text{treated}} - A_{\text{blank}}) / (A_{\text{control}} - A_{\text{blank}})] \times 100\%$$

Determination of LDH activity

HGC-27 cell culture media treated with oridonin (0, 1.25, 2.5, 5, 10 and 20 μ g/mL) for 24 h were collected. After centrifugation of the cell culture medium at 1000 g for 5 min at 4 °C, supernatants were carefully aspirated: these were marked "LDH_{necrosis}". Precipitated cells were rinsed three times in PBS (pH 7.4), and then lysed with 0.4% Triton X-100 for 30 min on ice. Cell lysate supernatants were centrifuged at 3000 g for 5 min at 4 °C: these were marked "LDH_{apoptosis}". Residual adherent HGC-27 cells were collected after treatment with different concentra-

tions of oridonin using 0.4% Triton X-100 for 30 min on ice. Cell lysate supernatants were centrifuged at 3000 g for 5 min at 4 °C: these were marked "LDH_{live}". LDH levels were measured in the three supernatants mentioned above (LDH_{necrosis}, LDH_{apoptosis} and LDH_{live}) on a fully automatic biochemical analyzer (LX20; Beckman Coulter, Brea, CA, United States). LDH levels in supernatants (LDH_{necrosis} and LDH_{apoptosis}) were applied for the analysis of oridonin-induced necrosis and apoptosis of HGC-27 cells. The percentages of apoptosis and necrosis were calculated using the following formulae:

$$\text{Necrosis (\%)} = [\text{LDH}_{\text{necrosis}} / (\text{LDH}_{\text{necrosis}} + \text{LDH}_{\text{apoptosis}} + \text{LDH}_{\text{live}})] \times 100\%$$

$$\text{Apoptosis (\%)} = [\text{LDH}_{\text{apoptosis}} / (\text{LDH}_{\text{necrosis}} + \text{LDH}_{\text{apoptosis}} + \text{LDH}_{\text{live}})] \times 100\%$$

DNA fragmentation (DNA ladder) assay

HGC-27 cells (including suspended cells and adherent cells) were collected after treatment with oridonin (0, 1.25, 2.5, 5, 10 μ g/mL) for 24 h. They were rinsed three times in PBS at 4 °C. This was followed by degradation using 100 μ g cell lysates (1% NP-40, 20 mmol/L EDTA, 50 mmol/L Tris-HCl, pH 7.5) at 4 °C for 10 min, followed by centrifugation at 15 000 g for 20 min. RNase A (20 μ g/mL) was added to the supernatant at 37 °C for 1 h. Then 20 μ L 0.5 mol/L NaCl and 120 μ L 50% isopropanol were added and the mixture left overnight at -20 °C. The supernatant was removed after centrifugation at 15 000 g for 15 min at 4 °C. The supernatant was allowed to dry naturally and was dissolved in TE buffer (10 mmol/L Tris-HCl pH 7.4, 10 mmol/L EDTA pH 8.0), followed by electrophoresis at 100 V for 40 min using 0.1 mg/L, 2% agarose gels. A gel imaging system (FluorChem FC2; Alpha Innotech, Palo Alto, CA, United States) was used for observation and taking photographs.

Flow cytometric analysis

HGC-27 cells were treated by oridonin (0, 1.25, 2.5, 5, 10 μ g/mL) as describe above and the cells were collected for annexin V-phycoerythrin and 7-amino-actinomycin D double staining. Briefly, the cells were washed with PBS for three times, and stained according to the manufacturer's instructions (Guava Nexin® Reagent kit, 4700-1140, Millipore, United States). Samples were then analyzed using Guava EasyCyte Plus (Millipore, United States) within 30 min after the staining.

Cell morphology

AO/EB apoptotic staining was used to detect the morphology of apoptotic cells. After treatment with 10 μ g/mL oridonin for 24 h and 48 h, the cells were washed three times in PBS at room temperature. The 80 μ L AO/EB cocktail (Solomon Bio-Sci and Tech Co, China) was added to the culture plates for 30 min prior to observation under an inverted fluorescence microscope (IX71; Olympus, Tokyo, Japan). Viable cells stained only by AO appeared bright green with intact structure, whereas cells in early apoptosis showed bright green nuclear staining. Late apop-

Table 1 Primers used in the study

	Primer (forward)	Primer (reverse)	Product size (bp)
β-actin	5-CGGGACCTGACTGACTACCTC	5-GGACTCGTGATACTCCTGCTTG	500
Apaf-1	5-TTAGGAGCCAGGTGCGGT	5-GCTTGTCTTTCTCCCATTTTC	148
Bcl-2	5-TCGCCCTGTGGATGACTG	5-CAGGAGAAATCAAACAGAGGC	124
Caspase-3	5-CATCCAGTCGCTTTGTGCC	5-TGCCACAGATGCCTAAGTTC	619
Bax	5-CCCAGAGGTCCTTTTTC	5-GCCTTGAGCACCAGTTG	108
Cytochrome c	5-GAGCGGGAGTGTTCGTTGT	5-GTCTGCCCTTCTTCCTTCT	327

otic cells stained by AO low and EB were red-orange with condensation of chromatin as dense orange areas. The experiment was repeated three times in each group.

Semi-quantitative RT-PCR analysis

HGC-27 cells were collected after treatment with 10 µg/mL oridonin for 24 h. Total cellular RNA was extracted using the Trizol Reagent Kit according to manufacturer's instructions. The concentration and quality of total RNA extracted were confirmed using a Protein-Nucleic Acid Analyzer (GeneQuant Pro DNA/RNA; GE Healthcare, Piscataway, NJ, United States) and RNA electrophoresis. cDNA synthesis and PCR detection were conducted using a PCR instrument (PTC-200; Bio-Rad, Hercules, CA, United States) according to the instructions given for the PrimeScript™ RT-PCR Kit. Primers were designed using the freely available primer design software Primer Premier 5.0. The primers of β-actin, Apaf-1, Bcl-2, Bax, caspase-3 and cytochrome c are shown in Table 1.

The reaction conditions were as follows: 94 °C for 4 min; 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 45 s, for 30 cycles; and followed by extension at 72 °C for 10 min before ending. Electrophoresis (1% agarose gel, 120 V for 30 min) was carried out. The gel imaging system (FluorChem FC2; Alpha Innotech) was used for observation and taking photographs.

Western blotting verification

Gastric cancer cell samples were homogenized in lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L, 1% Triton X-100, and 100 µg/mL Phenylmethanesulfonyl fluoride). The concentration of total protein was quantitated by bicinchoninic acid method. Sixty µg total proteins from each sample were loaded on a 15% sodium dodecylsulfonate-polyacrylate gel electrophoresis gel and the proteins were transferred to a polyvinylidene fluoride membrane (Bio-Rad, United States). Blotted membranes were blocked in 5% bovine serum albumin and subsequently exposed to primary antibodies specific for Apaf-1 (1:500, sc65891, Santa Cruz, United States), Bcl-2 (1:300, sc7382, Santa Cruz, United States), Bax (1:300, sc70406, Santa Cruz, United States), caspase-3 (1:300, sc7272, Santa Cruz, United States) and cytochrome c (1:800, sc13156, Santa Cruz, United States), respectively. After incubation with the appropriate secondary antibody, the membranes were treated with electrochemiluminescence reagent (Generay, China) and exposed to autoradiographic films. Beta-actin was also detected as an internal control.

Statistical analysis

The experiments were repeated three times independently. Data were presented as the mean ± SD. Data were analyzed using SPSS software ver13.0 (SPSS, Chicago, IL, United States). If the results were distributed normally, the two independent samples *t* test was used for comparison. For comparisons between groups of more than two unpaired values, one-way analysis of variance (ANOVA) was used. If an ANOVA *F* value was significant, post-hoc comparisons were performed between groups. If results were not normally distributed, the Mann-Whitney *U* test was used to compare two groups of unpaired values, whereas for comparisons between groups of more than two unpaired values, the Kruskal-Wallis *H* test was used. *P* < 0.05 was considered significant.

RESULTS

Inhibitory effect of oridonin on growth of HGC-27 cells

The HGC-27 cell-growth inhibition rate after treatment with different concentrations of oridonin (0, 1.25, 2.5, 5, 10 µg/mL) is shown in Figure 1A. The inhibition rates of HGC-27 treated with the four different concentrations of oridonin for 24 h (1.25, 2.5, 5, 10 µg/mL) were 1.78% ± 0.36%, 4.96% ± 1.59%, 10.35% ± 2.76% and 41.6% ± 4.29%, with a significant difference (*P* < 0.05). The inhibition rates of HGC-27 treated with oridonin for 48 h at the four concentrations were 14.77% ± 4.21%, 21.57% ± 3.75%, 30.31% ± 4.91% and 61.19% ± 5.81%, with a significant difference (*P* < 0.05). The inhibition rates of HGC-27 treated with oridonin for 72 h at the four concentrations were 25.77% ± 4.85%, 31.86% ± 3.86%, 48.30% ± 4.16% and 81.80% ± 6.72%, respectively, with a significant difference (*P* < 0.05). As the drug concentration increased, HGC-27 cell-growth inhibition was gradually enhanced.

Oridonin induced apoptosis of HGC-27 cells

HGC-27 cells were tested by LDH release assay after treated with oridonin for 24 h, and we found that apoptosis-induced LDH release increased from 22.94% ± 3.8% at 1.25 µg/mL to 52.68% ± 2.4% at 20 µg/mL (*P* < 0.001). However, the change in the release of LDH caused by necrosis was insignificant (*P* > 0.05, Figure 1B), suggesting that the major cause of oridonin-induced HGC-27 cell death was apoptosis.

DNA of HGC-27 cells was also extracted and tested by DNA ladder analysis after treatment with oridonin for 24

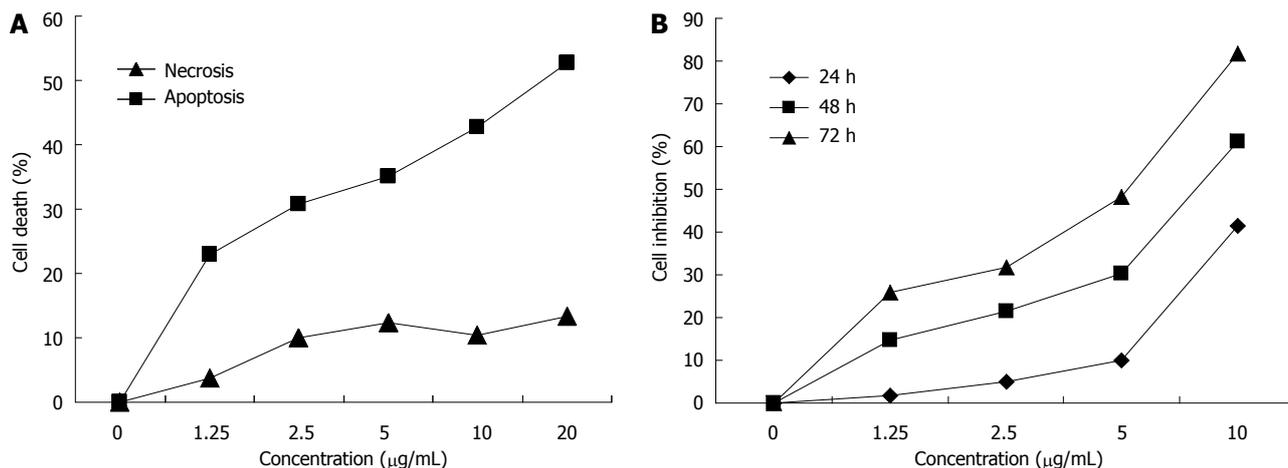


Figure 1 Inhibition of growth and lactate dehydrogenase release assay of HGC-27 cells after treatment with different concentrations of oridonin. A: Inhibition of growth HGC-27 cells; B: Lactate dehydrogenase (LDH) release assay of HGC-27 cells. The change in the release of LDH caused by apoptosis was significant.

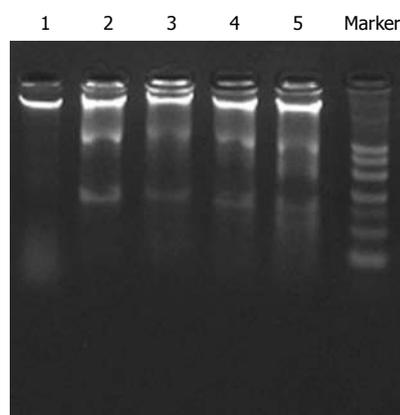


Figure 2 DNA ladder diagram after treatment with different concentrations of oridonin for 24 h. 1: Control; 2: 1.25 μg/mL oridonin; 3: 2.5 μg/mL oridonin; 4: 5 μg/mL oridonin; 5: 10 μg/mL oridonin.

h. As shown in Figure 2, oridonin induced a significant increase in DNA fragmentation in a dose-dependent manner.

Flow cytometric analysis also revealed that oridonin could induce significant apoptosis compared with the controls ($P < 0.05$, Figure 3). And the apoptosis rates of HGC-27 induced by oridonin at the four concentrations were $5.3\% \pm 1.02\%$, $12.8\% \pm 2.53\%$, $28.5\% \pm 4.23\%$ and $49.6\% \pm 3.76\%$, respectively, which were in a dose-dependent manner ($P < 0.05$).

Apoptotic morphology of HGC-27 cells after treated with oridonin

Apoptotic morphology was further observed under light microscopy and we found that, in the negative control group, HGC-27 cells were closely connected and uniform in size (Figure 4A). After treatment with oridonin, the cells became round (Figure 4B, black arrow), shrank, and developed small buds around the nuclear membrane while forming apoptotic bodies (Figure 4C, black arrow). AO/EB staining showed that, in the negative control group, cells were closely connected, uniform in size, and green in color (Figure 4D). After treatment with oridonin,

the typical features of cells at different phases of apoptosis could be seen. Live cytoplasm and nuclei appeared all green. Early apoptotic nuclei were yellowish-green and pyknotic, and the cytoplasm was stained green. In the mid-phase and late apoptosis, nuclei were pyknotic, and nuclei and cytoplasm were yellowish-green. Dying cells were pyknotic and red-orange (Figure 4E and F).

Effect of oridonin on HGC-27-induced gene expression

After treatment with oridonin for 24 h, gray ratio analyses of RT-PCR revealed that mRNA expression was up-regulated compared with control in caspase-3 (0.917 ± 0.103 vs 0.357 ± 0.019 , $P < 0.05$), cytochrome c (1.429 ± 0.111 vs 1.002 ± 0.014 , $P < 0.05$), Apaf-1 (0.688 ± 0.101 vs 0.242 ± 0.037 , $P < 0.05$) and Bax (0.856 ± 0.101 vs 0.278 ± 0.027 , $P < 0.05$), whereas down-regulated in Bcl-2 (0.085 ± 0.012 vs 0.175 ± 0.030 , $P < 0.05$). These findings suggested that oridonin-induced apoptosis of HGC-27 cells was correlated with changes in the expression of caspase-3, cytochrome c, Apaf-1, Bcl-2 and Bax. Agarose gel electrophoresis of the RT-PCR products is shown in Figure 5A, and the ratios between the indicators for the three PCR analyses and optical density of β-actin are shown in Figure 5B. Protein levels analyzed by Western blotting also confirmed this result (Figure 5C).

DISCUSSION

Apoptosis plays a key part in the evolution of organisms, homeostasis, and development of multiple systems, including cancer. Tumorigenesis occurs when a series of oncogenes and proto-oncogenes are activated and overexpressed within tumor cells. The genes and their products are important regulators of apoptosis. Their abnormal expression blocks the apoptotic process of tumor cells, increasing the number of tumor cells, thereby promoting tumor growth. The typical morphological features of apoptotic cells are: cell shrinkage; in some organelles, ribosomes and nuclear debris are “wrapped” by the cell membrane into apoptotic bodies, which bud off from the

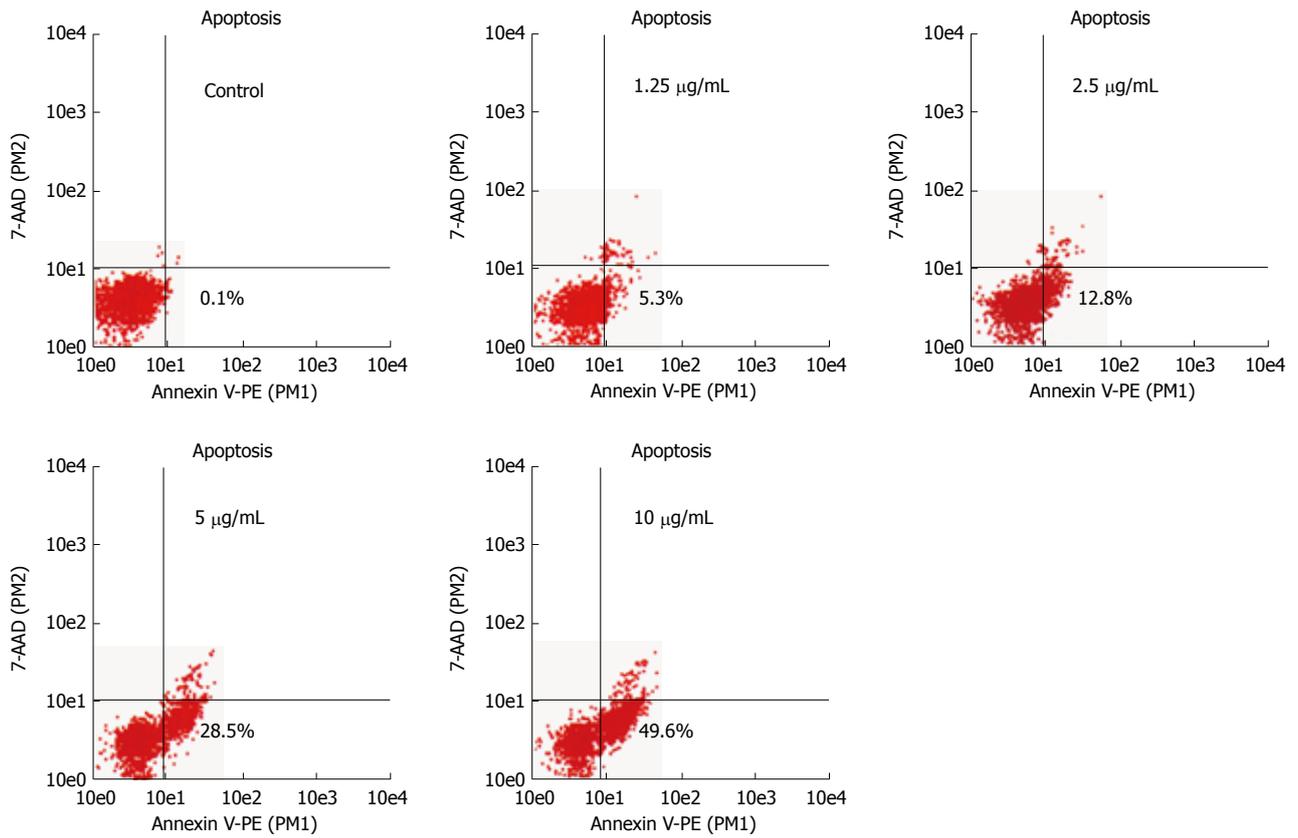


Figure 3 Analysis of apoptosis in HGC-27 cells. Flow cytometric analysis showed that oridonin induced the apoptosis of HGC-27 cells in a dose dependent manner. The x-axis indicates the Annexin V-positive populations and the y-axis indicates the 7-AAD-positive populations. The lower right was the early apoptotic cells.

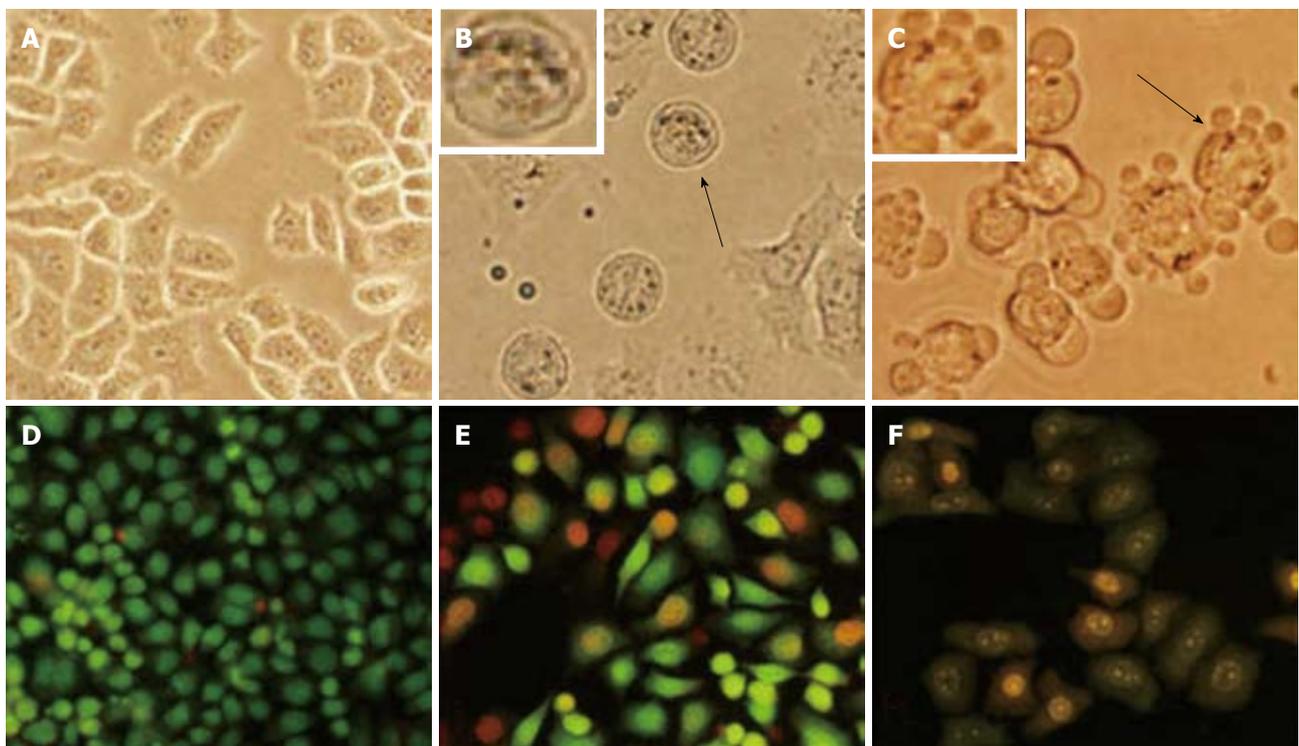


Figure 4 Morphological changes in HGC-27 cells after treatment with 10 µg/mL oridonin for 24 h and 48 h. A: Negative; B: Treatment for 24 h; C: Treatment for 48 h; D: Acridine orange/ethidium bromide (AO/EB) staining negative; E: Treatment for 24 h and AO/EB staining; F: Treatment for 48 h and AO/EB staining. Magnification ×200 in all images.

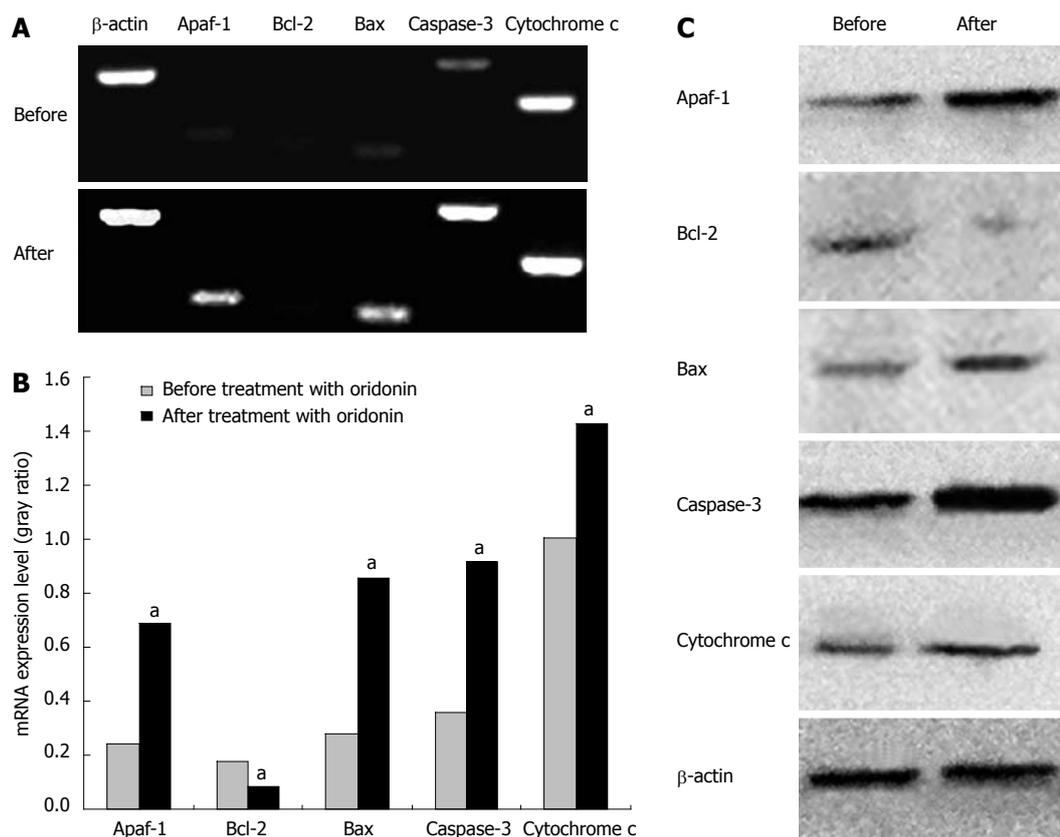


Figure 5 Changes in gene expression after treatment with 10 $\mu\text{g/mL}$ oridonin for 24 h. After treatment with oridonin for 24 h, the expression of caspase-3, cytochrome c, Apaf-1 and Bax was up-regulated, whereas that of Bcl-2 was down-regulated. A: Agarose gel electrophoresis of the reverse-transcript polymerase chain reaction products; B: Results of optical density analyses of Apaf-1/ β -actin, Bcl-2/ β -actin, Bax/ β -actin, caspase-3/ β -actin, and cytochrome c/ β -actin before and after treatment with oridonin (paired *t* test, ^a $P < 0.05$ vs before treatment with oridonin); C: Western blotting analysis.

cell surface and are finally “swallowed” by macrophages, epithelial cells and other phagocytic cells; phosphatidylserine eversion; condensation and marginalization of nuclear chromatin; and DNA fragmentation. Important molecules involved in apoptosis are: apoptosis-promoting molecules such as cysteine containing the aspartate-specific protease (caspase) family, and cytochrome c; and apoptosis-inhibitory molecules such as the Bcl-2 family. Apoptotic signaling pathways mainly involve the cell membrane receptor pathway [e.g., Fas/Fas ligand and tumor necrosis factors (TNF)/TNF receptor] or mitochondrial pathway conduction, which activates the caspases (caspase-8 or caspase-9) and key molecules of the downstream signal transduction pathway in succession to initiate caspase (caspase-3) and start apoptosis. In addition, apoptosis can also start in a caspase-independent manner^[21,22].

The Bcl-2 family contains molecules that can regulate apoptosis^[23]. These can be divided into two main categories: anti-apoptotic genes (particularly Bcl-2) and pro-apoptotic genes (particularly Bax). Bcl-2 can form protein dimers with the pro-apoptotic protein Bax and plays a decisive role in the apoptosis signaling pathway. If the Bcl-2/Bax ratio is decreased, apoptosis occurs^[24]. Changes in the expression of the Bcl-2 protein family lead to increased permeability of the outer membrane of mitochondria, thus triggering mitochondrial release of cy-

tochrome c. Apaf-1 can activate caspase-3. If cytochrome c is released into the cytoplasm, it forms the Apaf-1/cytochrome c complex with Apaf-1. After the Apaf-1/cytochrome c complex binds with ATP/dATP, Apaf-1 can “call” caspase-9 through its CARD domain to form apoptotic bodies, activate caspase-3, and start the caspase cascade reaction, thereby leading to apoptosis^[25].

Recent studies have shown that the anti-tumor activity of oridonin is related to its induction of apoptosis^[16,26-28]. In the present study, the MTT assay initially confirmed that oridonin can have a notable inhibitory effect on the proliferation of HGC-27 cells in a dose- and time-dependent manner. Our previous studies also suggested that oridonin has a notable inhibitory effect on the proliferation of gastric cancer^[20]. Previous studies also found the growth-inhibitory activity of oridonin on cancer cells^[29,30]. Light microscopy and AO/EB staining revealed that, after treatment with oridonin, HGC-27 cells became round, shrank, developed pyknosis, and formed small buds around the nuclear membrane as well as apoptotic bodies. The LDH release assay showed that oridonin could induce the death of gastric cancer cells mainly through its induction of apoptosis, and that the apoptotic effect was enhanced significantly as the concentration increased. These results were consistent with previous reports^[31-34]. DNA ladder analyses demonstrated that, after treatment

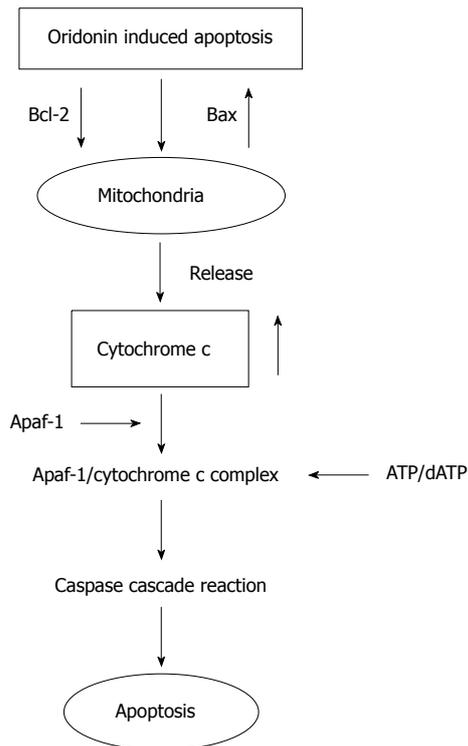


Figure 6 Possible mechanism by which oridonin induces the apoptosis of HGC-27 cells.

with different concentrations of oridonin, obvious DNA fragmentation could be seen. This finding indicated that oridonin could inhibit the proliferation of HGC-27 cells and induce their apoptosis. To further study the molecular mechanism of oridonin-induced apoptosis, semi-quantitative RT-PCR and Western blotting analysis were conducted to observe changes in expression of caspase-3, cytochrome c, Apaf-1, Bax and Bcl-2 mRNA after treatment with oridonin. Expression of caspase-3, cytochrome c, Apaf-1 and Bax was up-regulated, whereas that of Bcl-2 was down-regulated. The results showed that oridonin could inhibit the proliferation of HGC-27 cells, and that this effect was related to its induction of tumor-cell apoptosis. Liu *et al.*^[35] also found that oridonin induced a decrease in Bcl-2/Bax ratio and activation of caspase-3. Zhang *et al.*^[36] reported that regulation of the Bcl-2 and MAPK families may be the effector mechanisms of oridonin-induced L929 cell death, independent of the caspase pathway. So we speculated that oridonin may change expression of Bcl-2 and Bax, and then trigger the release of cytochrome c through the mitochondrial pathway to further activate the caspase cascade reaction and induce HGC-27 apoptosis (Figure 6).

In conclusion, oridonin significantly inhibited the proliferation and promoted apoptosis of gastric cancer cell line HGC-27. And the apoptosis of HGC-27 induced by oridonin may be associated with differential expression of Apaf-1, caspase-3 and cytochrome c, which are highly dependent upon the mitochondrial pathway.

COMMENTS

Background

Gastric cancer is a common cancer of the digestive system and the second most common type of cancer worldwide. It is important to seek and develop new and more effective anti-gastric cancer drugs. *Rabdosia rubescens*, a medicinal herb, has therapeutic actions. Studies have suggested that oridonin has certain anti-tumor effects in many kinds of tumors. However, the exact mechanism by which oridonin induces the apoptosis of gastric cancer cells remains undefined. In this study, the effect of oridonin on gastric cancer cells and its possible mechanism of action were explored.

Research frontiers

Studies have suggested that oridonin has certain anti-tumor effects on cervical cancer, human epidermal squamous cell carcinoma, leukemia, liver cancer, malignant melanoma, colon cancer, breast cancer, and other tumors. It has been found that the mechanism of oridonin-induced apoptosis and aging of colon cancer cells might lie in increased histone acetylation and changes in the expressions of p16, p21, p27 and c-myc. Oridonin can induce the apoptosis of hepatoma cells through the reactive oxygen species-mitogen-activated protein kinase-p53 pathway. In addition, some recent studies suggested that oridonin can also inhibit the proliferation of tumor cells by increasing the autophagy of tumor cells. All of these findings suggest that oridonin has good anti-tumor effects.

Innovations and breakthroughs

In the present study, the authors found that oridonin significantly inhibited the proliferation and promoted apoptosis of gastric cancer cell line HGC-27. And the apoptosis of HGC-27 induced by oridonin may be associated with differential expression of Apaf-1, caspase-3 and cytochrome c. The authors speculated that oridonin may change expression of Bcl-2 and Bax, and then trigger the release of cytochrome c through the mitochondrial pathway to further activate the caspase cascade reaction and induce HGC-27 apoptosis.

Applications

Oridonin possesses potent anti-gastric cancer activities associated with inhibition of proliferation and regulation of pathways critical for maintaining apoptosis induction. These results may lay the groundwork for further studies to establish the causal relationship between oridonin anti-tumor activity and specific genetic pathways and to identify molecular markers that will guide the development of future clinical therapies. Therefore, oridonin may represent a novel therapeutic option for gastric cancer.

Terminology

Gastric cancer is still the second leading cause of cancer-related death worldwide, particularly in Asian countries. Traditional Chinese medicine (TCM) has played an increasingly important part in the prevention and treatment of tumors. In particular, integration of TCM with Western medicine has appreciably improved the efficacy of drug combinations and prolonged patient survival. Oridonin (molecular formula: $C_{20}H_{20}O_6$; relative molecular weight: 364.42) is a tetracyclic diterpenoid compound, which was extracted from *Rabdosia rubescens*.

Peer review

The authors demonstrated the effect and mechanism of oridonin on HGC-27 gastric cancer cell line and suggested that oridonin may represent a novel therapeutic option for gastric cancer.

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Age-related symptom and life quality changes in women with irritable bowel syndrome

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Abstract

AIM: To explore age-related changes in symptoms and quality of life (QoL) of women with irritable bowel syndrome (IBS).

METHODS: Two-hundred and fifty-four female adult outpatients with IBS attending the Department of Gastroenterology at the First Affiliated Hospital of Nanjing Medical University between January, 2008 and October, 2008 were approached. Patients with a history of abdominal surgery, mental illness or those who had recently taken psychotropic drugs were excluded. A physician obtained demographic and abdominal symptom data. All patients were asked to complete the Zung Self-Rated Anxiety and Depression Scale

(SDS/SAS) and the IBS-specific QoL questionnaire. The patients were divided into six groups according to age, in 10-year increments: 18-27 years, 28-37 years, 38-47 years, 48-57 years, 58-67 years and 68-75 years (maximum 75 years). Age-related differences of abdominal pain or discomfort were analyzed using rank-sum tests. Differences in SDS/SAS and IBS-QoL scores between age groups were analyzed using one-way analysis of variance. Pearson's correlations evaluated potential associations between IBS symptoms, psychological factors and QoL in each age group.

RESULTS: There were no differences in the distribution of IBS subtypes between age groups ($\chi^2 = 20.516$, $P = 0.153$). Differences in the severity of abdominal pain/discomfort with age were statistically significant ($\chi^2 = 25.638$, $P < 0.001$); patients aged 48-57 years, 58-67 years or 68-75 years had milder abdominal pain/discomfort than those in the younger age groups. The severity of anxiety or depressive symptoms did not differ between age groups (SDS, $\chi^2 = 390.845$, $P = 0.110$; SAS, $\chi^2 = 360.071$, $P = 0.220$). Differences of IBS-QoL scores were statistically significant between age groups ($\chi^2 = 1098.458$, $P = 0.011$). The scores of patients in the 48-57-year group were lower than those in the 18-27-year and 28-37-year groups (48-57-year group vs 18-27-year group, 74.88 ± 8.76 vs 79.76 ± 8.63 , $P = 0.021$; 48-57-year group vs 28-37-year group, 74.88 ± 8.76 vs 79.04 ± 8.32 , $P = 0.014$). The scores in the 68-75-year group were lower than those in the 18-27-year, 28-37-year and 38-47-year groups (68-75-year group vs 18-27-year group, 71.98 ± 9.83 vs 79.76 ± 8.63 , $P = 0.003$; 68-75-year group vs 28-37-year group, 71.98 ± 9.83 vs 79.04 ± 8.32 , $P = 0.002$; 68-75-year group vs 38-47-year group, 71.98 ± 9.83 vs 76.44 ± 8.15 , $P = 0.039$). Anxiety and depression were negatively correlated with QoL in all age groups (SDS and QoL: 18-27-year group, $r = -0.562$, $P = 0.005$; 28-37-year group, $r = -0.540$, $P < 0.001$; 38-47-year group, $r = -0.775$, $P < 0.001$; 48-57-year group, $r = -0.445$, $P = 0.001$; 58-67-year group, $r =$

-0.692, $P < 0.001$; 68-75-year group, $r = -0.732$, $P < 0.001$. SAS and QoL: 18-27-year group, $r = -0.600$, $P = 0.002$; 28-37-year group, $r = -0.511$, $P < 0.001$; 38-47-year group, $r = -0.675$, $P < 0.001$; 48-57-year group, $r = -0.558$, $P = 0.001$; 58-67-year group, $r = -0.588$, $P < 0.001$; 68-75-year group, $r = -0.811$, $P < 0.001$). A negative correlation between abdominal pain severity and QoL was found in patients aged more than 58 years (58-67-year group, $r = -0.366$, $P = 0.017$; 68-75-year group, $r = -0.448$, $P = 0.048$), but not in younger patients (18-27-year group, $r = 0.080$, $P = 0.716$; 28-37-year group, $r = -0.063$, $P = 0.679$; 38-47-year group, $r = -0.029$, $P = 0.812$; 48-57-year group, $r = -0.022$, $P = 0.876$).

CONCLUSION: Factors affecting QoL should always be treated in IBS, especially emotional problems in young adults. Even mild abdominal pain should be controlled in elderly patients.

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Key words: Irritable bowel syndrome; Female; Age; Symptom; Quality of life

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INTRODUCTION

Irritable bowel syndrome (IBS) is the most common functional gastrointestinal disorders. It is common in Chinese people, representing approximately 11% of outpatient cases in gastroenterology departments^[1]. Patients with IBS have long-term symptoms including abdominal pain or discomfort related to defecation, accompanied by emotional disorders such as anxiety or depression, and often have a poor quality of life (QoL)^[2,3]. The abdominal symptoms of IBS may be related to changes of gastrointestinal motility, visceral sensitivity and other factors^[4,5], and affected by estrogen and progesterone levels and psychological factors^[6-8]. Anxiety or depression in IBS patients may be associated with physical discomfort and mental stress, and related to the patient's response to the disease and the degree of social support^[9,10].

It is widely recognized that gastrointestinal motility, visceral sensitivity, and estrogen and progesterone levels differ between people of different ages^[11,12], as do cognitive abilities and the response to disease^[13]. Do abdominal pain or discomfort, anxiety and depression vary with

age in IBS patients? Are there are age-related differences in the impact of these symptoms on QoL? In this study, we explored these two questions.

IBS is more common in women than in men, and female IBS patients report more severe symptoms and generally have lower QoL^[1]. Therefore, we investigated age-related changes of symptoms and QoL in female patients with IBS in a Chinese population, with the aim of improving individual treatment.

MATERIALS AND METHODS

Subjects

First-time outpatients who attended the Department of Gastroenterology at the First Affiliated Hospital of Nanjing Medical University between January, 2008 and October, 2008 and met the Rome III criteria for IBS were recruited^[14].

All patients were initially asked to undergo routine blood, urine and stool hemocult tests, stool form examination, and endoscopy or radiographic examination of the gastrointestinal tract. Patients younger than 18 years, with a structural bowel disease or a history of abdominal surgery, diagnosed with mental illness by a psychiatrist or who had recently taken psychotropic drugs were excluded^[1]. Pregnant patients were not included.

Measurements

The age of each subject was recorded. A physician obtained demographic and abdominal symptom data. All patients were asked to completed the Zung Self-Rated Anxiety and Depression Scale (SAS/SDS) and the IBS-specific QoL (IBS-QoL) questionnaire^[15-19].

IBS subtypes: Based on the Rome III diagnostic criteria and Bristol Stool Form Scale^[14], the patients were divided into the following groups: IBS with constipation (IBS-C), IBS with diarrhea (IBS-D), mixed IBS (IBS-M) and unsubtyped IBS (IBS-U).

IBS abdominal symptoms: The patients were asked about the severity of their abdominal pain/discomfort, which was rated on a three-point response scale as follows: mild ("can be ignored if I don't think about it"), moderate ("cannot be ignored, but does not affect my lifestyle") or severe ("affects my lifestyle")^[20].

SAS/SDS: Anxiety and depression were measured using the SAS and SDS assessment tools, respectively^[15-17]. Each of the SAS and SDS comprises 20 questions with four possible responses to each: never, rarely/sometimes, frequently and always. Higher SAS/SDS scores indicate a greater degree of anxiety/depression.

IBS-QoL: The IBS-QoL^[18,19] comprises 34 self-reported items; a higher total score indicates a better QoL. There are also eight subscale scores for dysphoria, interference with activities, body image, health concerns,

Table 1 Severity of abdominal pain/discomfort and Zung Self-Rated Anxiety and Depression Scale scores in each age group

Age group (yr)	Total (n)	Severity of abdominal pain/discomfort (n)			Mean rank	SDS score	SAS score
		Mild	Moderate	Severe			
18-27	23	12	5	6	150.74	52.17 ± 8.03	39.09 ± 8.18
28-37	46	29	10	7	133.84	51.83 ± 5.09	39.65 ± 6.16
38-47	68	34	22	12	149.66	54.03 ± 6.66	41.25 ± 7.17
48-57	52	45	9	1	106.90	54.24 ± 7.60	42.31 ± 7.37
58-67	42	33	8	1	110.82	54.60 ± 6.09	39.83 ± 6.82
68-75	20	17	3	0	102.53	56.15 ± 4.98	42.45 ± 7.81

The severity of abdominal pain/discomfort differed between age groups ($\chi^2 = 25.638, P < 0.001$). There were no differences in Zung Self-Rated Anxiety Scale (SAS) or Zung Self-Rated Depression Scale (SDS) scores between any age groups (SDS, $\chi^2 = 390.845, P = 0.110$; SAS, $\chi^2 = 360.071, P = 0.220$).

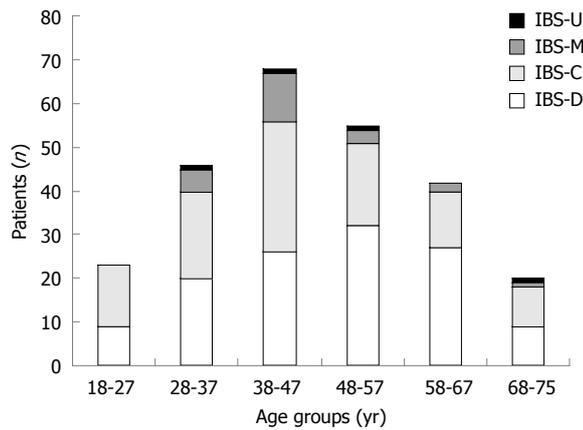


Figure 1 Distribution of irritable bowel syndrome subtypes in each age group. IBS: Irritable bowel syndrome; IBS-C: IBS with constipation; IBS-D: IBS with diarrhea; IBS-M: Mixed IBS; IBS-U: Unsubtyped IBS.

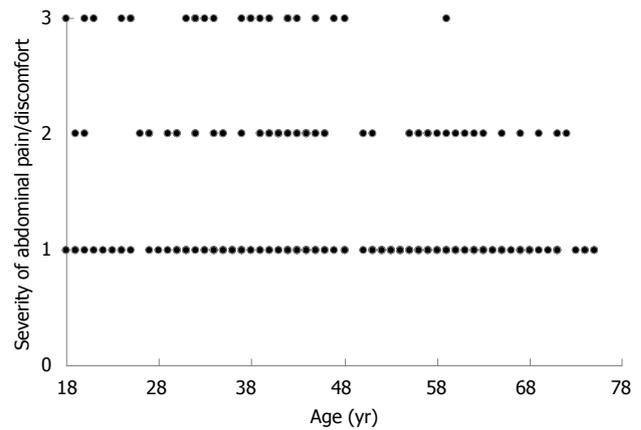


Figure 2 Severity of abdominal pain/discomfort by age. 1 = Mild; 2 = Moderate; 3 = Severe.

food avoidance, social reaction, sexual issues and relationship problems.

Age groups: The patients were divided into six groups according to age, in 10-year increments: 18-27 years, 28-37 years, 38-47 years, 48-57 years, 58-67 years and 68-75 years (maximum 75 years)^[21-23].

Statistical analysis

All data were analyzed using SPSS Version 19.0. Statistical significance was set at $P < 0.05$. Categorical data and ratios were analyzed using the χ^2 test. Rank-sum tests were used to analyze ranked data. All measurement data are reported as the mean \pm SD. Differences in SDS/SAS and IBS-QoL scores between age groups were analyzed using one-way analysis of variance. Pearson's correlations were used to evaluate the potential associations between IBS symptoms, psychological factors and QoL in each age group.

RESULTS

Patient background and clinical data

Two hundred and fifty-four women with IBS were approached for recruitment to this study. These patients were aged between 18 and 75 years (46.67 ± 14.26

years). The median duration of IBS was 3 years, and 8.4% of the patients had a history of IBS of more than 10 years. There were no differences in the duration of IBS between age groups (average duration of each group: 2.79 ± 2.48 years, 2.22 ± 2.27 years; 2.94 ± 3.29 years, 2.97 ± 3.32 years, 4.27 ± 4.79 years, 4.35 ± 6.55 years, respectively; $\chi^2 = 129.4, P = 0.101$). One hundred and twenty-three patients (48.4%) were diagnosed with IBS-D, 105 (41.3%) with IBS-C, 22 (8.7%) with IBS-M and 4 (1.6%) with IBS-U. There were no differences in the distribution of IBS subtypes between age groups ($\chi^2 = 20.516, P = 0.153$), as shown in Figure 1.

Symptoms by age

Abdominal pain/discomfort: Differences in the severity of abdominal pain/discomfort with age are shown in Figure 2. These differences were statistically significant ($\chi^2 = 25.638, P < 0.001$; Table 1); patients aged 48-57 years, 58-67 years or 68-75 years had milder abdominal pain/discomfort than those in the younger age groups.

Anxiety and depression: SAS and SDS scores with age are shown in Figure 3. There were no statistically significant differences between any age groups (SDS, $\chi^2 = 390.845, P = 0.110$; SAS, $\chi^2 = 360.071, P = 0.220$; Table 1), indicating that the severity of anxiety and depression does not vary with age.

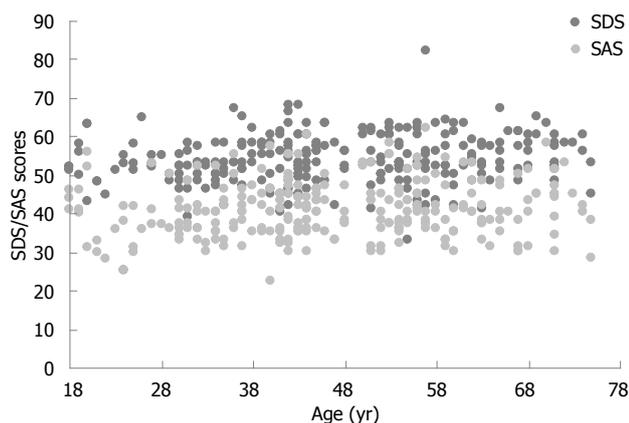


Figure 3 Zung self-rated anxiety and depression scale scores according to age. SAS/SDS: Zung self-rated anxiety and depression scale.

QoL: IBS-QoL scores with age are shown in Figure 4A. These differences were statistically significant between age groups ($\chi^2 = 1098.458$, $P = 0.011$; Figure 4B). The scores of patients in the 48-57-year group were lower than those in the 18-27-year and 28-37-year groups ($P = 0.021$, $P = 0.014$). The scores in the 68-75-year group were lower than those in the 18-27-year, 28-37-year and 38-47-year groups ($P = 0.003$, $P = 0.002$ and $P = 0.039$, respectively).

Association of abdominal pain, anxiety and depression with QoL

As shown in Figure 5, the severity of abdominal pain/discomfort was negatively correlated with IBS-QoL score in the 58-67-year and 68-75-year groups ($P < 0.05$), but there was no correlation in the 18-27-, 28-37-, 38-47- or 48-57-year groups. SAS and SDS scores were negatively correlated with IBS-QoL score in all age groups ($P < 0.01$).

DISCUSSION

In this study, most of the 254 women with IBS were middle-aged. IBS-D was the most common subtype, followed by IBS-C, IBS-M and IBS-U. The distribution of IBS subtypes showed no difference between any age groups, consistent with previous epidemiological studies^[1,23,24].

Our study suggests that the severity of abdominal pain/discomfort differs in patients of different ages, but anxiety and depressive symptoms do not. Patients aged 48-57 years or 68-75 years had the worst QoL. Anxiety and depression were negatively correlated with QoL in all age groups, and a negative correlation between abdominal pain severity and QoL was found in patients aged more than 58 years, but not in younger patients.

Defecation-related abdominal pain/discomfort is the principal symptom of IBS^[14]. In the present study, patients older than 48 years had milder abdominal pain/discomfort. Visceral hypersensitivity, psychological factors, motility, immunity and infection are the major

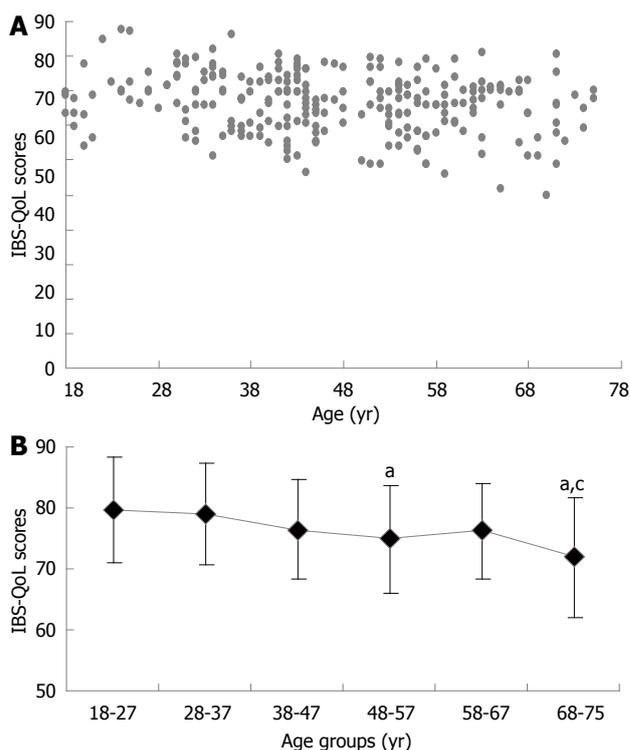
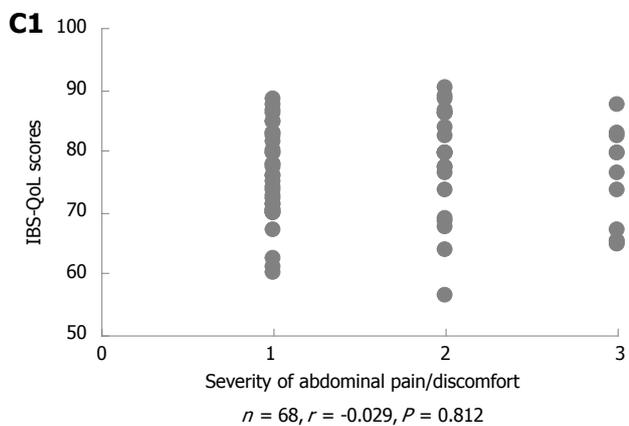
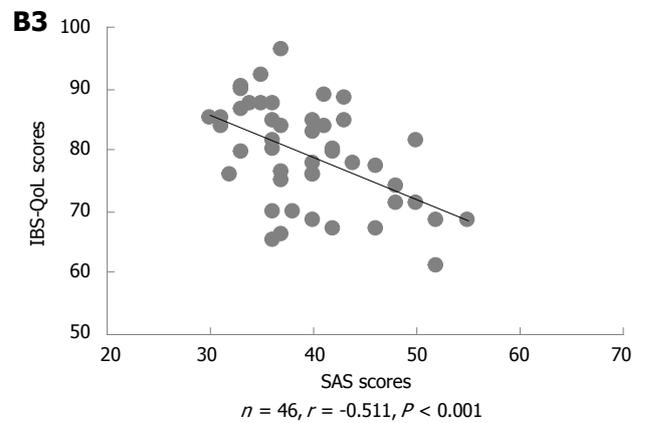
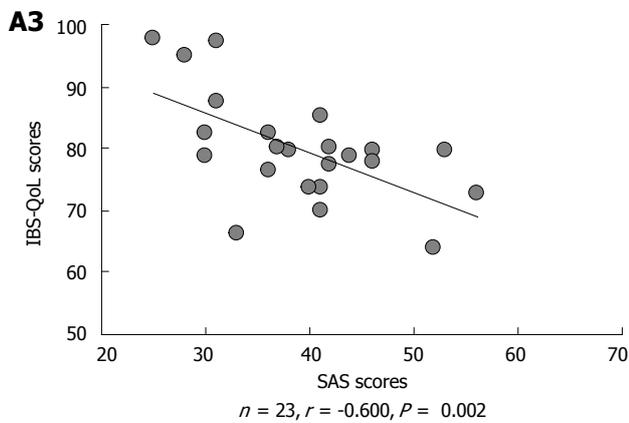
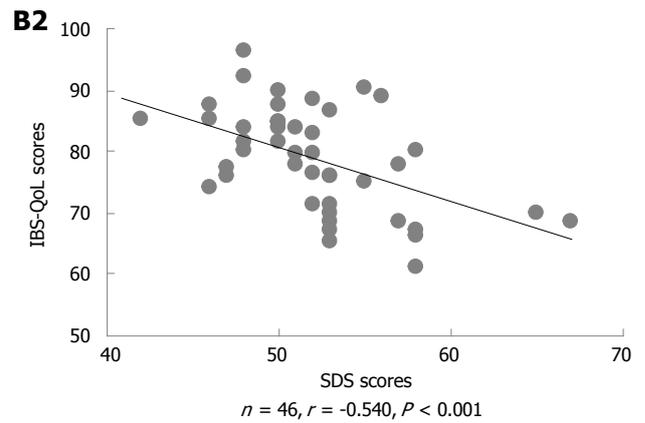
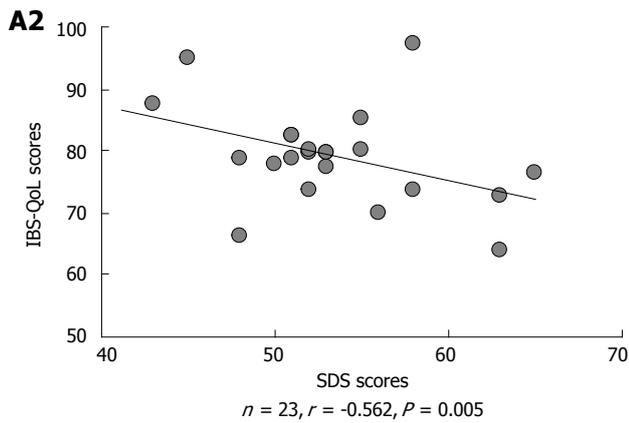
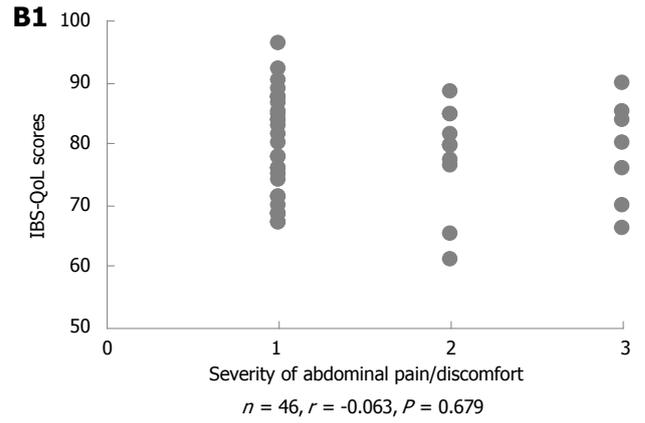
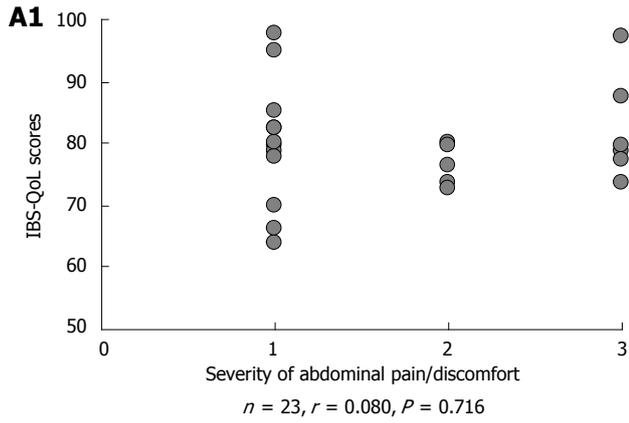


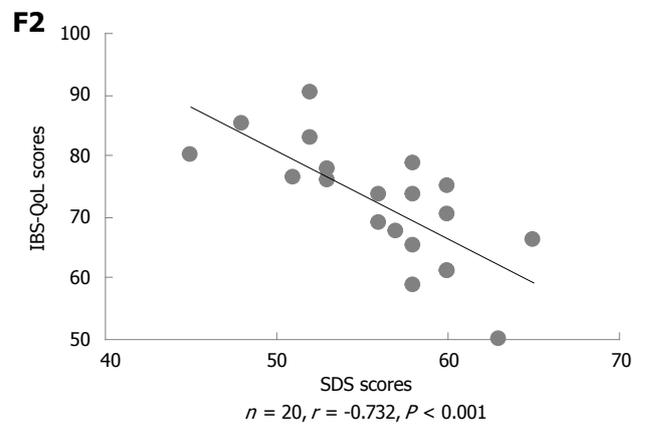
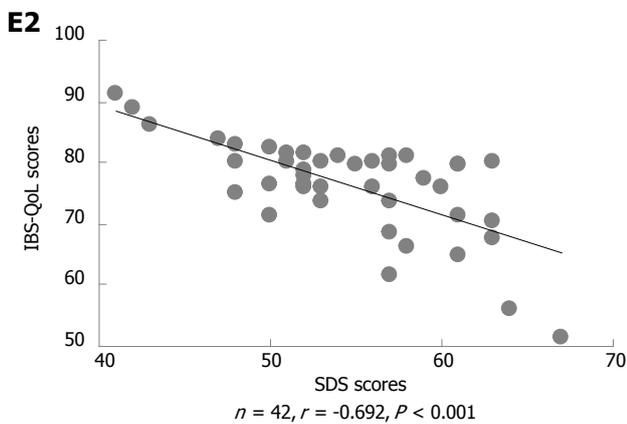
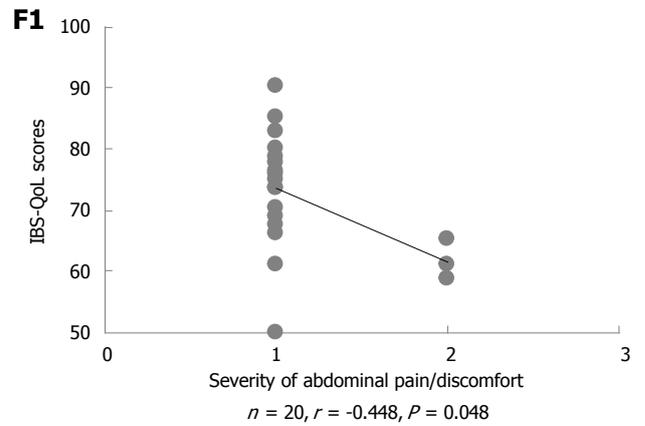
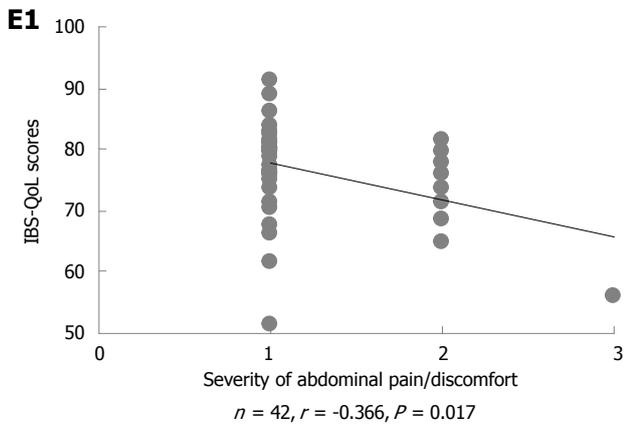
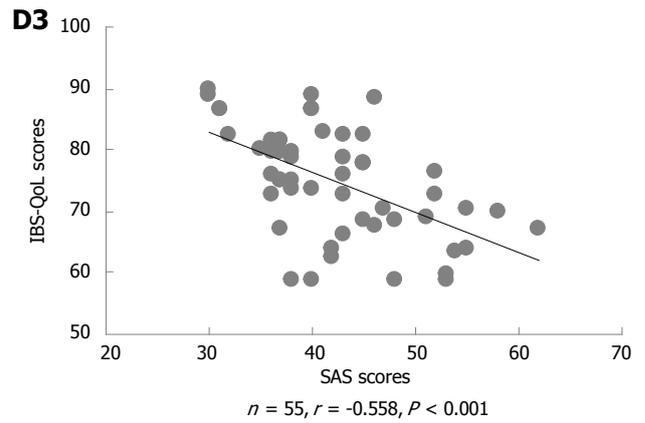
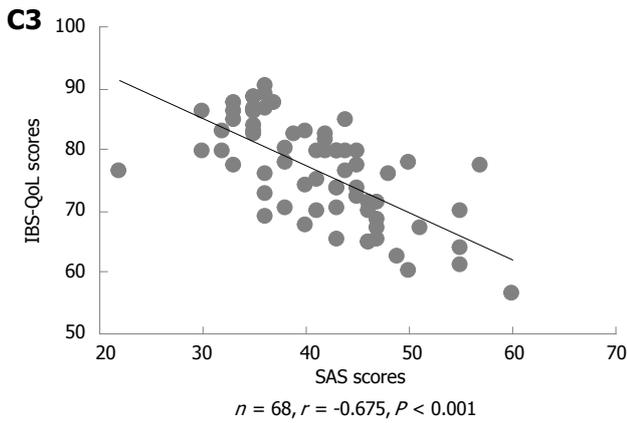
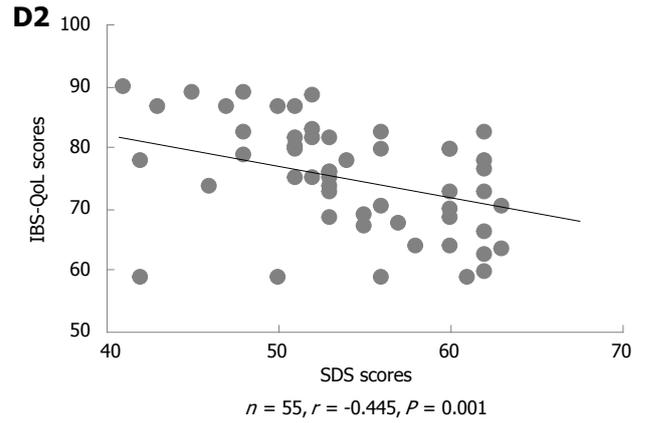
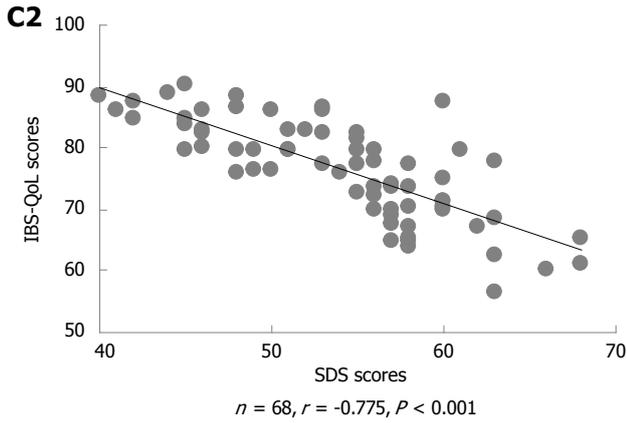
Figure 4 Specific quality of life questionnaire scores for patients with irritable bowel syndrome. A: Specific quality of life questionnaire scores for patients with irritable bowel syndrome (IBS) according to age; B: Specific quality of life questionnaire scores for patients with IBS in each age group. ^a $P < 0.05$ vs 18-27-year and 28-37-year groups; ^c $P < 0.05$ vs 48-57-year group. QoL: Quality of life; SAS/SDS: Zung self-rated anxiety and depression scale.

pathophysiologic factors in abdominal pain/discomfort in IBS^[4,5,25-27], and the role of visceral hypersensitivity in the pathogenesis of IBS has become commonly accepted in recent years. IBS patients have been reported to have a decreased intestinal pain perception threshold and are more likely to report feeling pain^[28,29]. Some studies have shown visceral sensitivity to decrease with age^[12]. Sanoja *et al*^[7] found that estrogen and progesterone can regulate sensitivity to pain and temperature sensation, and Heitkemper *et al*^[30], summarizing the results of recent research, found that estrogen and progesterone can affect the symptoms of IBS. Most women over the age of 48 years are in the menopause or perimenopausal period, and the decline of estrogen and progesterone levels may be accompanied by decreased visceral sensitivity.

In the modern bio/psychosocial medical model, IBS is recognized as a psychosomatic disorder accompanied by various emotional disorders, among which anxiety and depression are the most common. The present study found no differences in SAS/SDS scores between age groups, suggesting that the severity of anxiety and depression is similar in patients of differing ages.

Although it has been confirmed that coping capacity increases with age^[13], ageing is accompanied by reduced sleep quality and blood hemoglobin concentration, and both of these are closely related to depression^[31-33]. Negative life events, social support and other factors are also involved in the occurrence of anxiety and depres-





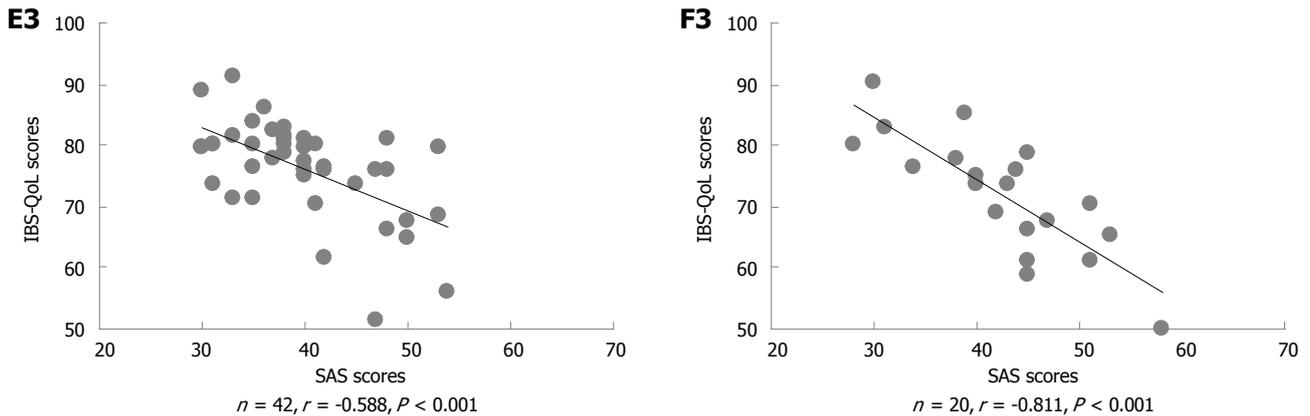


Figure 5 Association of abdominal pain/discomfort severity, and Zung self-rated anxiety and depression scale scores for patients with irritable bowel syndrome in each age group. A: Age 18-27 years; B: Age 28-37 years; C: Age 38-47 years; D: Age 48-57 years; E: Age 58-67 years; F: Age 68-75 years. A1 to F1, association of abdominal pain/discomfort with quality of life in each age group. Severity of abdominal pain/discomfort was negatively correlated with irritable bowel syndrome-specific quality of life questionnaire (IBS-QoL) scores in the 58-67-year and 68-75-year groups ($P < 0.05$), but there was no correlation in the 18-27-year, 28-37-year, 38-47-year or 48-57-year groups ($P > 0.05$); A2 to F2, association of Zung Self-Rated Depression Scale (SDS) scores with quality of life in each age group. SDS scores were negatively correlated with IBS-QoL scores in all age groups ($P < 0.01$); A3 to F3, association of Zung Self-Rated Anxiety Scale (SAS) scores with quality of life in each age group. SAS scores were negatively correlated with IBS-QoL scores in all age groups ($P < 0.01$).

sion^[10,34,35]. None of these factors showed any significant age-related differences in IBS patients.

QoL relates to the functional capabilities of individuals in social life and reflects subjective feelings. It is an important concept in biomedical and social psychology, but is of greatest concern in disease states and when evaluating treatments for disease-related symptoms. In patients with IBS, it is regarded as a major measure of clinical outcome and is widely used in clinical studies^[18,19]. Previous studies have shown that bowel symptoms, anxiety and depression are important factors in the QoL of IBS patients^[36-38]. The present study demonstrated age-related differences in the QoL of women with IBS; the QoL of patients aged 48-57 years or over 68 years was poorer than that of younger patients. However, the patients in the older age groups had milder abdominal pain, and their degree of anxiety and depression was similar to that of the other age groups. Thus, what caused the decline of their QoL? Pines *et al.*^[39] found that the reduction of hormone levels in menopausal women resulted in various physical symptoms that affected QoL, and hormone replacement therapy partially improved these symptoms. Menopause-related factors may have responsible for the poor QoL of the patients aged 48-57 years in our study. In addition, we speculate that multiple other factors such as decreased activity or social support, or impaired psychological status may lead to a poor QoL in older patients. It is necessary to identify other factors that affect QoL in these patients, in addition to bowel symptoms, anxiety and depression.

We also investigated changes with age in the correlation of abdominal pain, anxiety and depressive symptoms with QoL, and found that anxiety and depression were negatively correlated with QoL in all age groups.

Psychologists believe that IBS is a psychosomatic disorder; most IBS patients have core symptoms of anxiety or depression, while gastrointestinal symptoms such as abdominal pain and changes in bowel habit represent

somatization^[40]. In our previous study, we found that most IBS patients complained of nonspecific somatic symptoms such as dizziness, insomnia and fatigue^[1]. We should therefore pay attention to the treatment of anxiety, depression and other negative emotions in IBS patients. In the present study, found that, in young and middle-aged patients (18-57 years), QoL was negatively correlated with anxiety and depression, but not associated with the severity of abdominal pain/discomfort. Therefore, improving gastrointestinal symptoms only may not improve QoL in these age groups, and antidepressant treatment may be more important. In IBS patients aged over 58 years, abdominal pain/discomfort was mild but closely associated with QoL. Thus, even slight abdominal pain should be controlled in the treatment of older patients. Although age-related physiological and psychological changes may be directly or indirectly related to ovarian function and female hormone levels, this was a retrospective study and thus we lack information on the patients' hormone levels and menstrual history. Furthermore, the use of self-reported measures and individual differences in literacy and understanding may have affected the results. None of these limitations could be avoided.

In conclusion, the following points should be borne in mind when treating women with IBS. Firstly, in patients aged 48-57 years or over 68 years, factors other than IBS-related symptoms that affect QoL should be treated. Secondly, negative emotions should be treated in all patients; anti-anxiety and antidepressant treatment are especially important in young adults. Thirdly, to improve QoL, even mild abdominal pain should be controlled in elderly patients.

COMMENTS

Background

Irritable bowel syndrome (IBS) is the most common functional gastrointestinal

disorder. IBS is more common in women than in men, and female IBS patients report more severe symptoms and generally have a lower quality of life (QoL). Age-related changes, such as gastrointestinal motility, visceral sensitivity, estrogen and progesterone levels, cognitive abilities and the response to disease may cause differences in abdominal pain or discomfort, anxiety and depression and reduced QoL levels. Therefore, the authors investigated age-related changes of symptoms and QoL levels in female patients with IBS in a Chinese population, with the aim of improving individual treatment profiles.

Research frontiers

The symptoms of IBS may be related to changes in gastrointestinal motility and visceral sensitivity and are affected by estrogen and progesterone levels, psychological factors, patient response to pain and the degree of social support. All of the above factors differ between people of different ages. In this study, authors demonstrate that differences in abdominal pain or discomfort levels, but not anxiety or depression, and their association with QoL exist in patients in different age groups.

Innovations and breakthroughs

Recent reports have highlighted the differences in IBS abdominal and psychological symptoms in different genders and IBS subtypes, which can guide individual treatment regimens. Other reports have referred to the different age distributions of IBS patients. However, no study has investigated age-related changes in symptoms and their association with QoL in IBS patients, particularly in an exclusively female patient cohort or in a Chinese population.

Applications

In this study, the authors have demonstrated the difference of IBS symptoms and their association with QoL in each patient age group. With this knowledge, the authors may improve individual treatment regimens for IBS according to the patient age.

Terminology

Zung self-rated anxiety and depression scale (SAS/SDS) are assessment tools that are used to measure anxiety and depression, respectively. Higher SAS/SDS scores indicate a greater degree of anxiety/depression. IBS-specific QoL questionnaire (IBS-QoL) is a QoL assessment tool, which was specifically set up for patients with IBS. A higher IBS-QoL score indicates a better QoL.

Peer review

The authors explored the age-related changes of symptoms, QoL and the correlations between these two parameters in a population of Chinese women. The results demonstrated that factors that affect QoL should always be treated in IBS, especially emotional problems in young adults, and even mild abdominal pain should be controlled in elderly patients. These findings will help to improve the treatment of individuals with IBS.

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Mechanisms of cholecystokinin-induced calcium mobilization in gastric antral interstitial cells of Cajal

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Abstract

AIM: To investigate the effect of sulfated cholecystokinin-8 (CCK-8S) on calcium mobilization in cultured murine gastric antral interstitial cells of Cajal (ICC) and its possible mechanisms.

METHODS: ICC were isolated from the gastric antrum of mice and cultured. Immunofluorescence staining with a monoclonal antibody for c-Kit was used to identify ICC. The responsiveness of ICC to CCK-8S was measured using Fluo-3/AM based digital microfluorimetric measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). A confocal laser scanning microscope was used to monitor $[Ca^{2+}]_i$ changes. The selective CCK₁ receptor antagonist lorglumide, the intracellular Ca^{2+} -ATPase inhibitor thapsigargin, the type III inositol 1,4,5-triphosphate (InsP₃) receptor blocker xestospongin C and the L-type voltage-operated Ca^{2+} channel inhibitor nifedipine were used to examine the mecha-

nisms of $[Ca^{2+}]_i$ elevation caused by CCK-8S. Immunoprecipitation and Western blotting were used to determine the regulatory effect of PKC on phosphorylation of type III InsP₃ receptor (InsP₃R3) in ICC. Protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA) and inhibitor chelerythrine were used to assess the role of PKC in the CCK-8S-evoked $[Ca^{2+}]_i$ increment of ICC.

RESULTS: ICC were successfully isolated from the gastric antrum of mice and cultured. Cultured ICC were identified by immunofluorescence staining. When given 80 nmol/L or more than 80 nmol/L CCK-8S, the $[Ca^{2+}]_i$ in ICC increased and 100 nmol/L CCK-8S significantly increased the mean $[Ca^{2+}]_i$ by $59.30\% \pm 4.85\%$ ($P < 0.01$). Pretreatment of ICC with 5 μ mol/L lorglumide inhibited 100 nmol/L CCK-8S-induced $[Ca^{2+}]_i$ increment from $59.30\% \pm 4.85\%$ to $14.97\% \pm 9.05\%$ ($P < 0.01$), suggesting a CCK₁R-mediated event. Emptying of intracellular calcium stores by thapsigargin (5 μ mol/L) prevented CCK-8S (100 nmol/L) from inducing a $[Ca^{2+}]_i$ increase. Moreover, pretreatment with xestospongin C (1 μ mol/L) could also abolish the CCK-8S-induced effect, indicating that Ca^{2+} release from InsP₃R-operated stores appeared to be a major mechanism responsible for CCK-8S-induced calcium mobilization in ICC. On the other hand, by removing extracellular calcium or blocking the L-type voltage-operated calcium channel with nifedipine, a smaller but significant rise in the $[Ca^{2+}]_i$ could be still elicited by CCK-8S. These data suggest that the $[Ca^{2+}]_i$ release is not stimulated or activated by the influx of extracellular Ca^{2+} in ICC, but the influx of extracellular Ca^{2+} can facilitate the $[Ca^{2+}]_i$ increase evoked by CCK-8S. CCK-8S increased the phosphorylation of InsP₃R3, which could be prevented by chelerythrine. Pretreatment with lorglumide (5 μ mol/L) could significantly reduce the CCK-8S intensified phosphorylation of InsP₃R3. In the positive control group, treatment of cells with PMA also resulted in an enhanced phosphorylation of InsP₃R3. Pretreatment with various concentrations of PMA (10 nmol/L-10 μ mol/L) apparently inhibited the effect of CCK-8S and the effect of

100 nmol/L PMA was most obvious. Likewise, the effect of CCK-8S was augmented by the pretreatment with chelerythrine (10 nmol/L-10 μ mol/L) and 100 nmol/L chelerythrine exhibited the maximum effect.

CONCLUSION: CCK-8S increases $[Ca^{2+}]_i$ in ICC *via* the CCK₁ receptor. This effect depends on the release of InsP₃R-operated Ca²⁺ stores, which is negatively regulated by PKC-mediated phosphorylation of InsP₃R3.

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Key words: Cholecystokinin octapeptide; Interstitial cells of Cajal; Calcium mobilization; Protein kinase C

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INTRODUCTION

In the gastrointestinal (GI) tract, phasic contractions are caused by electrical activity termed slow waves, which are generated and propagated by the interstitial cells of Cajal (ICC)^[1,2]. The initiation of pacemaker activity in the ICC is caused by rhythmic cytoplasmic Ca²⁺ oscillation^[3,4]. The ICC can regulate slow-wave-driven peristaltic activity as well as mediate motor inputs from the GI nervous system^[5,6]. ICC abnormalities are associated with many GI motility disorders, such as achalasia of cardia, slow transit constipation and irritable bowel syndrome^[7,8]. Therefore, understanding the mechanisms underlying pacemaker activity and excitability of ICC is of crucial importance^[9]. Although neurohumoral regulation of GI function has been studied in some detail, less is known about the mechanisms of how neurohumoral factors regulate pacemaker activities in the ICC.

Cholecystokinin (CCK) is a bioactive peptide that regulates a variety of physiological functions, acting as both hormone and neurotransmitter in the GI tract^[10,11]. Sulfated CCK-8 (CCK-8S), which has frequently been used in research studies, is one of the main biologically active forms of CCK^[12]. The regulatory actions of CCK are mediated by two receptor subtypes, CCK₁ and CCK₂ receptors. CCK receptors are distributed in enteric nerves and smooth muscles^[13]. In addition, CCK₁ receptors are also found in the ICC, suggesting a role for the ICC in the mediation of CCK effects^[14].

CCK inhibits gastric emptying by relaxing the proxi-

mal part of the stomach and increasing pyloric pressure^[10,12]. However, the mechanisms of the regulatory effects of CCK on gastrointestinal motility are not clear. Previous studies have shown that CCK can activate phospholipase C (PLC) through binding to its distinct receptors^[15]. This activation leads to the production of diacylglycerol (DAG) and inositol 1,4,5- triphosphate (InsP₃), which in turn activates protein kinase C (PKC) and mobilizes intracellular calcium ($[Ca^{2+}]_i$)^[16]. Through this signaling pathway, CCK may participate in various physiological responses, such as secretion, neurotransmission and muscle contraction^[17]. However, the cross-talk of the CCK-8S triggered PKC and Ca²⁺ signaling pathways is not been well understood. The aims of this study were to investigate the effect of CCK-8S on $[Ca^{2+}]_i$ in the ICC and the respective contributions of InsP₃R-sensitive intracellular Ca²⁺ stores and extracellular Ca²⁺ sources in those responses. Additionally, the role of PKC in regulation of CCK-8S-triggered calcium signaling pathway was also studied.

MATERIALS AND METHODS

Preparation of cells and cell culture

All experiments were performed according to the guiding principles for the care and use of animals approved by Institutional Animal Use and Care Committee of the First Affiliated Hospital of Nanjing Medical University. Every effort was made to minimize both the number of animals used and their suffering.

Balb/c mice (5-6 wk) of either sex were purchased from the laboratory of the First Affiliated Hospital of Nanjing Medical University. The animals were anesthetized by chloroform inhalation and killed by cervical dislocation. The stomach was excised and the contents were washed away with ice-cold Krebs-Ringer bicarbonate (KRB). The mucosa was removed by peeling. In Sylgard dishes filled with Krebs solution, the tissues were washed three times and then cut into about 0.5 cm segments. The segments were transferred into a centrifuge tube and dispersed with an enzyme solution containing collagenase 1.3 mg/mL, trypsin inhibitor 2 mg/mL and ATP 0.27 mg/mL. The centrifuge tube was incubated at 37 °C for 30 min and the tissue segments were blown for 30 s with pipette every 5 min during incubation. An equal volume Medium 199 containing 10% fetal bovine serum was added to stop digestion. The tube was centrifuged at 1000 rpm for 3 min and then the supernatant was removed. The sediment was suspended with Medium 199 and cells were collected by pouring the suspension through a 200-mesh sieve. After centrifugation at 1000 rpm for 3 min, the cells were dispersed with Medium 199 and then plated onto 35 mm glass-bottom culture dishes (NEST Biotechnology Co., Ltd, China) coated with rat-tail tendon collagen (5 mg/mL). The cells were then cultured at 37 °C in a 95% O₂-5% CO₂ incubator in Medium 199 supplemented with 2% Penicillin-Streptomycin liquid and murine stem cell factor (5 ng/mL).

Labeling of cultured ICC by c-Kit immunofluorescence

Cultured ICC were fixed in acetone (4 °C, 8 min). Following fixation, preparations were washed for 40 min in phosphate buffered saline (PBS; 0.01 mol/L, pH 7.4) and then incubated in 10% goat serum for 1 h to reduce nonspecific antibody binding. To examine the ICC, we incubated cultured ICC overnight at 4 °C with a rabbit anti-mouse monoclonal c-Kit antibody (1:300 in PBS). Immunoreactivity was detected using Alexa Fluor 488 (1:1000 in PBS, 60 min, room temperature), and nuclei were stained with Hoechst 33 258. Cells were examined under a confocal laser scanning microscope (LSM710, Zeiss, Germany) at an excitation wavelength appropriate for Alexa Fluor 488 (488 nm).

Measurement of intracellular Ca²⁺ concentration

Changes in [Ca²⁺]_i were monitored using Fluo-3/AM, which was initially dissolved in dimethyl sulfoxide (DMSO) and stored at -20 °C. The cultured ICC grown on glass-bottom dishes were rinsed twice with PBS and then incubated in Medium 199 containing 5 μmol/L Fluo-3/AM in the 95% O₂-5% CO₂ incubator for 40 min. Following rinsing for two more times, the dishes were scanned every 2 s with a confocal laser scanning microscope. Fluorescence was excited at a wavelength of 488 nm and emitted light was observed at 515 nm. The variations of [Ca²⁺]_i fluorescence emission intensity were expressed as F/F₀, where F₀ is the intensity of the first imaging.

Immunoprecipitation, electrophoresis and immunoblotting

Prepared from gastric ICC using equal volumes of a cell suspension, isolated cells were assayed for protein concentration. Following appropriate treatments, cells were pelleted and resuspended in 0.5 mL of ice-cold lysis buffer. Cell samples were sonicated and left on ice for 30 min to solubilize. Immunoprecipitation of InsP₃R was performed using a 1:100 dilution of an InsP₃R3-specific monoclonal antibody. Following 2 h of incubation with the InsP₃R3 antibody at 4 °C, immobilized protein A beads were added to each sample for 1 h at 4 °C. As a control, samples were also prepared without immunoprecipitating antibody. Following immunoprecipitation of InsP₃R3, proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Membranes were probed with a 1:1000 dilution of phospho-(Ser/Thr) substrate antibody that specifically detected phosphorylated Ser/Thr residues with Arg at the -2 or -3 position within the PKC substrate sequence. Immunoreactivity was visualized using a 1:1000 dilution of peroxidase-conjugated secondary antibody. Where indicated, the nitrocellulose membrane was stripped of primary and secondary antibodies at 50 °C for 30 min.

Drugs and solutions

CCK-8S, collagenase, trypsin inhibitor, ATP, thapsigargin, chelerythrine, phorbol 12-myristate 13-acetate (PMA), nifedipine, Hoechst 33258, DMSO, HEPES were pur-

chased from Sigma (United States). SCF was purchased from Peprotech (United States). Rat-tail tendon collagen was purchased from Shengyou Biotechnology Co., Ltd (Hangzhou, China). C-Kit monoclonal antibody and phospho-(Ser/Thr) substrate antibody were from Cell Signaling Technology (United States). Type III InsP₃R-specific monoclonal antibody, protein A beads were from BD Biosciences Transduction Laboratories (United States). Alexa Fluor 488 (goat anti-rabbit secondary antibody) and Fluo-3/AM were from Invitrogen (United States). Lorglumide was purchased from Santa Cruz (United States). Xestospongin C was from Calbiochem (Germany). Medium 199, fetal bovine serum and Penicillin-Streptomycin liquid were purchased from Gibco (United States).

The KRB solution contains (mmol/L): NaCl 117, KCl 4.7, MgSO₄ 1.2, NaHCO₃ 25, KH₂PO₄ 1.2, Glucose 11 and CaCl₂ 2.6; pH was 7.4. Lysis buffer contains (mmol/L): NaCl 150, NaF 100, Tris 50, EDTA 10, Triton X-100 1%, and 1 complete EDTA-free protease inhibitor mixture tablet; pH was 7.4.

Statistical analysis

Data were expressed as mean ± SE. Differences in the data were evaluated by ANOVA or by Student's *t* test. Zeiss Zen 9.0 was used to analyze the calcium intensity data and GraphPad Prism 5.0 for charting. Differences between control and test values were considered significant when *P* < 0.05.

RESULTS**Identification of cultured ICC**

After the cells were isolated and plated onto culture dishes, it was initially difficult to identify the ICC. After prolonged culture (4-7 d), the cultured ICC, were identified by c-Kit immunofluorescence and showed distinctive shapes, such as spindle, triangular or stellar-like with two to five long processes (Figure 1).

Effects of CCK-8S on intracellular Ca²⁺ intensity in cultured ICC

Addition of CCK-8S produced substantial, dose-dependent elevations of Fluo-3/AM fluorescence in cytoplasm an nucleus of the ICC, indicating that free calcium level had increased compared with the control (Figure 2A). When given ≤ 50 nmol/L CCK-8S, the [Ca²⁺]_i did not increase (Figure 2B). As shown in Figure 2D, CCK-8S (100 nmol/L) significantly increased the mean [Ca²⁺]_i by 59.30% ± 4.85% (*P* < 0.01, *n* = 6) and CCK-8S (80 nmol/L and 500 nmol/L) also evoked [Ca²⁺]_i increases in the percentage of cells responding (20.22% ± 5.48% and 39.32% ± 2.51%, respectively, Figure 2C, E and F). Group data for the [Ca²⁺]_i changes in response to CCK-8S at different concentrations are shown in Figure 2F.

CCK-8S increases [Ca²⁺]_i in cultured ICC via CCK₁ receptor

The CCK₁ receptor (CCK₁R) has been reported to be

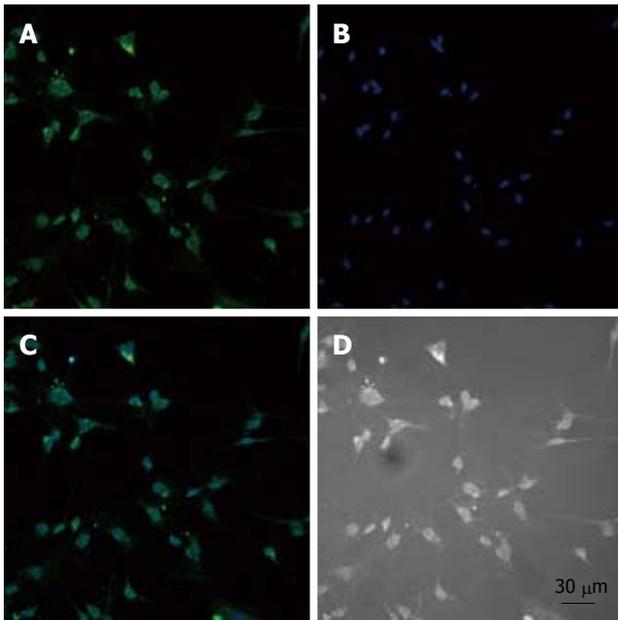


Figure 1 Identification of cultured interstitial cells of Cajal. A-C: Prolonging the culture to 4-7 d, the cultured interstitial cells of Cajal (ICC), which are identified by c-Kit immunofluorescence, had distinctive shapes such as spindle, triangular or stellar-like with two to five long processes. ICC were fixed with acetone and identified immunologically using a monoclonal c-Kit antibody and Alexa Fluor 488-conjugated secondary fluorescent antibody. Nuclei were stained with Hoechst 33258 dye (B, blue); C: A merged image of A and B; D: A light microscopic image of ICC.

expressed in GI ICC^[14]. To identify the subtype of CCK receptor involved in the CCK-8S-induced increase in $[Ca^{2+}]_i$, the CCK₁R selective antagonist lorglumide was employed. Pretreatment of ICC with 5 μ mol/L lorglumide for 2 min inhibited 100 nmol/L CCK-8S-induced $[Ca^{2+}]_i$ increment from 59.30% \pm 4.85% to 14.97% \pm 9.05% ($P < 0.01$, $n = 6$) (Figure 3), suggesting a CCK₁R-mediated event.

CCK-8S-induced calcium mobilization in cultured ICC

To determine the source of CCK-8S-induced calcium mobilization, intracellular calcium release and extracellular calcium influx were investigated. Firstly, the ICC were exposed to CCK-8S in a medium without extracellular calcium and subsequently to specific intracellular Ca^{2+} -ATPase inhibitor thapsigargin. Compared with control, emptying of intracellular calcium stores by thapsigargin (5 μ mol/L) prevented CCK-8S (100 nmol/L) to induce a $[Ca^{2+}]_i$ increase (Figure 4A), indicating that Ca^{2+} release from intracellular stores appeared to be a major mechanism responsible for CCK-8S-induced calcium mobilization in the ICC. This result was similar to the effect of CCK-8S in murine gastric smooth muscle cells^[18], but unlike that in murine myenteric neurons^[19]. To further understand the mechanisms of CCK-8S-induced intracellular calcium release, the specific $InsP_3R$ inhibitor xestospongine C was used. Xestospongine C (1 μ mol/L) completely abolished $[Ca^{2+}]_i$ increases triggered by CCK-8S (Figure 4B).

Removing extracellular Ca^{2+} or blocking L-type voltage-operated calcium channel by nifedipine partly decreased the effect of CCK-8S (Figure 4C-E). These data indicate that the $[Ca^{2+}]_i$ release is not stimulated or activated by the influx of extracellular Ca^{2+} in gastric antrum ICC, while the influx of extracellular Ca^{2+} can facilitate the $[Ca^{2+}]_i$ increase evoked by CCK-8S.

CCK-8S stimulation results in PKC-dependent phosphorylation of $InsP_3R_3$

Experiments were undertaken to determine whether CCK-8S could evoke PKC to increase phosphorylation of $InsP_3R_3$. Samples containing equal amounts of protein were stimulated with CCK-8S (100 nmol/L) before or following administration of chelerythrine. PMA was used as a positive control. All drug/agonist treatments were 5 min in duration. CCK-8S resulted in a markedly increased phosphorylation of $InsP_3R_3$ in the ICC as compared with unstimulated ICC. When pretreated with chelerythrine for 5 min, CCK-8S-induced $InsP_3R_3$ phosphorylation was completely inhibited. Pretreatment with CCK₁R lorglumide (5 μ mol/L) significantly reduced the CCK-8S intensified phosphorylation of $InsP_3R_3$. As a positive control, PMA also enhanced phosphorylation of $InsP_3R_3$ in ICC (Figure 5).

Effect of PKC on CCK-8S-evoked response in cultured ICC

To investigate functional consequence of $InsP_3R_3$ phosphorylation by PKC on $[Ca^{2+}]_i$ changes of the ICC, the following experiments were performed. Followed by CCK-8S (100 nmol/L), PMA at various concentrations could significantly reduce the CCK-8S-evoked $[Ca^{2+}]_i$ response ($P < 0.01$, $n = 6$, Figure 6), and the effect of 100 nmol/L PMA was most obvious. Under the same conditions, chelerythrine showed the opposite effect to PMA ($P < 0.01$, $n = 6$, Figure 7), and 100 nmol/L chelerythrine exhibited the maximum effect.

DISCUSSION

In recent years, many studies have focused on the effects of CCK receptor ligands on GI motor functions, as the pharmacological characterization of these agents in humans is of potential therapeutic value^[20,21]. CCK and its related peptides have been implicated in the pathophysiology of functional digestive diseases, such as functional dyspepsia, achalasia of cardia and irritable bowel syndrome^[22,23]. Previous studies suggested that either an altered release of CCK or abnormal responses to this peptide could contribute to symptoms of GI dysmotility^[24,25]. However, the mechanisms of the effects of CCK on GI motility are unclear. Previous studies revealed that CCK could evoke calcium signaling in cultured myenteric neurons and activate them^[19,26]. The GI ICC also express CCK₁R and play an important role in regulating the GI motility; therefore, it is meaningful and necessary to understand the effect of CCK on the ICC in

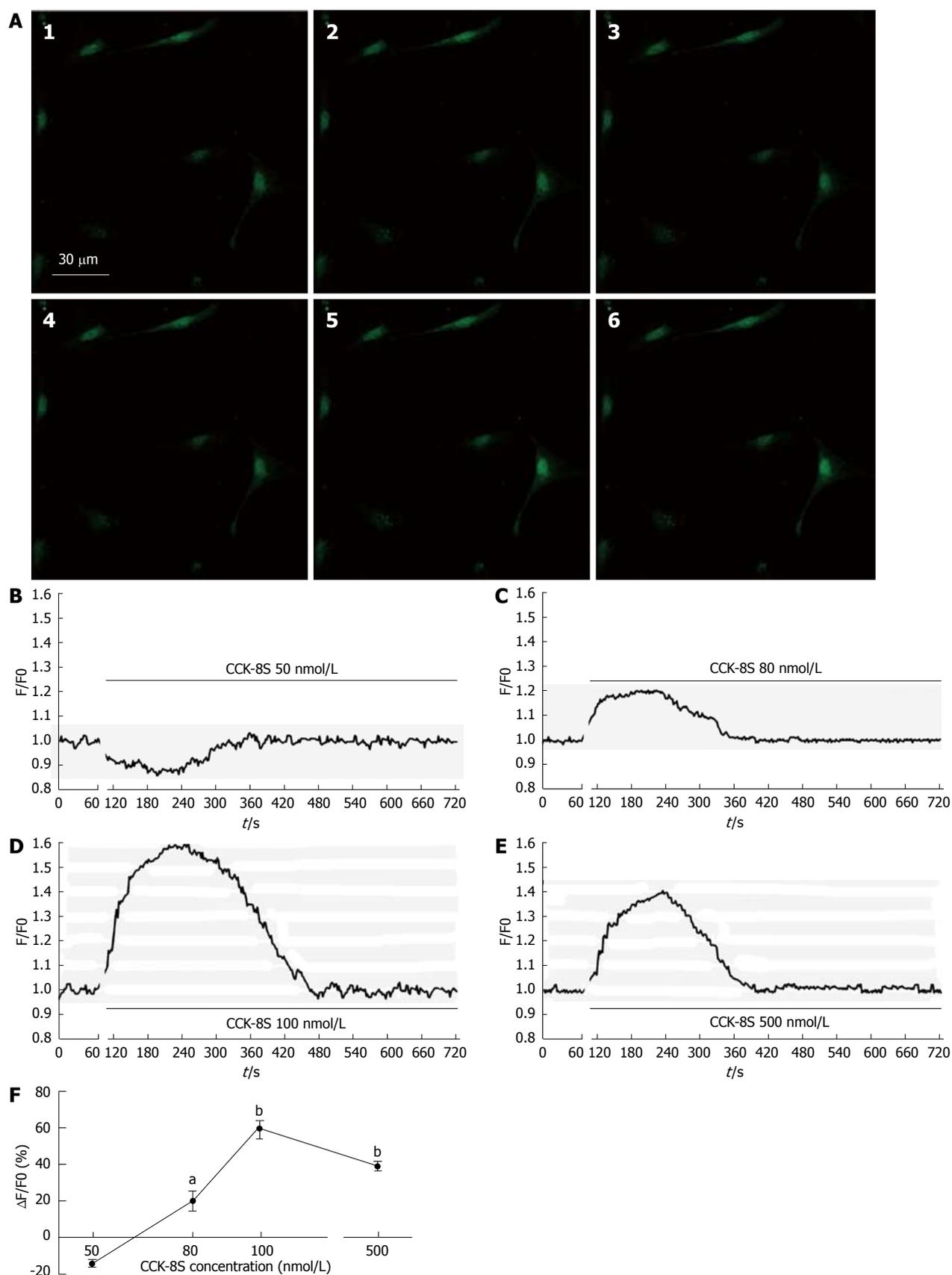


Figure 2 The regulation of sulfated cholecystokinin-8 on $[Ca^{2+}]_i$ in cultured interstitial cells of Cajal from the murine gastric antrum. A1: Fluorescent intensity image of Fluo-3/AM loaded cultured interstitial cells of Cajal (ICC) under normal conditions; A2-6: Fluorescent intensity gradually increased in the presence of cholecystokinin-8 (CCK-8S) (100 nmol/L); B-E: Effects of different concentrations of sulfated CCK-8S on mean $[Ca^{2+}]_i$. In each case, cells from at least five different cell cultures; F: Effects of CCK-8S were estimated as percentage of $\Delta F/F_0$, where F_0 was derived from the averaged intensity of the first 10-30 frames minus the background in the cell-free region and ΔF is fluorescent intensity of the response minus F_0 . ^a $P < 0.05$, ^b $P < 0.01$ vs control.

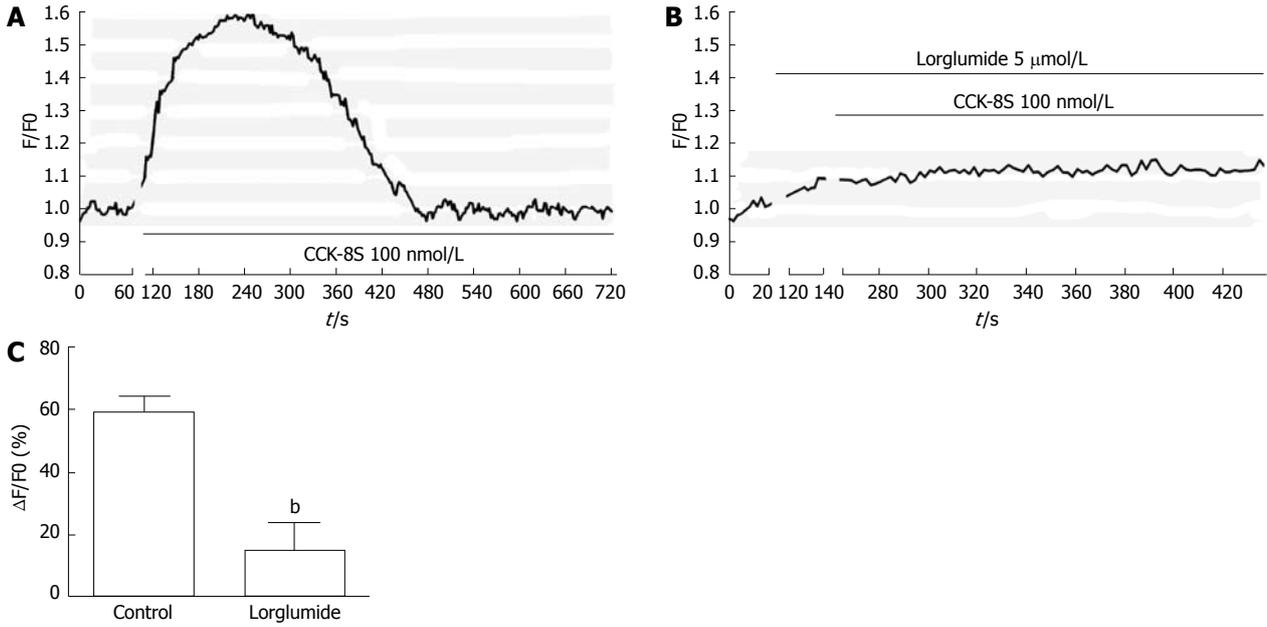


Figure 3 Sulfated cholecystokinin-8 activates interstitial cells of Cajal through the cholecystokinin1 receptor. A: Compared with the control; B: Lorglumide significantly inhibited cholecystokinin-8 (CCK-8S)-induced increase in $[Ca^{2+}]_i$ of interstitial cells of Cajal; C: Quantification of $[Ca^{2+}]_i$ changes shown in A and B. Each experiment was repeated at least three times. ^b $P < 0.01$ vs control.

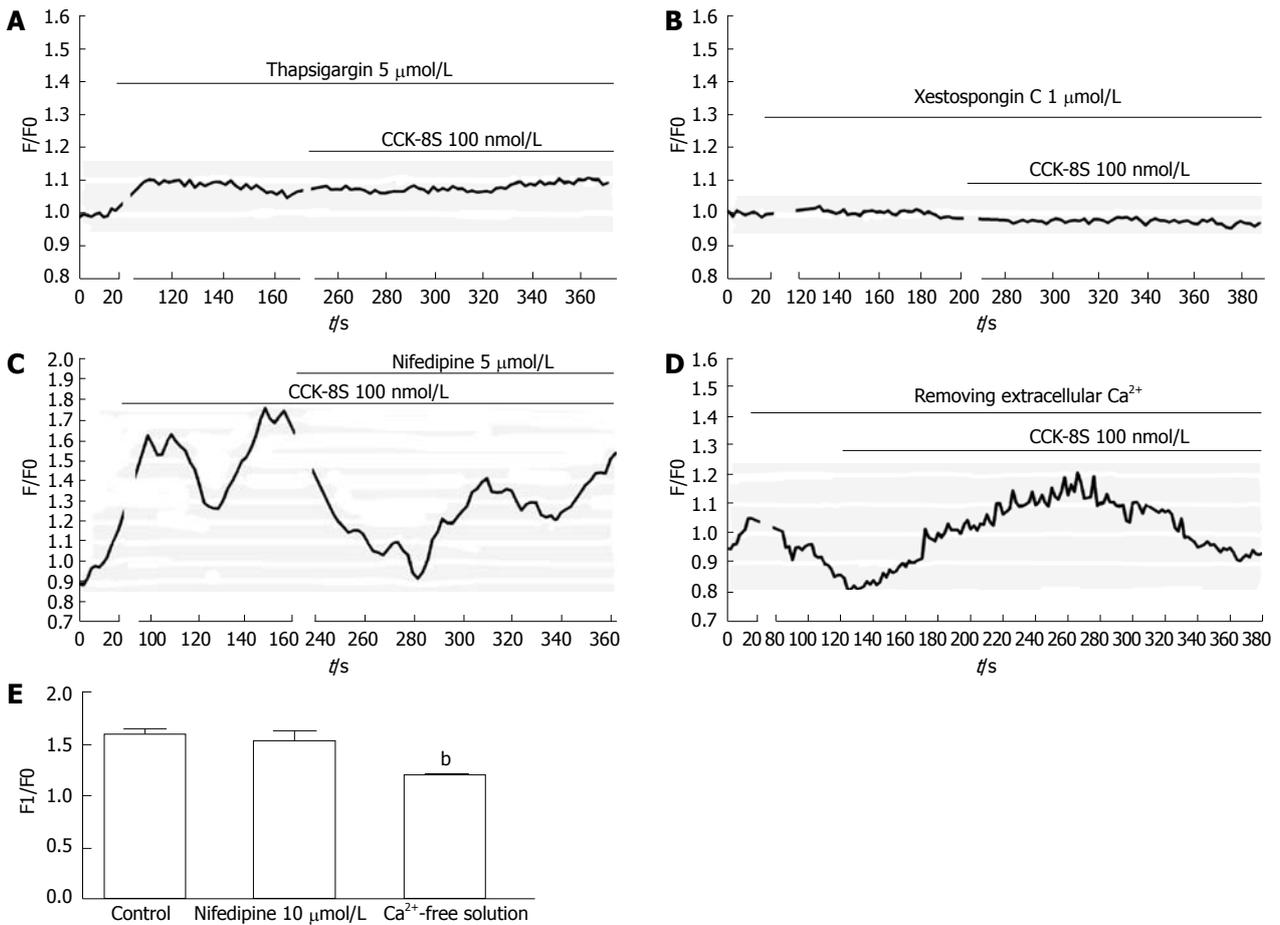


Figure 4 Sulfated cholecystokinin-8 induced calcium mobilization in cultured interstitial cells of Cajal. A, B: Pretreatment with 5 $\mu\text{mol/L}$ thapsigargin (A) or 1 $\mu\text{mol/L}$ xestospongin C (B) completely abolished sulfated cholecystokinin-8 (CCK-8S)-induced $[Ca^{2+}]_i$ increases; C: Addition of nifedipine resulted in a smaller peak of $[Ca^{2+}]_i$ in comparison with normal conditions; D: The CCK-8S-elicited $[Ca^{2+}]_i$ increase in the calcium-free medium was lower than that in the calcium-containing buffer; E: Quantification of $[Ca^{2+}]_i$ changes following addition of nifedipine or removal of extracellular Ca^{2+} . The fluorescence was normalized as F1/F0 (F1: Maximal fluorescence after drug addition; F0: Basal fluorescence before drug addition). ^b $P < 0.01$ vs control.

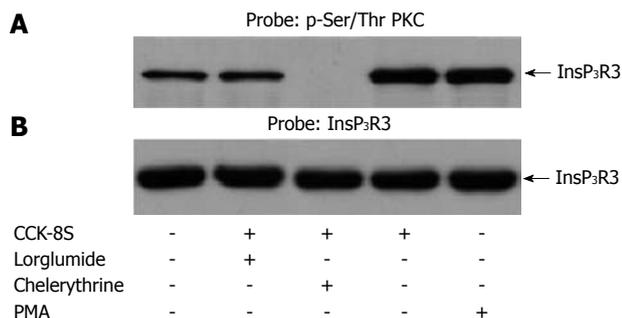


Figure 5 Sulfated cholecystokinin-8 stimulation of interstitial cells of Cajal resulted in the protein kinase C-dependent phosphorylation of type III inositol 1,4,5-triphosphate receptor. A: Western blots of proteins were immunoprecipitated with type III inositol 1,4,5-triphosphate receptor (InsP₃R3)-specific antibody. The immunoprecipitated proteins were probed with antibody specific for phosphorylated Ser/Thr protein kinase C (PKC) substrate sequences. The sulfated cholecystokinin-8 (CCK-8S)-induced phosphorylation of InsP₃R3 was apparently inhibited by pretreatment with chelerythrine. Pretreatment with lorglumide (5 μmol/L) could significantly reduce the CCK-8S intensified phosphorylation of InsP₃R3. In the positive control group, treatment of cells with phorbol-12-myristate-13-acetate (PMA) also resulted in an enhanced phosphorylation of InsP₃R3; B: The nitrocellulose membrane in A was stripped and reprobed with InsP₃R3 (1:1000) to determine the levels of InsP₃R3 immunoprecipitated. Each cell sample contained nearly equal amounts of InsP₃R3.

controlling GI motility. Our study indicated that CCK-evoked gastric contraction is probably mediated through direct action on CCK₁R located on the ICC. However, with respect to GI motility, both the ICC network and the gastrointestinal nervous system play essential roles in producing highly coordinated peristalsis^[27,28]. Additional studies are needed to investigate the role of interactions between the enteric neurons and the ICC in CCK-evoked effect.

In the GI ICC, the cytoplasmic Ca²⁺ oscillation is responsible for the pacemaker activity. The pacemaker activity is generated in the ICC and then transferred to smooth muscle cells through the gap junctions^[1,29]. In this study, we proved that CCK-8S markedly increased [Ca²⁺]_i in cultured gastric antrum ICC, and the biological effects of CCK-8S were mainly mediated *via* CCK₁R located on the ICC. Two major mechanisms are involved in [Ca²⁺]_i increment during the contraction: calcium release of endoplasmic reticulum Ca²⁺ store, and/or calcium influx from the extracellular space through activation of calcium channels^[29]. We have shown that emptying of the intracellular calcium stores by thapsigargin completely blocked the enhancement effect of CCK-8S on the [Ca²⁺]_i level of ICC. Removing extracellular calcium could also inhibit the effect of CCK-8S, but not abolish it. Therefore, both mechanisms mediate the CCK-8S action and Ca²⁺ release from intracellular stores appears to be the major mechanism. The CCK-8S-induced [Ca²⁺]_i increment could be abolished by blockage of InsP₃R, suggesting a predominant role of InsP₃R-operated stores in CCK-8S-induced intracellular Ca²⁺ release. However, the CCK-8S-evoked [Ca²⁺]_i increment was persistent in the presence of L-type calcium channel blocker nifedipine, indicating that the [Ca²⁺]_i release is not activated by the influx of extracel-

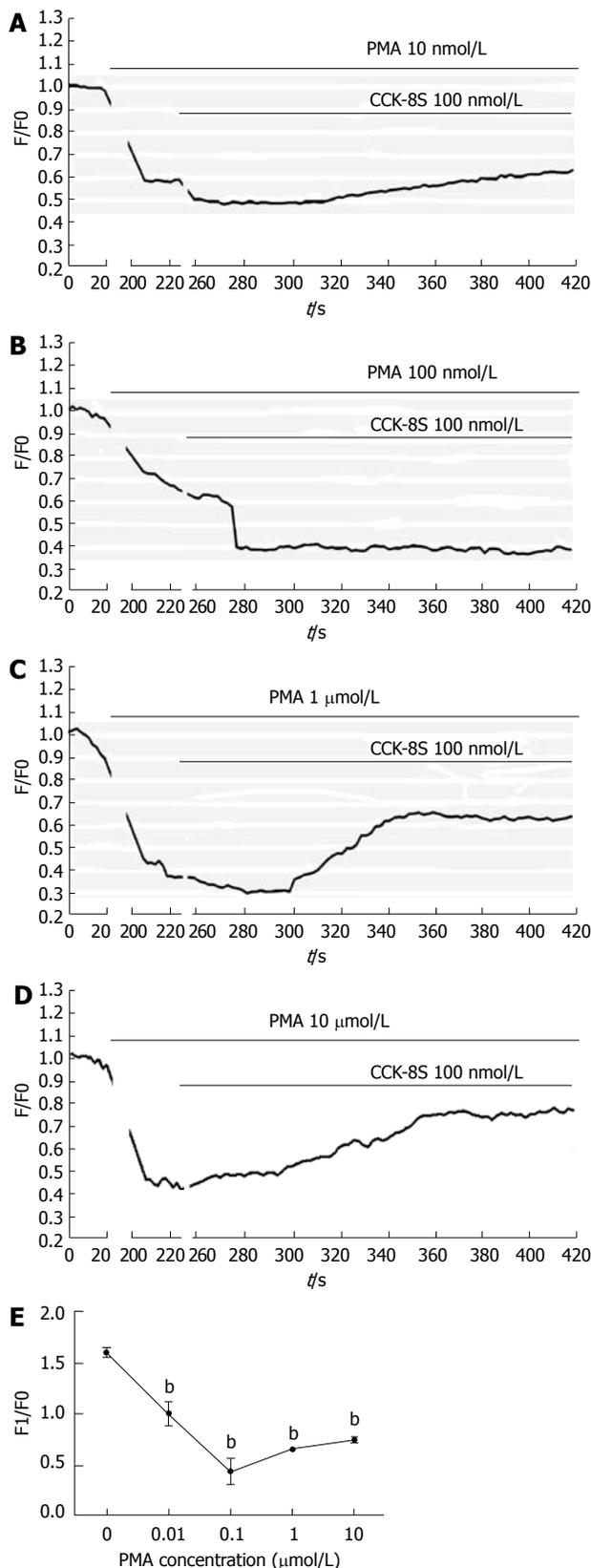


Figure 6 Effects of phorbol-12-myristate-13-acetate on sulfated cholecystokinin-8-evoked Ca²⁺ signaling in interstitial cells of Cajal. Fluo-3-loaded interstitial cells of Cajal were pretreated with various concentrations of Phorbol-12-myristate-13-acetate (PMA) (A: 10 nmol/L; B: 100 nmol/L; C: 1 μmol/L; D: 10 μmol/L) for 4 min before addition of sulfated cholecystokinin-8 (CCK-8S) (100 nmol/L). All could significantly reduce the CCK-8S-evoked [Ca²⁺]_i response; E: Quantification of [Ca²⁺]_i changes shown in A-D. Each experiment was repeated at least three times. ^bP < 0.01 vs control.

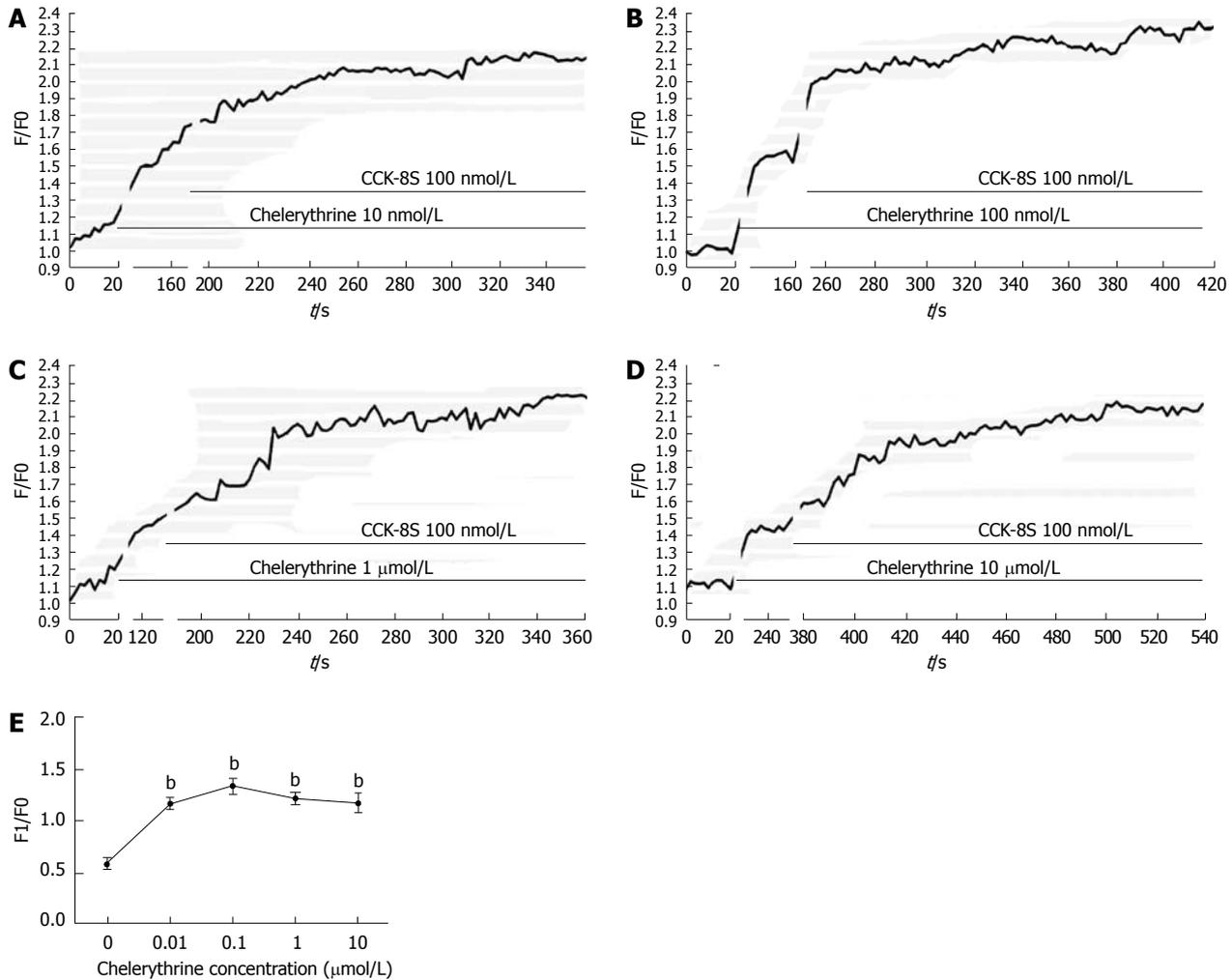


Figure 7 Effects of chelerythrine on Sulfated cholecystokinin-8-evoked Ca²⁺ signaling in interstitial cells of Cajal. Fluo-3-loaded interstitial cells of Cajal were pretreated with various concentrations of chelerythrine (A: 10 nmol/L; B: 100 nmol/L; C: 1 μmol/L; and D: 10 μmol/L) for 2 min before administration of cholecystokinin-8 (CCK-8S) (100 nmol/L). All could significantly increase the CCK-8S-evoked [Ca²⁺]_i response; E: Quantification of [Ca²⁺]_i changes shown in A-D. Each experiment was repeated at least three times. ^bP < 0.01 vs control.

lular calcium, while the influx of extracellular calcium can facilitate the [Ca²⁺]_i increase evoked by CCK-8S.

InsP₃R_s are a family of Ca²⁺ channels of the endoplasmic reticulum (ER) that are widely distributed in different tissues^[30,31]. InsP₃ triggers opening of the InsP₃R, which can rapidly release Ca²⁺ stored in the ER into the cytosol, generating a transient increase of [Ca²⁺]_i^[32]. The kinetics of [Ca²⁺]_i response depends on the amount of InsP₃, but many other signaling pathways participate in modulating the response. Among them, phosphorylation of InsP₃R by a series of kinases has been reported^[16,33,34]. In the present study, increased phosphorylation of InsP₃R3 in gastric ICC induced by CCK-8S was significantly prevented by pretreatment with the PKC specific inhibitor chelerythrine, while treatment of cells with the PKC activator PMA alone resulted in an enhanced phosphorylation of InsP₃R3, indicating an important regulatory role of PKC activation in this event. In addition, PMA potently reduced the peak of CCK-8S-induced calcium oscillation, while chelerythrine exhibited an opposite

effect. Thus, we might conclude that the activation of PKC negatively regulates CCK-8S-evoked calcium mobilization by phosphorylation of InsP₃R3. It is consistent with our previous study in other cells^[18]. The inhibition of Ca²⁺ release by PKC may be very useful to avoid full ER-Ca²⁺ emptying after agonist stimulation, which could have deleterious effects for the cell, first because of the waste of energy that would result from having a full ER-Ca²⁺ emptying after each agonist stimulation and second because of the effects of ER-Ca²⁺ depletion in terms of triggering the activation of stress signaling pathways^[35,36].

In summary, data obtained in our study suggest that: (1) CCK-8S could evoke calcium mobilization in cultured ICC; (2) the biological effects of CCK-8S are probably *via* CCK₁R located on the ICC; (3) this process is mainly mediated by the release of InsP₃-dependent intracellular Ca²⁺ from ER; and (4) CCK-8S could activate PKC simultaneously, which increases the phosphorylation of InsP₃R3 to negatively regulate the intracellular Ca²⁺ release in the ICC.

COMMENTS

Background

The interstitial cells of Cajal (ICC) are pacemaker cells in the gastrointestinal (GI) tract; their pacemaker activity is mediated by rhythmic intracellular Ca^{2+} oscillation. Cholecystokinin (CCK) contributes to the regulation of GI motility. To date, the mechanisms of the regulatory effects of CCK remain unclear. The finding that ICC express CCK₁ receptor (also known as CCK-AR) suggests a role for ICC in the mediation of CCK effects. It is worth investigating the direct effect of CCK on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in the ICC.

Research frontiers

Many neurotransmitters, including circulating hormones, modulate the ICC pacemaker activity. However, the effect of CCK on GI ICC remains unclear.

Innovations and breakthroughs

In the present study, the authors found that CCK evoked the calcium signaling in the ICC mainly *via* the Ca^{2+} release of $InsP_3R$ -operated stores and CCK activated protein kinase C simultaneously to negatively regulate the release of Ca^{2+} stores. The findings provide a novel insight into the mechanisms of the regulatory effects of CCK on GI motility.

Applications

The findings may provide clues to the mechanisms of the effects of CCK and its analogs in the treatment of GI motility disorders to some extent.

Peer review

The authors investigated the effect of sulfated cholecystokinin octapeptide on calcium mobilization in cultured murine gastric antral interstitial cells of Cajal and its possible mechanisms. They found that cholecystokinin octapeptide could evoke the $[Ca^{2+}]_i$ signaling in interstitial cells of Cajal through the Ca^{2+} release of $InsP_3R$ -operated stores. Additionally, CCK-8S activated PKC simultaneously to negatively regulate the release of $[Ca^{2+}]_i$ stores. The manuscript is highly interesting.

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Establishment of a rat liver transplantation model with prolonged biliary warm ischemia time

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Abstract

AIM: To investigate the impact of different time points of secondary warm ischemia on bile duct in a rat autologous liver transplantation model with external bile drainage.

METHODS: One hundred and thirty-six male inbred SD rats were randomly assigned to one of four groups (I-IV) according to the secondary warm ischemia time of 0, 10, 20 and 40 min. A rat model of autologous liver transplantation with continuous external biliary drainage under ether anesthesia was established. Ten rats in each group were used to evaluate the one-week survival rate. At 6 h, 24 h, 3 d and 7 d after reperfusion of the hepatic artery, 6 rats were killed in each group to collect the blood sample via the infrahepatic vena cava and the median lobe of liver for assay. Warm ischemia time of liver, cold perfusion time, anhepatic

phase, operative duration for biliary external drainage and survival rates in the four groups were analyzed for the establishment of models.

RESULTS: No significant difference was shown in warm ischemia time, anhepatic phase and operative duration for biliary external drainage among the four groups. Five of the 40 rats in this study evaluated for the one-week survival rate died, including three deaths of severe pulmonary infection in group IV. A significant decrease of one-week survival rate in group IV was noted compared with the other three groups. With the prolongation of the biliary warm ischemia time, the indexes of the liver function assessment were significantly elevated, and biliary epithelial cell apoptosis index also increased. Pathological examinations showed significantly aggravated inflammation in the portal area and bile duct epithelial cell injury with the prolonged secondary warm ischemia time. Microthrombi were found in the micrangium around the biliary tract in some sections from groups III and IV.

CONCLUSION: The relationship between secondary warm ischemia time and the bile duct injury degree is time-dependent, and 20 min of secondary warm ischemia time is feasible for the study of bile duct injury.

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Key words: Bile duct; Liver; Transplantation; Warm ischemia; Rat

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INTRODUCTION

Liver transplantation has dramatically improved the prognosis, morbidity and mortality of the patients with end-stage liver diseases. Of all liver transplant recipients, 10%-40% develop biliary complications associated with a mortality rate of 8%-15%^[1,2]. With the improvement of surgical techniques, the incidence of anastomotic biliary strictures decreased remarkably, whereas the nonanastomotic biliary strictures (NAS) have become the major type of biliary complications of liver allograft. NAS, which is also called ischemic cholangiopathy, appears early during the immediate postoperative period, and it is characterized by biliary strictures and dilatations at any location in the biliary system of the transplanted liver^[3].

Knowledge about the pathogenesis of NAS is slowly emerging from clinical and experimental studies performed during the last decade. The cause of NAS is multifactorial, and ischemia/reperfusion injury of the biliary epithelium is considered as one of the major causes^[4]. The most commonly used procedure for revascularization of the liver graft in clinical practice is initial portal reperfusion and subsequent reconstruction of the hepatic artery. Compared with liver cells, the bile duct epithelial cells experience an extra ischemia process [the time from portal vein (PV) recanalization to hepatic artery (HA) recanalization], which is "secondary warm ischemia time in the biliary tract (SWIT)" or "relative warm ischemia time in the biliary tract". This is the special phase of biliary tract warm ischemia in the graft.

During the transplantation process, the warm ischemia of biliary tract includes temporal warm ischemia of liver graft during procurement and secondary warm ischemia in the biliary tract. Because warm ischemic time in the harvesting of donor liver after cardiac death (DCD) is inevitable, more and more studies focus on the effect of secondary warm ischemia time on bile duct injury^[5]. In this study, we investigated the impact of different time points of secondary warm ischemia in a rat autologous liver transplantation model with external bile drainage.

MATERIALS AND METHODS

Animals and experimental groups

One hundred and thirty-six male inbred SD rats weighing 220-250 g were purchased from the Animal Center of Yangzhou University (Yangzhou, China). The rats were housed and fed at the Animal Center of Drum Tower Hospital for least 7 d before transplantation to acclimate them to the environment. All rats were provided with standard laboratory chow and water and housed in accordance with institutional animal care policies. Prior to the study, the rats were fasted for 8 h and were allowed free access to water.

The following experimental protocol was approved by the Animal Care and Use Committee of the Drum Tower Hospital and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

All rats were randomly assigned to four experimental groups according to the secondary warm ischemia time. Group I had no SWIT, in which simultaneous reperfusion was performed through the PV and HA after cold perfusion. The SWIT in groups II-IV was 10, 20 and 40 min, in which blood reperfusion of HA was performed 10, 20 or 40 min after PV reperfusion, respectively. Ten rats in each group were used to evaluate the one-week survival rate.

Surgical procedures

A rat model of autologous liver transplantation with continuous external biliary drainage under ether anesthesia was established in this study. It was described as follows:

A midline incision was made on the abdomen. The ligaments around the liver were dissociated and severed. The left diaphragm vein, hepatoesophageal ligament vein and right adrenal vein were separated, ligated and severed. The liver was turned left, and the suprahepatic vena cava (SHVC) was anatomized. And the common bile duct, HA, and PV were anatomized over the margin of the duodenal bulb. The infrahepatic vena cava (IHVC) was dissociated downwards about 6-8 mm. The liver was dissociated completely except for the hepatic blood vessels in and out, and the common bile duct.

A clamp was placed on the crossing of the cranial mesenteric vein and splenic vein, and the PV was pricked with a transfixion pin and fixed by another clamp. Then heparin saline (35 U/mL, 3 mL) was injected to make the rat heparinized and allow liver blood to enter the general circulation.

The abdominal aorta was pricked between the common iliac artery and left renal artery with pinhead and fixed by a clamp, and it was blocked from the area above the celiac trunk to the area below the puncture point by a clamp. The clamps were placed on the SHVC and IHVC with IHVC outflow tract left before cold perfusion. Lactated Ringer's solution of 20 mL (4 °C, containing heparin 12.5 U/mL) was perfused at 2.5 mL/min through the PV by an infusion pump and the abdominal aorta, respectively. The liver surface was covered with 4 °C lactated Ringer's solution to lower the temperature. The normal anatomical position of liver was maintained to avoid uneven perfusion. Subsequently, the color of the liver faded (Figure 1A).

The transfixion pin was removed after the perfusion, the PV and abdominal aorta were repaired using 9-0 prolene suture, and the IHVC outflow tract was restored with 8-0 prolene suture. The clamp was loosened to check whether the restoration was successful. The HA was clamped for the removal of the pre-remained line in groups II-V, then the PV, abdominal aorta, SHVC and IHVC were unclamped to end the anhepatic phase. The

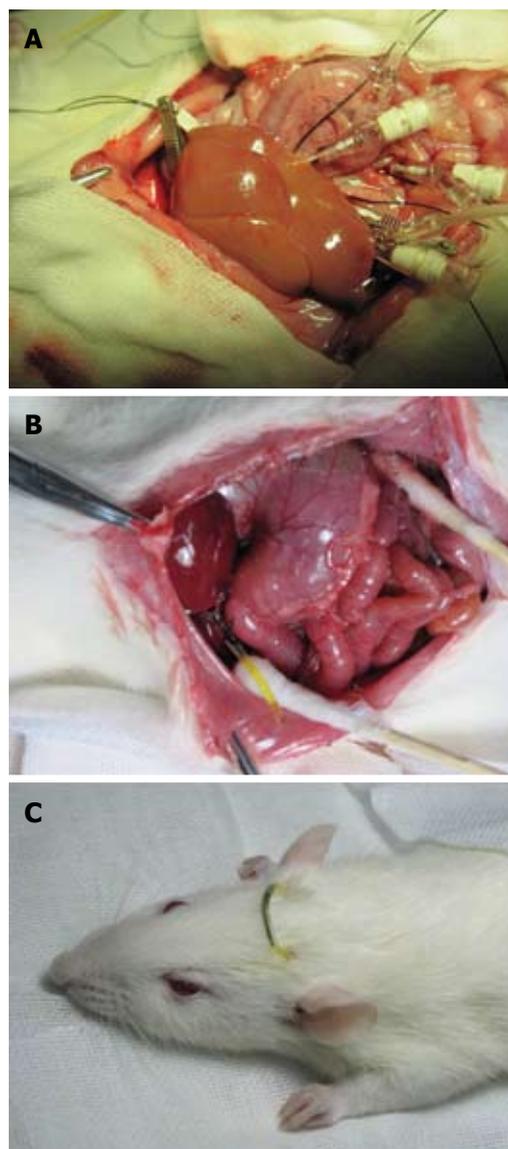


Figure 1 Surgical procedure for a rat model of autologous liver transplantation with continuous external biliary drainage. A: Lactated Ringer's solution was perfused by an infusion pump through the portal vein and the abdominal aorta; B: A polyethylene catheter was inserted into the common bile duct for external biliary drainage; C: The free ends of external biliary drainage tube and jejunal fistula tube were connected.

liver surface was covered with 38 °C normal saline (20 mL) for rapid rewarming. Thus the liver was filled with blood and turned red. The intestinal tract was placed back to the abdominal cavity during the secondary warm ischemia-reperfusion. The abdominal cavity was covered with wet gauze and heated by a lamp to maintain normal temperature.

The distal end of the bile duct was ligated and a polyethylene catheter (inner diameter 0.8 mm, outer diameter 1.2 mm) was inserted into the common bile duct for external biliary drainage. A jejunal fistula was established to return the remaining bile into the enterohepatic circulation. A 15-cm portion of polyethylene catheter (inner diameter 1.2 mm, outer diameter 1.5 mm) was inserted approximately 2 cm into jejunum through a stab in the je-

junum wall and fixed to the peritoneum (Figure 1B). The free ends of external biliary drainage tube and jejunal fistula tube were brought out of the body through a stab on the back of the rat's neck and connected with a short hypodermic needle (Figure 1C).

Sample collection

At 6 h, 24 h, 3 d and 7 d after reperfusion of the hepatic artery, six rats were killed in each group to collect blood sample *via* the infrahepatic vena cava and the median lobe of liver for assay. The serum was separated and stored at -70 °C until analysis. Washed with cold saline solution, the liver samples were stored immediately in liquid nitrogen until analysis.

Analysis of establishment of models

Analysis of establishment of models included warm ischemia time of liver (the time from abdominal aorta clamping to cold perfusion of liver), cold perfusion time, anhepatic phase (the time from PV clamping to PV reperfusion), operative duration for biliary external drainage and survival rates in the four groups.

Liver function assessment

The liver function assessment included measurements of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AKP), total bilirubin (TB) and direct bilirubin (DB). The serum samples were collected at 6 h, 24 h, 3 d and 7 d after reperfusion of HA and liver function test was conducted by automatic biochemistry analyzer (HITACHI 7600).

Apoptosis assay of bile duct epithelia

Apoptosis of biliary tract epithelia was identified by detecting DNA fragmentation *in situ* in serial sections at 6 and 24 h after HA reperfusion. DNA fragmentation was detected by TUNEL staining, which was performed on deparaffinized and dehydrated sections using the In Situ Cell Death Detection kit (Zhongshan Biomedical Technology Co., Beijing, China) according to the manufacturer's instructions. TUNEL-positive cholangiocytes displayed a characteristic morphology of apoptosis, such as chromatin condensation, cell fragmentation and apoptotic bodies. Apoptotic cells were examined at original magnification $\times 400$ in 10 randomly chosen fields per section. The apoptotic index was calculated as percentage of apoptotic cells related to the total number of cholangiocytes.

Histological evaluation of bile duct injury

Six hepatic specimens were collected at 24 h after reperfusion of HA in each group. The liver specimens for light microscopy were fixed with 10% formalin and then embedded in paraffin. The sections were stained with hematoxylin and eosin for histological examination. Bile duct injury in specimens was semi-quantified by calculating a bile duct injury severity score (BDISS)^[6] based on the following three components: bile duct damage (graded

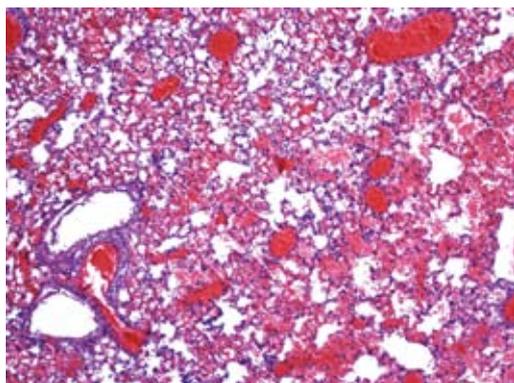


Figure 2 Pathological examination of the lung after the death of three rats in group IV (hematoxylin-eosin; original magnification $\times 400$).

as 0, absent; 1, mild; 2, moderate; and 3, severe; modified from the Banff criteria for defining acute rejection); ductular proliferation (graded 0-3, using a similar scale as stated earlier); and cholestasis (graded 0-3, using a similar scale as stated earlier). This resulted in a minimal BDISS of zero and a maximum score of nine points. All examinations were conducted by an experienced pathologist who was unaware of the other study data.

Statistical analysis

Results were expressed as mean \pm SD. Numerical data was analyzed with Statistical Analysis System. One-way repeated measures analysis of variance with the Student-Newman-Keuls test was performed for multiple comparisons to test the effect of time, groups, and the interaction between the time and groups. A *P* value of < 0.05 was considered significant.

RESULTS

Analysis of establishment of models

The cold perfusion time in each group was eight minutes with the use of an infusion pump. No significant difference was shown in warm ischemia time, anhepatic phase and operative duration for biliary external drainage among the four groups ($P > 0.05$). Five of the 40 rats in this study evaluating the one-week survival rate died, including one death of bleeding caused by unsuccessful abdominal aorta repair (group III), one of sudden respiratory arrest after HA reperfusion (group IV) and three of severe pulmonary infection (group IV). The result of autopsy after the death of three rats in group IV showed serious pulmonary vascular exudation and edema accompanied with pulmonary consolidation. Pathological examination revealed obvious leucocyte infiltration in alveolar cavity and pulmonary interstitium, numerous inflammatory exudates around bronchi, and alveolar wall damage with microvascular endothelial cell injury and bleeding (Figure 2). A significant decrease of one-week survival rate in group IV was noted compared with the other three groups ($P < 0.05$, Table 1).

Table 1 Analysis of establishment of models

Group	Warm ischemia time (min)	Anhepatic phase (min)	Operative duration for biliary external drainage (min)	1-wk survival rate (%)
I	3.1 \pm 0.5	18.1 \pm 1.2	6.2 \pm 1.2	100
II	3.0 \pm 0.4	17.6 \pm 1.5	7.1 \pm 1.4	100
III	3.3 \pm 0.3	18.5 \pm 1.1	6.7 \pm 1.6	90
IV	3.2 \pm 0.5	17.9 \pm 1.7	6.5 \pm 2.0	60

Liver function assessment

A significant increase of ALT, AST, AKP, TB and DB was observed at 6 h, 24 h, day 3 and day 7 ($P < 0.05$) after operation in the three groups compared with the results in group I. The increased indexes of liver function test in group IV were higher than in groups II and III, and there were significant differences among these three groups at 6 h, 24 h, day 3 and day 7 after operation ($P < 0.05$).

ALT and AST reached the peak at 6 h after autologous liver transplantation, and very significant decrease was observed on post-operative days 3 and 7 ($P < 0.01$). Other liver function indexes, including AKP, TB and DB, reached the peak at post-operative 24 h, and there was significant decrease on post-operative days 3 and 7 ($P < 0.05$, Table 2).

Apoptosis assay of bile duct epithelia

The apoptosis index of bile duct epithelia in groups I at 6 and 24 h after hepatic artery reperfusion was 5.87 ± 0.50 and 7.13 ± 0.60 . Compared with group I, there was a significant increase of apoptosis index at 6 and 24 h in groups II and III ($P < 0.05$), and a very significant increase in group IV ($P < 0.01$). The increased apoptosis index in group IV was higher than in groups II and III, and there were significant differences among these three groups at post-operative 6 and 24 h ($P < 0.05$). In all groups, the apoptosis index at 24 h after hepatic artery reperfusion was higher than at 6 h, but no significant differences were noted ($P > 0.05$) (Table 3; Figure 3A and B).

Histological evaluation of bile duct injury

The predominant injuries of bile duct in group I included cholangiocytes lined in disorder, diversified morphous and edema, inflammatory cell infiltration, migrated chromatin, and the necrotic and caducous cell debris in the lumen under light microscope. The bile duct showed more histological changes in groups II and III, and the most significant injury occurred in group IV (Figure 4A and B). Microthrombi were found in the micrangium around the biliary tract in some sections from groups III and IV.

Compared with group I, there was a significant increase of BDISS at 6 and 24 h in groups II and III ($P < 0.05$), and a very significant increase of apoptosis in group IV ($P < 0.01$). The BDISS in group IV was higher than in groups II and III, and there were significant differences among the three groups at post-operative 6 and

Table 2 Effect of different secondary warm ischemia time on liver function (mean ± SD)

Liver function	Normal value	Group	6 h	24 h	3 d	7 d
ALT (U/L)	5-40	I	165.71 ± 31.42	140.22 ± 21.08	24.30 ± 12.04	20.15 ± 8.12
		II	356.15 ± 52.52 ^a	246.31 ± 22.93 ^a	46.43 ± 4.69 ^a	38.24 ± 3.52 ^a
		III	632.23 ± 32.28 ^{b,c}	444.32 ± 27.80 ^{b,c}	79.58 ± 17.93 ^{a,c}	56.12 ± 10.23 ^{a,c}
		IV	931.27 ± 20.21 ^{b,d,e}	801.81 ± 25.17 ^{b,d,e}	108.95 ± 11.81 ^{b,d,e}	71.26 ± 9.14 ^{b,d,e}
AST (U/L)	8-40	I	855.11 ± 28.20	553.62 ± 17.66	82.02 ± 21.14	52.13 ± 17.26
		II	1027.05 ± 42.02 ^a	837.31 ± 47.72 ^a	117.33 ± 30.86 ^a	84.24 ± 18.92 ^a
		III	1560.46 ± 68.39 ^{b,c}	1129.26 ± 137.09 ^{b,c}	173.48 ± 33.61 ^{a,c}	113.13 ± 25.72 ^{a,c}
		IV	2620.13 ± 123.68 ^{b,d,e}	2194.53 ± 297.70 ^{b,d,e}	250.10 ± 48.54 ^{b,d,e}	165.31 ± 41.26 ^{b,d,e}
AKP (U/L)	47-185	I	124.52 ± 26.26	154.13 ± 32.32	121.22 ± 14.21	91.52 ± 11.56
		II	206.54 ± 27.69 ^a	270.85 ± 28.48 ^a	189.74 ± 24.28 ^a	126.51 ± 16.15 ^a
		III	297.40 ± 34.14 ^{b,c}	363.36 ± 30.73 ^{b,c}	295.90 ± 22.04 ^{b,c}	163.26 ± 13.29 ^{b,c}
		IV	417.06 ± 10.82 ^{b,d,e}	445.81 ± 33.49 ^{b,d,e}	327.92 ± 12.36 ^{b,c,e}	227.84 ± 32.16 ^{b,c,e}
TB (mg/dL)	0.3-1.2	I	0.82 ± 0.26	1.42 ± 0.27	0.82 ± 0.24	0.70 ± 0.21
		II	1.13 ± 0.17 ^a	1.52 ± 0.10	0.93 ± 0.11	0.81 ± 0.16
		III	2.12 ± 0.20 ^{a,c}	2.54 ± 0.19 ^{a,c}	1.37 ± 0.20 ^{a,c}	1.04 ± 0.18 ^{a,c}
		IV	2.64 ± 0.28 ^{b,c,e}	3.43 ± 0.19 ^{b,d,e}	2.34 ± 0.27 ^{b,c,e}	1.96 ± 0.28 ^{b,c,e}
DB (mg/dL)	0.1-0.4	I	0.83 ± 0.17	1.06 ± 0.11	0.64 ± 0.11	0.52 ± 0.21
		II	1.02 ± 0.11	1.33 ± 0.12 ^a	0.83 ± 0.12	0.71 ± 0.16
		III	2.05 ± 0.22 ^{a,c}	2.36 ± 0.20 ^{a,c}	1.45 ± 0.22 ^{a,c}	1.32 ± 0.19 ^{a,c}
		IV	2.36 ± 0.12 ^{b,c,e}	2.75 ± 0.12 ^{a,c,e}	2.23 ± 0.20 ^{b,d,e}	2.02 ± 0.26 ^{b,d,e}

^a*P* < 0.05, ^b*P* < 0.01 vs group I; ^c*P* < 0.05, ^d*P* < 0.01 vs group II; ^e*P* < 0.05 vs group III. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AKP: Alkaline phosphatase; TB: Total bilirubin; DB: Direct bilirubin.

Table 3 Effect of different timing of secondary warm ischemia on apoptosis index of bile duct epithelia and bile duct injury severity score (mean ± SD)

Group	Apoptosis index of bile duct epithelia		Bile duct injury severity score	
	6 h	24 h	6 h	24 h
I	5.87 ± 0.50	7.13 ± 0.60	2.5 ± 0.3	2.9 ± 0.2
II	7.58 ± 0.31 ^a	8.96 ± 0.67 ^a	3.6 ± 0.3 ^a	3.7 ± 0.4 ^a
III	8.98 ± 0.69 ^{a,c}	9.90 ± 0.48 ^{a,c}	4.7 ± 0.2 ^{a,c}	5.1 ± 0.3 ^{a,c}
IV	11.99 ± 0.49 ^{b,c,e}	13.08 ± 0.65 ^{b,d,e}	6.1 ± 0.4 ^{b,e}	6.5 ± 0.2 ^{b,c,e}

^a*P* < 0.05, ^b*P* < 0.01 vs group I; ^c*P* < 0.05, ^d*P* < 0.01 vs group II; ^e*P* < 0.05 vs group III.

24 h (*P* < 0.05). In all groups, BDISS at 24 h after hepatic artery reperfusion was higher than at 6 h, but no significant differences were noted (*P* > 0.05, Table 3).

DISCUSSION

Grafts from DCD are used to increase the number of organs available for liver transplantation, and warm ischemic time in the donor in addition to subsequent cold ischemia-reperfusion injury is believed to result in increased damage to biliary epithelial cells^[7,8]. It is associated with a higher risk of biliary strictures, and the incidence of NAS after DCD ranged from 10% to 30% compared with an incidence of 1%-10% after brain death^[9-11]. The present experiment used a rat model of autologous orthotopic liver transplantation to simulate I/R injury of the biliary tract, which simulated the whole process of clinical liver transplantation. This model decreased the possibility of blood vessel or vascular anastomosis damage compared with allogeneic orthotopic liver transplantation, and it avoided the effects of immuno-

logic rejection. This model is simple and has a high successful rate, better reflects the pathophysiologic process of bile ducts, and provides an approach for investigating the intrahepatic bile duct injury in liver transplantation caused directly by I/R injury^[12].

More and more studies have focused on the role of bile salt toxicity in the development of bile duct injury after transplantation, and bile composition analysis may be important in evaluating the liver function^[13-15]. Establishing a stable model of orthotopic liver transplantation with external biliary drainage in rats is needed to provide the possibility for dynamic detection of bile characteristics after transplantation. But the lack of bile in the gut lumen is one of the factors associated with an increase in translocation of bacteria through the intestinal mucosa. It has been proposed that the intestinal bile flow is important to the immunity because bile salt has a dispersion effect on lipopolysaccharide and endotoxin, which had been proved to correlate with suppression of cellular immunity^[16]. In experimental models, internal biliary drainage resulted in better systemic immune status, and improved intestinal barrier and mucosal immune functions^[17]. The operative duration for biliary external drainage in this study was 6.7 ± 1.7 min, and no rat died of biliary complication. The biliary extra-drainage model in rat autologous liver transplantation in this study provides a simple and reliable method for dynamic collection of bile, and it could be applied in various experimental studies.

Hepatocytes are supplied by both the hepatic artery and the portal vein, but bile ducts entirely depend on arterial blood supply for oxygenation. The terminal arteriole of hepatic artery branches off into the peribiliary plexus (PBP), which supplies the intrahepatic bile ducts. Therefore, the changes of PBP usually altered the intra-

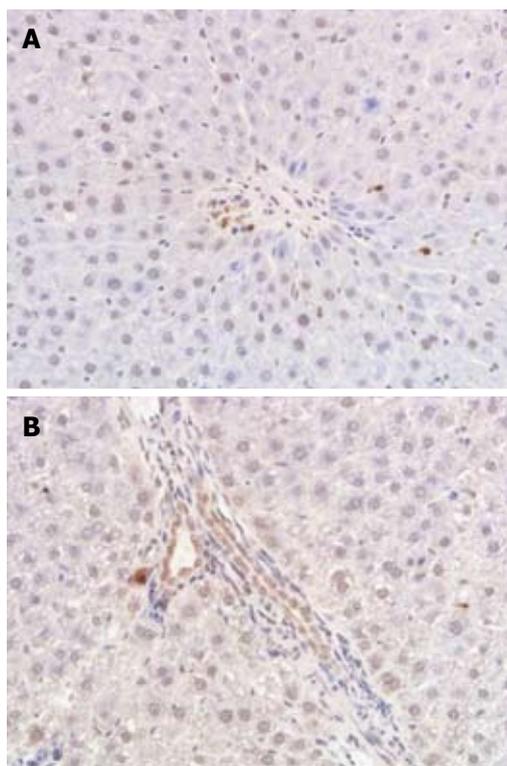


Figure 3 With the prolongation of secondary biliary warm ischemia time, more biliary epithelial cells became apoptotic. A: Biliary epithelial cell apoptosis in group I at 24 h after hepatic arterial (HA) reperfusion; B: Biliary epithelial cell apoptosis in group IV at 24 h after HA reperfusion (hematoxylin-eosin; original magnification $\times 400$).

hepatic bile duct structure. Post-transplantational hepatic artery ischemia induces ischemia and occlusion of PBP, which is the vital reason leading to aggravation of ischemia of intrahepatic bile ducts^[18-20]. The pathomorphologic changes of biliary tract showed that the relationship between secondary ischemia time and pathological structural injury was time-dependent, and the biliary tract injury in group IV was most serious among the four groups in this study. Microthrombi were found in the microcirculation around the biliary tract in some sections from groups III and IV. Accordingly, the serum levels of liver function increased with the prolonged second warm ischemia time. The levels of AKP, TB and DB, in particular, revealed the injury of the bile duct, which became worse with the longer second warm ischemia time.

Bile duct epithelia are highly susceptible to reoxygenation after anoxia^[21]. The increased susceptibility to reoxygenation injury by cholangiocytes is associated with increased production of toxic reactive oxygen species by cholangiocytes during reoxygenation with concomitant low basal levels of the antioxidant glutathione in these epithelial cells^[22]. Apoptosis is one of the two mechanisms by which cell death occurs (the other is the pathological process of necrosis). Accumulating evidence suggests that apoptosis plays an important role in ischemia-reperfusion injury in organ transplantation^[23,24] and it is widely taken as a reference index to evaluate bile duct epithelial injury. With the prolongation of the biliary warm ischemia time,

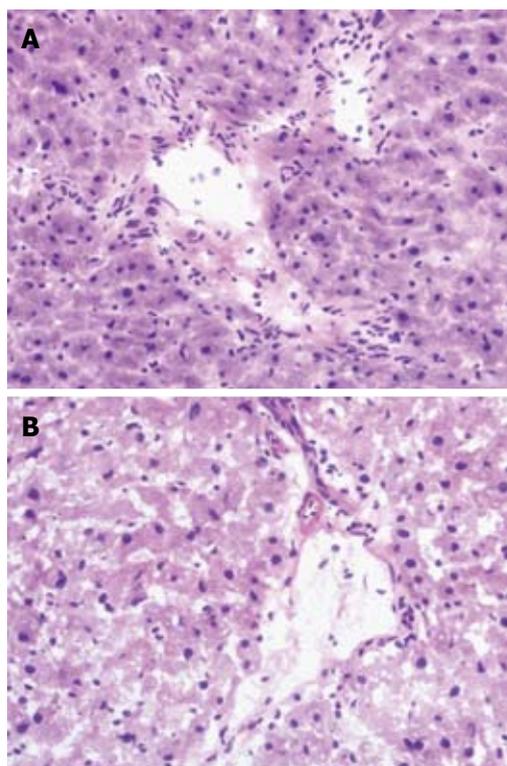


Figure 4 Histological examination of the liver at 24 h after hepatic arterial reperfusion. A: Cholangiocyte injury can be found in group I; B: More marked injury occurs in group IV (hematoxylin-eosin; original magnification $\times 400$).

the biliary epithelial cell apoptosis index was significantly elevated, and the apoptosis index at 24 h after hepatic artery reperfusion was higher than at 6h, but with no significant differences.

In conclusion, the relationship between secondary warm ischemia time and the bile duct injury degree is time-dependent. Because of a lower one-week survival rate in the 40 min group, 20 min of secondary biliary warm ischemia time is feasible for the study of bile duct injury in a rat autologous liver transplantation model with external bile drainage.

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COMMENTS

Background

With the improvement of surgical techniques, the incidence of anastomotic biliary strictures after liver transplantation decreased remarkably, whereas the nonanastomotic biliary strictures became the major type of biliary complications of liver allograft. Diffuse non-anastomotic biliary strictures remain the most challenging type of biliary complication as they are frequently therapy-resistant and often associated with long-term consequences.

Research frontiers

Warm ischemic time in donation after cardiac death in addition to subsequent cold ischemia-reperfusion injury is believed to result in increased damage to biliary epithelial cells. Compared with liver cells, the bile duct epithelial cells experience an extra ischemia process, which includes the time from portal vein

recanalization to hepatic artery recanalization, and this is "secondary warm ischemia time in the biliary tract" or "relative warm ischemia time in the biliary tract". This is the special phase of biliary tract warm ischemia in the grafts, and more and more studies have focused on the effect of secondary warm ischemia time on bile duct injury.

Innovations and breakthroughs

Because warm ischemic time in the harvesting of donor liver after cardiac death is inevitable, more and more studies have investigated the effect of secondary warm ischemia time on bile duct injury. In this study, the authors investigated the impact of different time points of secondary warm ischemia in a rat autologous liver transplantation model with external bile drainage. This model provides a simple and reliable method for dynamic collection of bile, and can be applied in various experimental studies.

Applications

This study demonstrated that the relationship between secondary warm ischemia time and the bile duct injury degree is time-dependent. Because of the lower one-week survival rate in the 40 min group, the authors proposed that 20 min of secondary biliary warm ischemia time should be feasible for the study of bile duct injury in a rat autologous liver transplantation model with external bile drainage.

Terminology

This is a well designed and conducted experimental study with very interesting results. The literature review was adequate. The materials and methods were adequate and coincident to the objective of the study. Newer studies with your rat model with prolonged secondary biliary will help us to elucidate unanswered questions in hepatobiliary surgery.

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Electroacupuncture alleviates stress-induced visceral hypersensitivity through an opioid system in rats

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Abstract

AIM: To investigate whether stress-induced visceral hypersensitivity could be alleviated by electroacupuncture (EA) and whether EA effect was mediated by endogenous opiates.

METHODS: Six to nine week-old male Sprague-Dawley rats were used in this study. Visceral hypersensitivity was induced by a 9-d heterotypic intermittent stress (HIS) protocol composed of 3 randomly stressors, which included cold restraint stress at 4 °C for 45 min, water avoidance stress for 60 min, and forced swimming stress for 20 min, in adult male rats.

The extent of visceral hypersensitivity was quantified by electromyography or by abdominal withdrawal reflex (AWR) scores of colorectal distention at different distention pressures (20 mmHg, 40 mmHg, 60 mmHg and 80 mmHg). AWR scores either 0, 1, 2, 3 or 4 were obtained by a blinded observer. EA or sham EA was performed at classical acupoint ST-36 (Zu-San-Li) or BL-43 (Gao-Huang) in both hindlimbs of rats for 30 min. Naloxone (NLX) or NLX methiodide (m-NLX) was administered intraperitoneally to HIS rats in some experiments.

RESULTS: HIS rats displayed an increased sensitivity to colorectal distention, which started from 6 h (the first measurement), maintained for 24 h, and AWR scores returned to basal levels at 48 h and 7 d after HIS compared to pre-HIS baseline at different distention pressures. The AWR scores before HIS were 0.6 ± 0.2 , 1.3 ± 0.2 , 1.9 ± 0.2 and 2.3 ± 0.2 for 20 mmHg, 40 mmHg, 60 mmHg and 80 mmHg distention pressures, respectively. Six hours after termination of the last stressor, the AWR scores were 2.0 ± 0.1 , 2.5 ± 0.1 , 2.8 ± 0.2 and 3.5 ± 0.2 for 20 mmHg, 40 mmHg, 60 mmHg and 80 mmHg distention pressures, respectively. EA given at classical acupoint ST-36 in both hindlimbs for 30 min significantly attenuated the hypersensitive responses to colorectal distention in HIS rats compared with sham EA treatment [AWRs at 20 mmHg: 2.0 ± 0.2 vs 0.7 ± 0.1 , $P = 4.23 \times 10^{-4}$; AWRs at 40 mmHg: 2.6 ± 0.2 vs 1.5 ± 0.2 , $P = 0.00163$; AWRs at 60 mmHg: 3.1 ± 0.2 vs 1.9 ± 0.1 , $P = 0.003$; AWRs at 80 mmHg: 3.6 ± 0.1 vs 2.4 ± 0.2 , $P = 0.0023$; electromyographic (EMG) at 20 mmHg: 24 ± 4.7 vs 13.8 ± 3.5 ; EMG at 40 mmHg: 60.2 ± 6.6 vs 30 ± 4.9 , $P = 0.00523$; EMG at 60 mmHg: 83 ± 10 vs 39.8 ± 5.9 , $P = 0.00029$; EMG at 80 mmHg: 94.3 ± 10.8 vs 49.6 ± 5.9 , $P = 0.00021$]. In addition, EA at the acupuncture point BL-43 with same parameters did not alleviate visceral hypersensitivity in HIS rats. EA in healthy rats also did not have any effect on AWR scores to colorectal distention at distention pressures

of 20 and 40 mmHg. The EA-mediated analgesic effect was blocked by pretreatment with NLX in HIS rats [AWR scores pretreated with NLX *vs* normal saline (NS) were $2.0 \text{ vs } 0.70 \pm 0.20$, $2.80 \pm 0.12 \text{ vs } 1.50 \pm 0.27$, $3 \text{ vs } 2.00 \pm 0.15$ and $3.60 \pm 0.18 \text{ vs } 2.60 \pm 0.18$ for 20 mmHg, 40 mmHg, 60 mmHg and 80 mmHg; $P = 0.0087$, 0.0104 , 0.0117 and 0.0188 for 20, 40, 60 and 80 mmHg, respectively]. Furthermore, EA-mediated analgesic effect was completely reversed by administration of m-NLX, a peripherally restricted opioid antagonist (EMG pretreated with m-NLX *vs* NS were $30.84 \pm 4.39 \text{ vs } 13.33 \pm 3.88$, $74.16 \pm 9.04 \text{ vs } 36.28 \pm 8.01$, $96.45 \pm 11.80 \text{ vs } 50.19 \pm 8.28$, and $111.59 \pm 13.79 \text{ vs } 56.42 \pm 8.43$ for 20 mmHg, 40 mmHg, 60 mmHg and 80 mmHg; $P = 0.05026$, 0.00034 , 0.00005 , 0.000007 for 20 mmHg, 40 mmHg, 60 mmHg and 80 mmHg, respectively).

CONCLUSION: EA given at classical acupoint ST-36 alleviates stress-induced visceral pain, which is most likely mediated by opioid pathways in the periphery.

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Key words: Irritable bowel syndrome; Visceral pain; Electroacupuncture; Opioid pathway; Stress

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INTRODUCTION

Irritable bowel syndrome (IBS) is a common gastrointestinal disorder characterized by chronic visceral pain and bloating in association with altered bowel movements^[1-3]. Chronic visceral pain, the cardinal feature of IBS, has been difficult to treat^[4-6]. Acupuncture is an ancient form of traditional Chinese medicine that can be traced back for more than 3000 years. The acupuncture procedure involves the insertion of thin needles into the skin and underlying muscle layer, which are termed acupuncture loci, or "acupoints". In traditional acupuncture, the needles are twisted right and left at 0.5- to 1-s intervals. More recently, acupuncture needles are stimulated by electricity at various frequencies (1-100 Hz), which is termed electroacupuncture (EA). EA has been used extensively for treatment of various painful

conditions and gastrointestinal diseases, including IBS, functional dyspepsia, constipation, and diarrhea^[7-9]. It has been shown that EA treatment results in a significant improvement both in general conditions and in symptoms of bloating^[10,11]. Combined EA at the acupuncture points ST-36 and PC-6 significantly increases the threshold of rectal sensations induced by rectal distension in IBS patients^[8,12,13], suggesting that EA might be a promising method to treat visceral pain in patients with IBS. However, research into EA for chronic visceral pain is still in its infancy, and much of the limited scientific evidence surrounding it is fragmentary and often contradictory^[14-16]. Thus, further investigations of EA efficacy and its mechanisms are definitely merited. Recently, we have developed a rat model of visceral hypersensitivity induced by heterotypic intermittent stress (HIS)^[17]. These rats displayed no robust inflammation or injury in the colon, but a significantly higher visceromotor response (VMR) to colorectal distention (CRD) compared with age-matched controls. Thus, the animal model resembles some characteristics of IBS seen in human patients. The aim of this study was to investigate whether EA has therapeutic benefits on visceral hypersensitivity induced by HIS, and if so, what is the underlying mechanism. We found that EA treatment at acupoint ST-36 significantly attenuated abdominal withdrawal reflexes (AWRs) in HIS rats at distention pressures of 20 and 40 mmHg, and in both stressed and non-stressed rats at distention pressures of 60 and 80 mmHg. Pretreatment with naloxone (NLX), an opioid receptor antagonist, or NLX methiodide (m-NLX), a selectively peripherally acting opioid receptor antagonist, completely reversed the EA effect in HIS rats.

MATERIALS AND METHODS

Animals

Six to nine week-old male Sprague-Dawley rats ($n = 94$) housed at approximately 22 °C with a 12-h light/dark cycle were used in this study. Care and handling of these animals were approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch and at Soochow University. The animals were euthanized by decapitation at various times indicated in the Result section after the end of *in vivo* behavioral studies. Compared with our previous report that HIS-induced visceral hypersensitivity returned to normal level 24 h after termination of the last stressor in Wistar rats^[17], HIS-induced visceral hyperalgesia lasted longer in SD rats than in Wistar rats. Therefore, SD rats were used in this experiment. Rats were grouped before experiment and were single-housed during experiments.

Heterotypic intermittent stress protocol

Rats were subjected to 9 consecutive days of a HIS protocol comprised of 3 randomly selected stressors, which included cold restraint stress (CRS) at 4 °C for 45 min, water avoidance stress (WAS) for 60 min, and forced

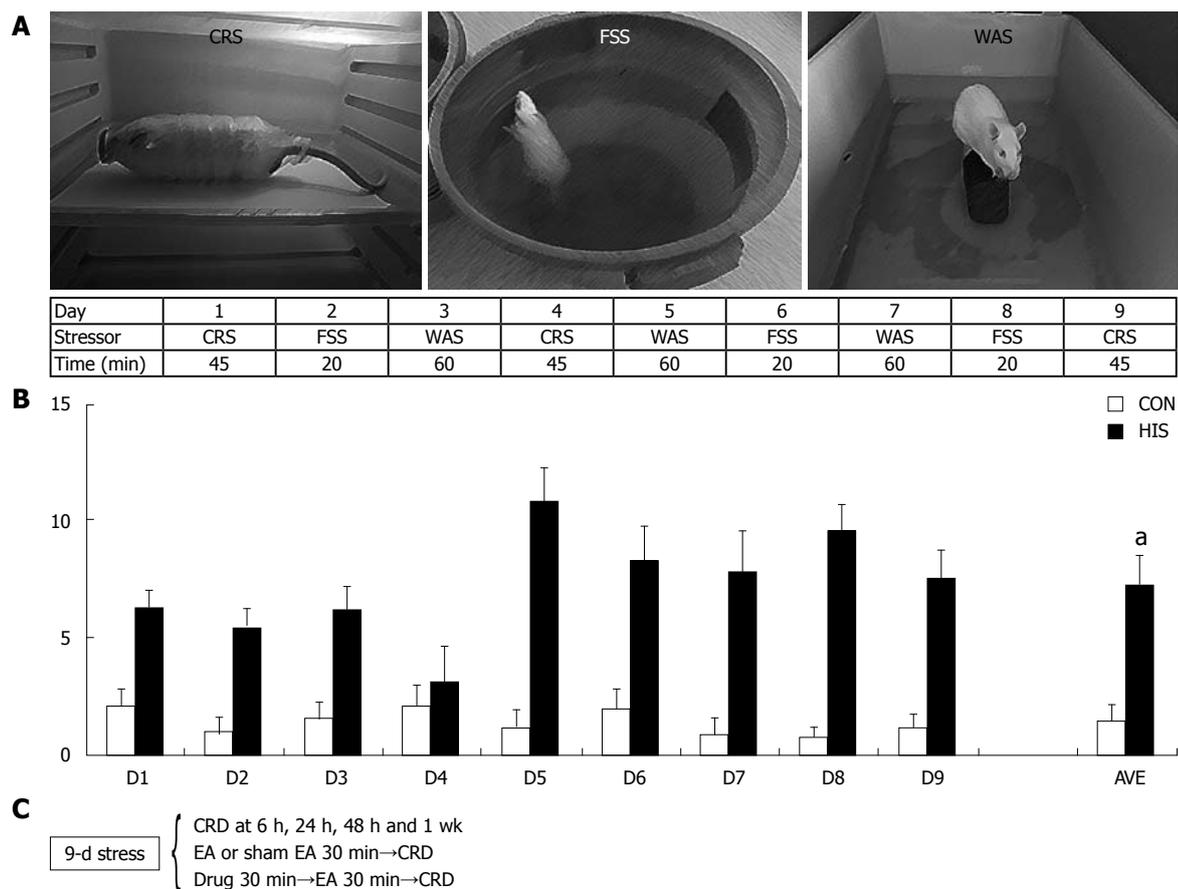


Figure 1 Heterotypic intermittent stress protocol and pellets count. **A:** Nine-day heterotypic intermittent stress (HIS) protocol comprising three different randomly arranged stressors; **B:** Stress accelerated colonic transit ($n = 10$ for control, $n = 8$ for HIS), which was accompanied by an increase in visceral hypersensitivity ($^{\#}P < 0.05$). **C:** A schematic representation of the various treatments: stressors, colorectal distention (CRD) and drug treatment with time sequence. CON: Control; AVE: The mean number of pellets for all 9-d stress protocol or from respective control rats; CRS: Cold restrained stressor; FSS: Forced swimming stressor; WAS: Water avoidance stressor.

swimming stress (FSS) for 20 min, as described previously^[17] and in Figure 1A. In brief, each stressor was applied between 8 am and 11 am. For CRS, the rats were restrained in a clear plastic container (6 cm in diameter \times 18 cm in length). The container had 2-cm diameter openings at each end for the rat to breathe normally. The restraining container was placed in cold room at 4 °C for 45 min. The CRS was given to rats on days 1, 4 and 9. For WAS, the rat was placed for 60 min on a brick (12 cm high \times 6 cm wide \times 8.5 cm long) in the middle of a plastic container (14 cm high \times 36 cm wide and \times 54 cm long) filled with water at room temperature (approximately 22 °C) within 1 cm from the top. The rats were subjected to WAS on days 3, 5 and 7. For FSS, the rat was forced to swim for 20 min in a plastic container (38 cm high \times 24 cm wide \times 32 cm long) filled to a depth of 12 cm below the top with water at room temperature (approximately 22 °C). The FSS was given to rats on days 2, 6 and 8. Age-matched control rats were brought to the laboratory and handled identically without the stress protocol.

Heterotypic intermittent stress was chosen because clinical findings suggest that long-term stress, rather than short-term stress, exacerbates symptoms of IBS. In

addition, variable stressors are less likely to produce adaptation when compared to repeated applications of the same stressor. The number of pellets during each stress protocol was counted for each rat in order to measure colonic transit during stress protocol (Figure 1B). It is remarkable that stress accelerated defecation rate when compared with controls (two sample *t* test, $P < 0.001$). The number of pellets in the stress situations is not time dependent as it is in the control situation. This is most likely due to the different stressors used. The increase in number of pellets and visceral hypersensitivity in the stress situations indicates that this model can mimic the major characteristics of patients with IBS and thus it is a suitable rat model for study of the effect and mechanisms of EA treatment.

Measurement of visceral hypersensitivity to graded colorectal distention

Electromyographic recordings. Visceral hypersensitivity was measured by electromyographic (EMG) measurements of VMR to CRD as described previously^[18]. Briefly, under anesthesia with isoflurane, 2 electrodes were implanted in the external oblique muscle and externalized behind the head. Rats were allowed 1 wk to

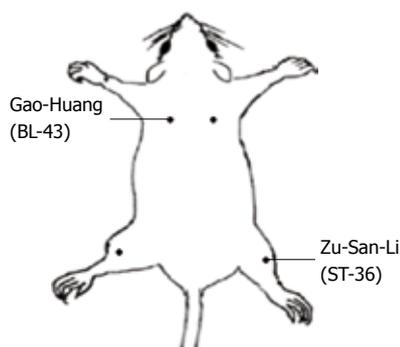


Figure 2 Schematic representation of electroacupuncture points. ST-36 (Zu-San-Li) is thought to be relevant to gastrointestinal tract while Gao-Huang is not.

recover from the surgery. After recovery, baseline EMG measurement was recorded, and then 9-d stress protocol was applied. After termination of last stressor, the EMGs were recorded again from these rats. In some cases, EA or sham EA was applied during the stress protocol. Under anesthesia with isoflurane, a flexible balloon (5 cm) constructed from a surgical glove finger attached to a tygon tubing was inserted 8 cm into the descending colon and rectum *via* the anus and held in place by taping the tubing to the tail. Rats were placed in small Lucite cubicles (20 cm × 8 cm × 8 cm) (Bioengineering Department, University of Texas Medical Branch, Galveston, TX) and allowed to adapt for 30 min. CRD was performed by rapidly inflating the balloon to constant pressure. Pressure was measured using a sphygmomanometer connected to a pressure transducer. The balloon was inflated to various pressures (20 mmHg, 40 mmHg, 60 mmHg and 80 mmHg) for a 20 s stimulation period followed by a 2 min rest. EMG was recorded continuously during the experiment on a Biopac System EMG 100 °C. The EMG signal was amplified, filtered at 300 Hz and digitized using Acknowledge software (Biopac Systems, Inc., CA, United States). The area under the curve (AUC) for EMG activities during each 20 s of distention was calculated using an in-house written computer program^[18]. The net value for each distention was calculated by subtracting the baseline value derived from the AUC for the 20 s pre-distention period. EMG was measured before HIS and 6 h, 1 d, 2 d and 7 d after termination of last stressor. Each rat was tested for EMG twice for each distention pressure and the mean AUC of EMG calculated from the two repeated measurements was used for each rat for each pressure in the following statistical analysis.

Abdominal withdrawal reflex scores: Visceral hypersensitivity was also measured by grading behavioral response of rats to CRD as described previously^[3,19]. In brief, under anesthesia with isoflurane, a flexible latex balloon (5 cm) attached to a tygon tube was inserted 8 cm into the descending colon and rectum *via* anus and held in place by taping the tube to tail. Rats were placed in small lucite cubicles and allowed to adapt for 30 min. CRD

was performed by rapidly inflating the balloon to constant pressure using a sphygmomanometer. The balloon was inflated to 20 mmHg, 40 mmHg, 60 mmHg and 80 mmHg for 20 s followed by 2 min rest. Behavioral response to CRD was measured by visual observation of AWR by a blinded observer and AWR was scored either 0 (normal behavior), 1 (slight head movement), 2 (contraction of abdominal muscles), 3 (lifting of abdominal wall) or 4 (body arching and lifting of pelvic structures). AWR scores were measured before HIS and 6 h, 1 d, 2 d and 7 d after termination of last stressor. The experimenter, who assigned the AWR scores and performed the EMG analysis, was masked to the control or stressed group assignment, to the sham or EA treatment, and to the drug applied (saline or NLX/m-NLX). Each rat was tested twice for AWR score for each distention pressure and the mean AWR score from the two repeated measurements was used for each rat for each pressure in the following statistical analysis.

Electroacupuncture treatment

EA was applied by a pair of stainless steel suture needles. Hook-shaped needles were used to avoid spontaneous removal of inserted acupuncture needles from rat body^[20,21]. Two acupoints were used in this study: ST-36 (Zu-San-Li) or BL-43 (Gao-Huang). ST-36, equivalent to the human acupoint ST-36 (Figure 2), is located at 5 mm lateral to the anterior tubercle of the tibia and 10 mm below the knee joint^[21-23]. BL-43, equivalent to the human acupoint BL-43 (Figure 2), was used as an irrelevant acupuncture point to the colon. The needles were inserted bilaterally at a depth of 5 mm into the skin and underlying muscles at acupuncture point. To compare the effect of EA at an irrelevant acupoint, the same stimulation parameters were used to stimulate the BL-43. The needles inserted into acupoints were stimulated by an EA apparatus (Model G-6805-2, Shanghai Medical Electronic Apparatus Company, China) with a constant rectangular current of alternating trains of dense-sparse frequencies (100 Hz for 1.05 s and 2 Hz for 2.85 s alternately, pulse width, 0.1 ms). This combination of dense-sparse frequency would maximally induce opioid release of met-enkephalin and dynorphin A^[24]. Electrical stimulus intensity was set at the threshold for a detectable muscle twitch (approximately 1 mA). The stimulation was delivered for 30 min. For sham EA group, the needle set was inserted into the ST-36, but no electrical stimulation was applied. Behavioral tests were performed immediately after termination of EA.

Drug administration

After finishing the first distention series, NLX (0.1 mg/kg, Sigma) or m-NLX (1 mg/kg, Sigma) was administered intraperitoneally to HIS rats on the second day. Thirty min after the administration of NLX or m-NLX, EA at ST-36 was given for 30 min. The second distention series was performed immediately after termination of EA. The AUCs for the EMG signals or AWR scores

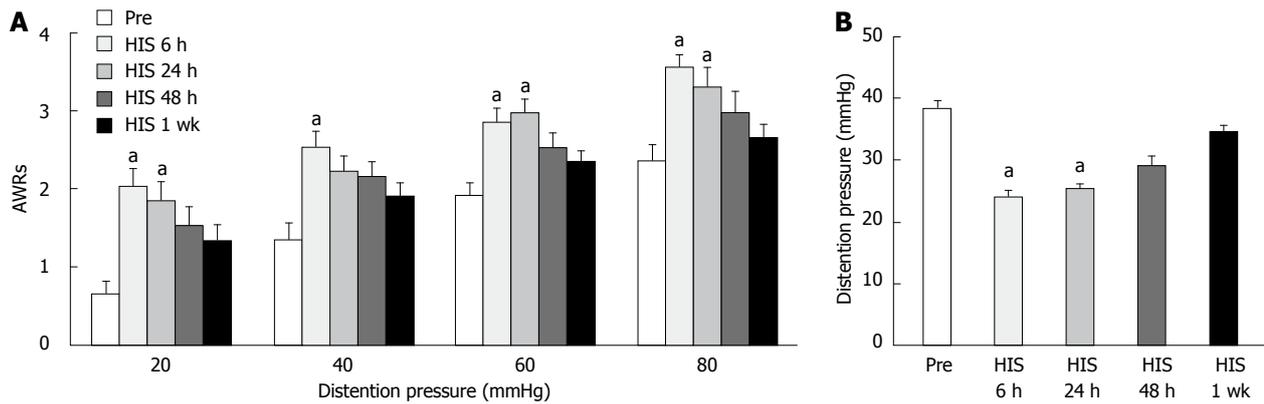


Figure 3 Heterotypic intermittent stress increased abdominal withdrawal reflex scores to colorectal distention. A: Abdominal withdrawal reflex (AWR) scores were used as a function of distention pressure (20 mmHg, 40 mmHg, 60 mmHg and 80 mmHg). Heterotypic intermittent stress (HIS) significantly enhanced AWR scores measured at 6 h and 24 h after termination of last stressor when compared with baseline (Pre) under 20 mmHg, 60 mmHg and 80 mmHg distention pressure, while HIS significantly enhanced AWR scores measured at 6 h after termination of last stressor when compared with Pre group under 40 mmHg distention pressure [Tukey *post hoc* test following Friedman analysis of variance (ANOVA)]. Therefore, HIS can enhance visceral hypersensitivity in rats at 6 h and 24 h after termination of last stressor generally when compared with baseline (Pre). AWR scores returned to normal level 48 h after termination of last stressor ($n = 8$ rats for each group; $^{\circ}P < 0.05$ vs Pre); B: HIS remarkably reduced distention threshold. Distention threshold was the minimal distention pressure to evoke abdominal movement. In agreement with AWR scores, distention threshold started to reduce at 6 h and maintained at a low level at 24 h and returned to normal level 48 h after termination of last stressor (Friedman ANOVA followed by Tukey *post hoc* test, $n = 8$ rats for each group; $^{\circ}P < 0.05$ vs Pre).

from two distention series were calculated. m-NLX is a selectively peripherally acting opioid receptor antagonist. The time schedule of experimental protocol is shown in Figure 1C.

Statistical analysis

All data were expressed as mean \pm SE. Statistical analysis were conducted using commercial software OriginPro 8 (OriginLab, United States) and Matlab (Mathworks, United States). Normality was checked for all analyses. Significance between groups was determined using two sample *t* test, Friedman analysis of variance (ANOVA) or two-way repeated-measures ANOVA followed by Tukey *post hoc* test or Mann-Whitney test where appropriate. The level of significance was set at $P < 0.05$.

RESULTS

HIS produced visceral hypersensitivity

To determine whether HIS induces visceral hypersensitivity, AWR scores to CRD were measured in rats before and after the 9-d HIS protocol. The AWR scores before HIS were 0.6 ± 0.2 , 1.3 ± 0.2 , 1.9 ± 0.2 and 2.3 ± 0.2 for 20, 40, 60 and 80 mmHg distention pressures, respectively. Six hours after termination of the last stressor, the AWR scores were 2.0 ± 0.1 , 2.5 ± 0.1 , 2.8 ± 0.2 and 3.5 ± 0.2 for 20, 40, 60 and 80 mmHg distention pressures, respectively (Figure 3A). Since the first measurement was at 6 h, the time when it started was unknown. To determine the time course of stress-induced visceral hypersensitivity, AWR scores were recorded 6, 24, 48 h, and 7 d after the HIS protocol. There was clear time effect for HIS on AWR scores for all distention pressures (Friedman ANOVA; $n = 8$ rats for each group). The increase in AWR scores started at 6 h, maintained for 24 h, and AWR scores returned to

basal levels at 48 h and 7 d after HIS compared with pre-HIS baseline at different distention pressures ($P < 0.05$, Tukey *post hoc* test following Friedman ANOVA, Figure 3A). In addition, the distention threshold was measured in these rats before and after HIS protocol. Distention threshold was the minimal distention pressure to evoke abdominal movement. The distention threshold was 38.3 ± 1.5 mmHg before HIS and was 23.9 ± 1.3 , 25.3 ± 0.8 , 29.0 ± 1.8 , 34.7 ± 0.9 mmHg at 6, 24, 48 h and 1 wk after termination of the last stressor, respectively. There was significant time effect on the distention threshold (Friedman ANOVA, $P < 0.001$, $n = 8$ rats for each group). In agreement with the AWR scores, the distention threshold was significantly lower at 6 h and 24 h, and returned to the baseline level at 48 h and 7 d after HIS protocol compared with pre-HIS baseline ($P < 0.05$, Tukey *post hoc* test following Friedman ANOVA, Figure 3B).

To further confirm the visceral hypersensitivity induced by HIS, EMG measurements were performed on rats before and after HIS. The AUC of EMG recordings before HIS was 10.6 ± 1.6 , 29.2 ± 3.3 , 41.3 ± 4.3 and 52.4 ± 5.1 for 20 mmHg, 40 mmHg, 60 mmHg and 80 mmHg distention pressures, respectively. Six h after termination of the HIS protocol, the AUCs were 24.0 ± 4.7 , 60.2 ± 6.6 , 83.0 ± 10.0 and 94.3 ± 10.9 for 20 mmHg, 40 mmHg, 60 mmHg and 80 mmHg distention pressures, respectively. There was significant time effect of HIS on EMG for all pressures [$P < 0.001$, two-way repeated measures ANOVA, with significant time \times pressure interaction ($P < 0.001$); $n = 6$ rats for each group]. The AUCs were higher at 6, 24 and 48 h, and returned to baseline values one week after termination of HIS compared with pre-HIS baseline for 40, 60 and 80 mmHg, with no significant difference between AUC of EMG of different time points at 20 mmHg ($P < 0.05$,

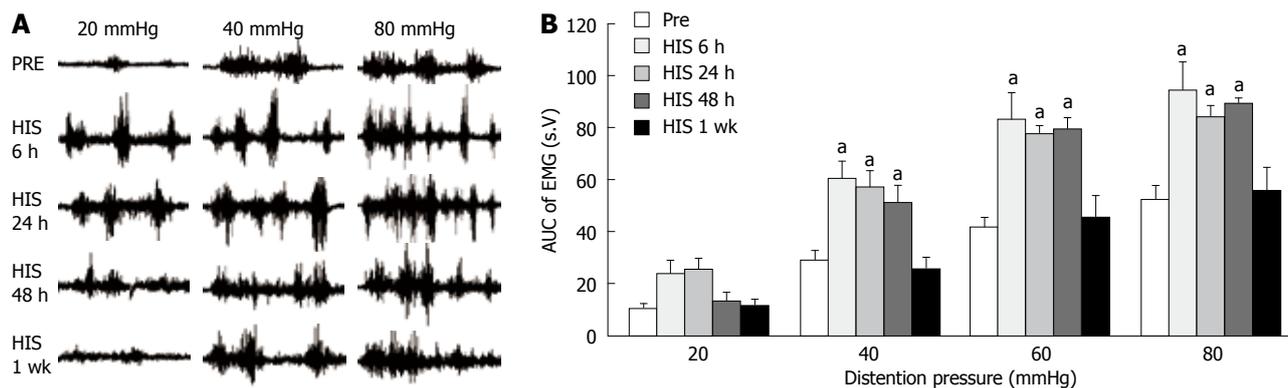


Figure 4 Heterotypic intermittent stress enhanced electromyographic activities in response to colorectal distention. Electromyographic (EMG) activities in the external oblique muscle in response to graded colorectal distention (CRD) were measured before stress and 6 h, 24 h, 48 h and 7 d after termination of heterotypic intermittent stress (HIS). The magnitude of EMG activity was expressed as area under curve (AUC). A: Examples of EMG activities recorded from baseline (Pre), 6 h, 24 h, 48 h and 7 d after termination of HIS in rats responding to distention pressures of 20 mmHg, 40 mmHg and 80 mmHg; B: Bar graph shows the changes in average of AUC before and after HIS protocol. There was no significant difference between EMGs of different time points at 20 mmHg. The magnitude of EMG activity of 6 h, 24 h and 48 h was significantly larger than that of Pre at distention pressures of 40 mmHg, 60 mmHg and 80 mmHg (Tukey *post hoc* test following two-way repeated measures analysis of variance, $n = 6$ rats for each group; $^aP < 0.05$ vs Pre).

Tukey *post hoc* test following two-way repeated measures ANOVA, Figure 4A and B).

EA treatment suppressed visceral hypersensitivity in HIS rats

To determine whether EA suppressed visceral hypersensitivity induced by HIS, AWR scores and AUCs of EMG recordings after EA treatment were compared with those after sham EA treatment. To define the specificity of EA-mediated analgesic effect in rats, we also examined EA effect on age-matched healthy rats (controls). Since AWR scores returned to the baseline level 48 h and EMG data returned to baseline level one week after termination of last stressor (Figures 3A and 4B), EA at acupoints ST-36 (Figure 2) was applied to control and HIS rats for 30 min within 48 h after termination of last stressor. For sham EA group, the needle set was inserted into the ST-36, but no electrical stimulation was applied. AWR scores and EMG activities were recorded immediately after termination of EA. Both distention stress and EA treatment affected AWRs ($n = 8$ rats for each group, two-way repeated measures ANOVA: under 20 mmHg, stress effect, $P < 0.001$; EA treatment effect, $P < 0.01$; under 40 mmHg, stress effect, $P < 0.001$; EA treatment effect, $P < 0.05$), with significant stress \times EA treatment interaction for 20 mmHg and 40 mmHg pressures ($P < 0.05$ for 20 mmHg and 40 mmHg). HIS sham group showed a significant increase in AWR scores compared with control sham group under 20 mmHg and 40 mmHg distention pressures (HIS sham *vs* control sham, for 20 mmHg: 2 ± 0.2 *vs* 0.6 ± 0.1 ; for 40 mmHg, 2.6 ± 0.2 *vs* 1.4 ± 0.2 ; $P < 0.05$, Tukey *post hoc* test following two-way repeated measures ANOVA), while there was no significant difference in AWR scores between control and HIS groups after EA treatment. EA treatment at ST-36 point significantly decreased AWR scores in HIS rats (sham EA *vs* EA, AWRs at 20 mmHg: 2.0 ± 0.2 *vs* 0.7 ± 0.1 ; at 40 mmHg: 2.6 ± 0.2 *vs* 1.5 ± 0.2 ; at 60 mmHg: 3.1 ± 0.2

vs 1.9 ± 0.1 ; at 80 mmHg: 3.6 ± 0.1 *vs* 2.4 ± 0.2 ; $P < 0.05$, Tukey *post hoc* test following two-way repeated measures ANOVA, Figure 5A), but had no effect on control rats under 20 mmHg and 40 mmHg pressures. Under 60 mmHg and 80 mmHg pressures, stress \times EA treatment interaction was not significant ($n = 8$ rats for each group, two-way repeated measures ANOVA); EA treatment significantly decreased AWR scores in both control and HIS rats under 60 mmHg and 80 mmHg pressures ($P < 0.05$, EA effect, two-way repeated measures ANOVA, Figure 5A).

To further confirm the EA effect on stressed rats, EMGs were performed before and after EA or sham EA treatment (Figure 5B and C). Both distention pressure and EA treatment affected AUCs of HIS rats significantly ($n = 6$ rats for each group, two-way repeated measures ANOVA: pressure effect, $P < 0.001$; EA treatment effect, $P < 0.05$), with significant pressure \times EA treatment interaction ($P < 0.001$). Rats that received EA treatment showed a significant decrease in their AUCs compared to rats that received sham EA under 40 mmHg, 60 mmHg and 80 mmHg distention pressures 6 h after HIS (EMG for sham EA *vs* EA, at 20 mmHg: 24 ± 4.7 *vs* 13.8 ± 3.5 ; EMG at 40 mmHg: 60.2 ± 6.6 *vs* 30 ± 4.9 , $P = 0.00523$; EMG at 60 mmHg: 83 ± 10 *vs* 39.8 ± 5.9 , $P = 0.00029$; EMG at 80 mmHg: 94.3 ± 10.8 *vs* 49.6 ± 5.9 , $P = 0.00021$; $P < 0.05$, Tukey *post hoc* test following two-way repeated measures ANOVA, Figure 5B and C), without significant effect under 20 mmHg pressure. This demonstrates that EA suppressed visceral hypersensitivity, and is in agreement with previous studies that EA treatment attenuated chronic visceral hyperalgesia induced by neonatal colonic injection of acetic acid^[23]. It is of note that results from EMG recordings at 20 mmHg were different from those of AWR scores after EA treatment.

To exclude the non-specific effect of EA treatment, EA was applied at Gao-Huang. Gao-Huang, an equivalent to the human acupoint BL-43 (Figure 2), was

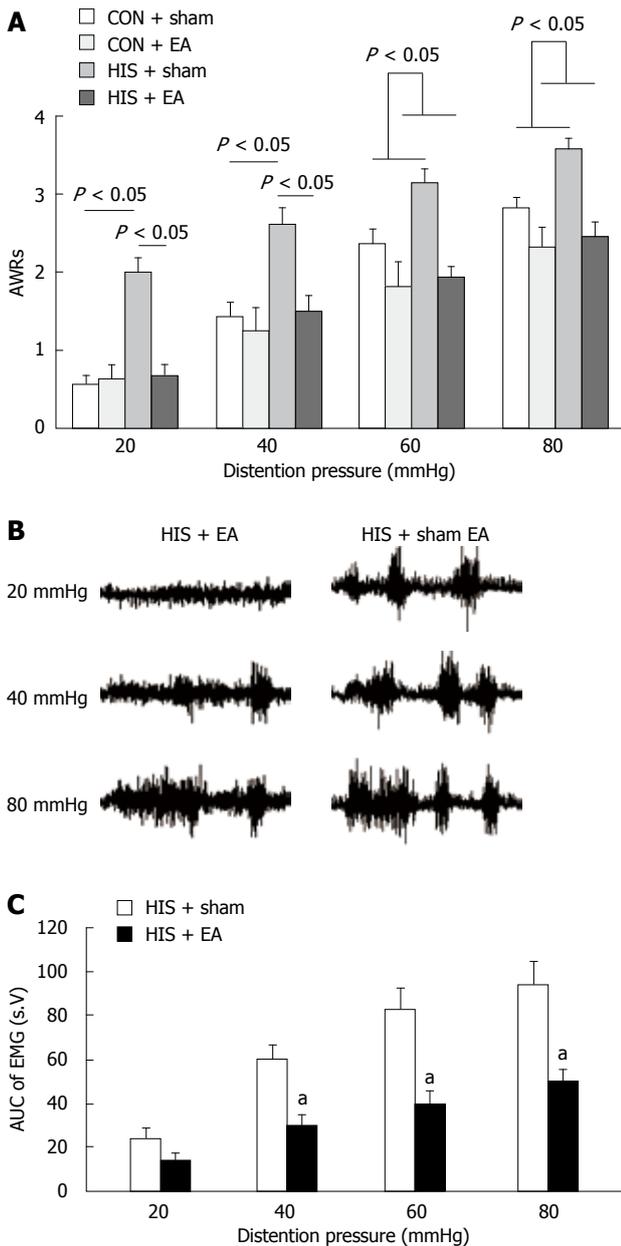


Figure 5 Electroacupuncture treatment attenuated the abdominal withdrawal reflex scores and electromyographic activities. Electroacupuncture (EA) treatments were delivered for 30 min within 24 h after termination of last stressor. For sham EA group, the needle set was inserted into the ST-36 but no electrical stimulation was applied. A: The effects of stress exposure and EA treatment on abdominal withdrawal reflex (AWR) scores. Heterotypic intermittent stress (HIS) sham group showed a significant increase in AWR scores compared to control sham group under 20 mmHg and 40 mmHg distention pressures, while there was no significant difference in AWR scores between control and HIS groups after EA treatment. EA treatment at ST-36 point significantly decreased AWR scores in HIS rats, but had no effect on control rats under 20 mmHg and 40 mmHg pressures [Tukey *post hoc* test following two-way repeated measures analysis of variance (ANOVA)]. EA treatment significantly decreased AWR scores in both control and HIS rats under 60 mmHg and 80 mmHg pressures (two-way repeated measures ANOVA, $n = 8$ rats for each group; $P < 0.05$); B: Representative electromyographic (EMG) traces recorded immediately after termination of EA (left) or sham EA (right); C: Bar graph showing effects of EA treatment and sham EA on EMG recordings. The EMG was significantly decreased by EA treatment at pressure of 40 mmHg, 60 mmHg and 80 mmHg in stressed rats compared to sham EA groups (Tukey *post hoc* test following two-way repeated measures ANOVA, $n = 6$ rats for each group; ^a $P < 0.05$ vs HIS + sham group). CON: Control.

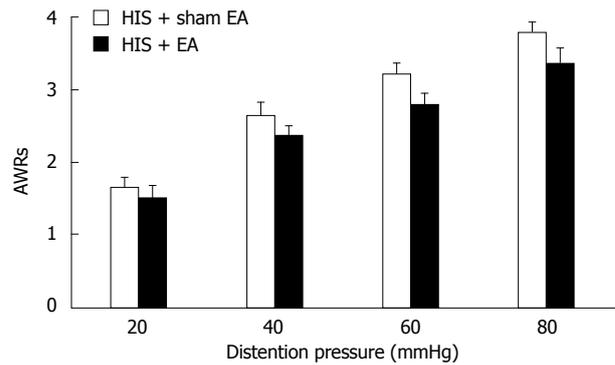


Figure 6 Effect of electroacupuncture at Gao-Huang. Same electroacupuncture (EA) parameters as EA at ST-36 (Zu-San-Li) were used for BL-43 (Gao-Huang) treatment in heterotypic intermittent stress (HIS) rats. Although the abdominal withdrawal reflexes (AWRs) to colorectal distention heavily depend on the pressure level (two-way repeated measures analysis of variance, pressure effect, $P < 0.001$), EA treatment at BL-43 point on HIS rats showed no significant effect on pain perception; $n = 7$ rats for each group.

chosen as an irrelevant acupuncture point to the colon. EA at BL-43 for 30 min did not produce any effect on AWR scores in HIS rats [$n = 7$ rats for each group. Two-way repeated measures ANOVA (EA treatment effect, $P > 0.05$; pressure \times EA interaction effect, $P > 0.05$), Figure 6].

NLX reversed EA-induced analgesic effects

To explore the possible involvement of endogenous opioid system in EA-induced antihyperalgesia, we then examined the effect of systemically injected NLX on EA-induced analgesia. After the 9-d HIS protocol, rats received intraperitoneal injection of 0.1 mg/kg of NLX in 1 mL 30 min before the beginning of EA at ST-36 points. Equal volume of normal saline (NS) was used as control. Immediately after EA termination, the AWR scores to CRD were determined (Figure 7A). The AWR scores from HIS rats pretreated with NS were 0.70 ± 0.20 , 1.50 ± 0.27 , 2.00 ± 0.15 and 2.60 ± 0.18 for 20 mmHg, 40 mmHg, 60 mmHg and 80 mmHg distention pressures, respectively. The AWR scores in HIS rats pretreated with NLX were 2.0 , 2.80 ± 0.12 , 3 and 3.60 ± 0.18 for 20 mmHg, 40 mmHg, 60 mmHg and 80 mmHg distention pressures, respectively. Pretreatment of NLX significantly reduced the EA-induced suppression of AWR scores of HIS rats under all distention pressures when compared with NS group [$n = 5$ rats for each group, Friedman ANOVA, NLX effect, $P < 0.001$; $P < 0.05$, Mann-Whitney test following Friedman ANOVA, Figure 7A]. This indicates the involvement of endogenous opioid system in mediating EA analgesic effects on HIS-induced visceral hyperalgesia. To exclude the non-specific effect of NLX, we further determined whether NLX itself produced any effect on AWR scores in control rats without EA treatment (Figure 7B). The AWR scores before 0.1 mg/kg of NLX in 1 mL treatment in control rats were 1.00 ± 0.16 , 1.90 ± 0.10 , 2.50 ± 0.22 and 2.70 ± 0.20 for 20 mmHg, 40 mmHg, 60 mmHg and 80 mmHg distention pressures, respectively. The

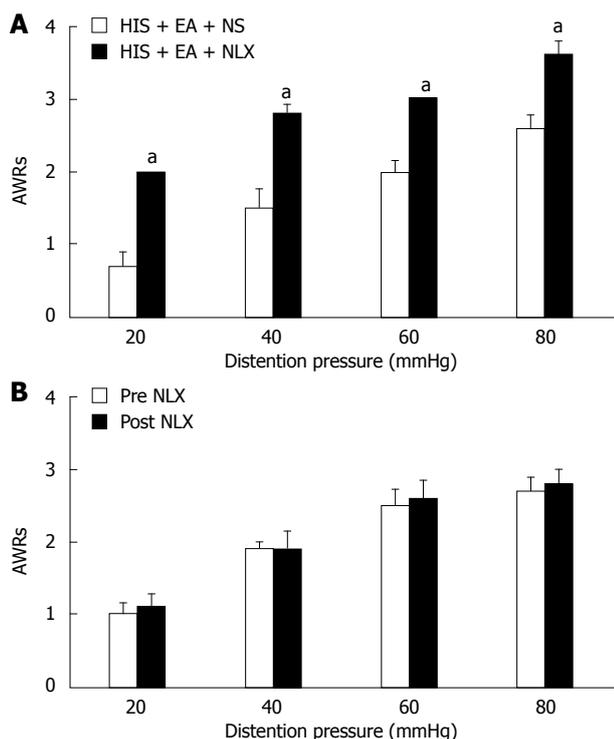


Figure 7 Reversal of electroacupuncture-mediated analgesic effect by naloxone. A: The opioid receptor antagonist, naloxone (NLX) (0.1 mg/kg body weight, $n = 5$), or normal saline (NS) was administrated intraperitoneally 30 min before electroacupuncture (EA) application. Abdominal withdrawal reflex (AWR) scores were recorded immediately after EA termination in rats pretreated with NLX or NS. Both pressure and NLX injection significantly affected the AWRs to colorectal distention in rats [Friedman analysis of variance (ANOVA), $P < 0.001$ for pressure effect and for NLX injection effect]. Bar graph showed that NLX (0.1 mg/kg body weight) completely blocked the EA-induced analgesic effect when compared with NS treatment at all pressures (Mann-Whitney test following Friedman ANOVA). $n = 5$ rats for each group. ^a $P < 0.05$ vs NS-EA group; B: Same dose of NLX treatment did not produce any effect on AWR scores in control rats without EA treatment (Friedman ANOVA). AWRs were only significantly affected by different pressure levels ($P < 0.001$). $n = 5$ rats for each group. Control rats were not exposed to heterotypic intermittent stress (HIS).

AWR scores after 0.1 mg/kg of NLX in 1 mL treatment were 1.10 ± 0.19 , 1.90 ± 0.24 , 2.60 ± 0.24 and 2.80 ± 0.20 for 20 mmHg, 40 mmHg, 60 mmHg and 80 mmHg distention pressures, respectively. This suggests that NLX itself did not produce any effect on AWR scores in control rats ($n = 5$ rats for each group, Friedman ANOVA, NLX effect, $P > 0.05$). Similarly, we did not observe any effect of NLX on AWR scores in HIS rats without EA treatment (data not shown). These data suggest that the inhibitory effect of NLX on visceral analgesia is associated with EA treatment.

m-NLX inhibited EA-induced analgesic effects

To further determine whether peripheral opioid system is involved in the EA-induced analgesic effect, m-NLX was administrated prior to EA treatment. m-NLX is an opioid receptor antagonist that can not cross the blood-brain barrier, thereby only acting at peripheral nervous system. After the 9-d HIS protocol, rats received intraperitoneal injections of 1 mg/kg of m-NLX in 1 mL 30 min before

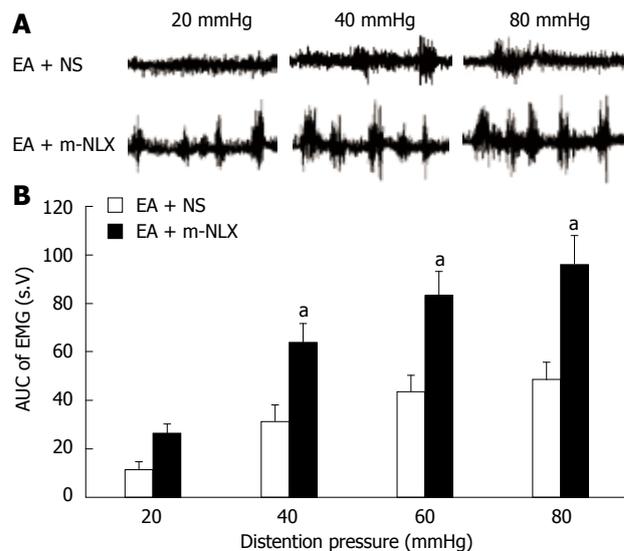


Figure 8 Reversal of electroacupuncture-mediated analgesic effect by naloxone methiodide. The opioid receptor antagonist, naloxone methiodide (m-NLX), or normal saline (NS) was administrated intraperitoneally (5 mg/kg body weight) 30 min before electroacupuncture (EA) application. Electromyographic (EMG) activities were recorded immediately after EA from rats pretreated with m-NLX and NS. A: Examples of EA effects on EMG activities pretreated with NS (top) or m-NLX (bottom); B: Bar graph showing that m-NLX completely blocked the EA-induced analgesic effect. The magnitude of EMG activity was significantly increased by m-NLX treatment at pressure of 40 mmHg, 60 mmHg and 80 mmHg (Tukey *post hoc* test following two-way repeated measures analysis of variance, $n = 6$ rats for each group, ^a $P < 0.05$ vs EA + NS group).

the beginning of EA at ST-36 points. Equal volume of NS was used as control. EMG recordings were measured immediately after EA termination. The AUCs of EMG activities from HIS rats pretreated with NS were 13.33 ± 3.88 , 36.28 ± 8.01 , 50.19 ± 8.28 and 56.42 ± 8.43 for 20 mmHg, 40 mmHg, 60 mmHg and 80 mmHg distention pressures, respectively. The AUCs of EMG activities in HIS rats pretreated with m-NLX were 30.84 ± 4.39 , 74.16 ± 9.04 , 96.45 ± 11.80 and 111.59 ± 13.79 for 20 mmHg, 40 mmHg, 60 mmHg and 80 mmHg distention pressures, respectively. Pretreatment of m-NLX (1 mg/kg, *i.p.*) significantly reduced the analgesic effects induced by EA in a pressure-dependent manner ($n = 6$ rats for each group, two-way repeated measures ANOVA, NLX effect, $P < 0.01$; pressure \times NLX interaction, $P < 0.01$). The effect of pretreatment of m-NLX was significant under 40 mmHg, 60 mmHg and 80 mmHg, but not under 20 mmHg ($P < 0.001$, Tukey *post hoc* test following two-way repeated measures ANOVA, Figure 8). To further determine the specificity of m-NLX, a low dose of m-NLX (0.1 mg/kg) was injected intraperitoneally. Pretreatment with a low dose of m-NLX did not affect the EA-induced suppression of EMG activities in HIS rats (data not shown). In addition, systemic injection of m-NLX (1 mg/kg, *i.p.*) did not produce any effect on the AUCs of EMG activities in normal or HIS rats without EA treatment (data not shown). These data suggest that EA may affect the peripheral opioid system to induce analgesia in HIS rats.

DISCUSSION

The present study examined the mechanisms involved in EA-induced analgesia in a rat model of visceral hypersensitivity induced by HIS. EA treatment significantly reduced AWR scores (Figure 5A) and suppressed EMG responses (Figure 5C) to colorectal distention in the stressed rats, but not in non-stressed rats, at the pressures of 20 mmHg and 40 mmHg, indicating that EA had an analgesic effect in this model. Although EA has been used clinically for alleviation of various types of pain^[25,26], there is not enough scientific validation for the use of EA in visceral pain. Together with our previous report that EA attenuated visceral pain induced by neonatal acetic acid infusion^[23], we have provided additional evidence for EA treatment for visceral pain in different models.

Acupuncture is being increasingly accepted by practitioners and patients, especially during the last three decades. EA is a modification of this technique that stimulates acupoints (or called acupuncture points) with electrical current instead of manual manipulations. The EA procedures may stimulate the somatic afferent nerves innervating the skin and muscles of the body, which was different from transcutaneous electrical nerve stimulation (TENS). EA typically involves penetration of the skin by fine, solid metallic needles, which are manipulated by electrical stimulation. TENS is a treatment that has been shown to be effective for pain relief in a variety of conditions. Electrodes for TENS are placed on the skin. Electric current applied at different pulse rates (frequencies) and intensities is used to stimulate these areas so as to provide pain relief. In this study, we focused on the EA-mediated effect in stressed animal models. We showed that EA at ST-36, but not at BL-43, significantly suppressed the visceral motor responses to CRD (Figure 6). Koo *et al.*^[27] reported that ankle sprain pain was relieved by EA at SI-6, but not at nearby LI-4. More recently, they reported that EA-induced analgesic effects in capsaicin-induced hyperalgesia are produced only by stimulation at SI-3/TE-8 of the forelimb, but not at nearby points (LI-3/LI-6) or several other points (GB-30/GB34, BL-40/BL-60, GV-2/GV-6)^[28]. Kim *et al.*^[29] demonstrated that acupuncture at HT-7, but not at nearby point TE-8, inhibited dopamine release in the nucleus accumbens and suppressed behavioral hyperactivity in the morphine addiction model, thus suggesting the acupoint-specificity. However, the acupoint specificity of EA effect in visceral pain remains unknown. In the present study, two acupoints, ST-36 and BL-43, were selected. The acupoint ST-36 has been used empirically for the treatment of gastrointestinal diseases for many years, while acupoint BL-43 is not thought to be related to the GI tract (Figure 2). EA at ST-36, but not at BL-43, significantly suppressed the visceral motor responses to CRD (Figure 6), suggesting that the EA effect is not a non-specific effect and it may be associated with acupoint and its related afferent fibers. This finding is consistent with a previous study which found that EA at ST-36, but not at BL-21, significantly reduced the increase in mean arterial blood pressure in response to

rectal distension in dogs^[30]. Since the parameter used for EA treatment at BL-43 is the same as at ST-36, further experiments are needed to investigate whether different stimulation parameters used to stimulate BL-43 produce the analgesic effect in this model. In addition, it is worth noting that our results showed that EA produced a significant analgesic effect only on the HIS-induced visceral hyperalgesia, but not on the age-matched healthy rats, at the distention pressures of 20 mmHg and 40 mmHg (Figure 5A). This further indicates that EA-produced analgesic effect is not a non-specific effect and that it may be disease-related under low stimulation intensity. The reason why EA did not produce inhibitory effect under low distention pressure remains unknown. It is most likely that low distention pressure produced the low responses that are not sensitive to EA treatment. However, there has been a certain degree of skepticism about acupoint and disease specificity. There was no statistical difference between acupoint and nonacupoint acupuncture in an experimental human pain model, thus suggesting no acupoint specificity. Rong *et al.*^[31] reported that manual acupuncture at ST-36 produced anti-nociceptive effect on CRD in healthy rats. This discrepancy may be due to the application of different methods of acupuncture and distention pressure as well. Therefore, the acupoint and disease specificity of EA treatment is still a controversial issue and is a subject of further study of pain.

EA analgesic effect in various conditions may be mediated by different mechanisms. These include opioid and non-opioid mechanisms^[32-35]. The involvement of the endogenous opioid system is a well-established hypothesis for explanation of EA effects. The involvement of non-opioid mechanisms in EA analgesia was confirmed by experiments in which administration of 5-HT or catecholamine or adrenoceptor antagonists or depletion of cellular monoamine content blocked the EA-induced analgesic effect^[36,37]. It appears that the underlying mechanisms of EA analgesic effect in various conditions may depend on the specific conditions^[28]. In this study, the systemic application of opioid receptor antagonist NLX completely blocked the EA-mediated analgesic effects, indicating that endogenous opioid pathways were involved in EA-mediated analgesia in the rat model of visceral pain. This was consistent with the reports of different animal models of visceral pain^[22,38]. In the present study, we focused on the role of opioid system because previous studies indicate that opioid system was sensitive to environmental factors, and changes in its expression attenuated the pain sensitivity in different models^[23,39-43]. However, we cannot rule out that other systems may also be affected by EA treatment. The net result of regulatory changes in cell signaling proteins induced by EA, however, is to attenuate visceral hypersensitivity.

The processing of pain information occurs at central (spinal and supraspinal) and peripheral sites, and thus modification of pain levels can be achieved through interventions at multiple sites. Although the exact locations where EA modifies pain are not clearly identified, EA is thought to activate the ascending sensory pathways such

as spinal dorsal horn and thalamus, or the descending pain inhibitory mechanisms, such as opioid, adrenergic and serotonergic pathways. Most of previous studies have resolved only the central mechanism of EA analgesia, and only a few reports have investigated the peripheral mechanism of EA effect. Somatic inputs (i.e., EA-activated input) and noxious visceral signals (i.e., CRD) might converge and interact in the neurons at spinal dorsal horn level. Their interactions were manifested as that when the cutaneous stimuli were applied, the neuronal response to CRD was reduced in most cases^[31]. Kim *et al.*^[28] showed that EA suppresses capsaicin-induced secondary hyperalgesia through an endogenous spinal opioid mechanism, thus indicating EA modulation of pain through a central, but not peripheral, mechanism. The anti-nociceptive effect of EA at ST-36 was abolished by pretreatment with NLX but not by m-NLX by observation of mean arterial blood pressure in response to rectal distension in dogs^[30]. These results suggest that EA at ST-36 may reduce visceral pain via central opioid pathway. In this study, we demonstrated that EA-mediated analgesia was completely blocked by m-NLX. m-NLX is unable to cross the blood-brain barrier and blocks only peripheral opioid receptors^[30,44], thus indicating that EA analgesia is likely mediated by peripheral opioid receptors. Recent studies showed that peripheral opioid receptors were activated by EA at the inflammatory site on the CFA model^[45]. NMDA receptor expression in primary sensory neurons is inhibited by EA^[46], suggesting the peripheral effect of EA analgesia. Again, this discrepancy points out the fact that EA-mediated analgesic effect may be disease and/or organ-specific. Since previous studies have shown that stimulation frequencies of EA determine the types of opioid peptides released in the nervous system^[24], further experiments are needed to explore which subtypes of opioid receptors are involved in mediating EA antihyperalgesic effects and to identify the location from which the opioids are released, e.g., the peripheral nerve terminals *vs* immunocytes, the mucosa/submucosa *vs* muscle layer of the colon.

In conclusion, together with our previous report^[23], the present study demonstrated that EA treatment given at classical acupoint ST-36 produces an analgesic effect on visceral hyperalgesia in a rat model of IBS. The EA effect is mediated by endogenous opioid pathways, possibly at peripheral sites, thus providing scientific evidence for the treatment of visceral pain in functional gastrointestinal disorders using EA.

COMMENTS

Background

Irritable bowel syndrome (IBS) is a common gastrointestinal disorder characterized by chronic visceral pain and bloating in association with altered bowel movements. Acupuncture is an ancient form of traditional Chinese medicine that has been used to treat diseases. Recently, acupuncture needles are stimulated by electricity at various frequencies, which is termed electroacupuncture (EA). However, the mechanism underlying EA-induced analgesia in visceral pain remains unknown.

Research frontiers

The present study used EA to treat visceral hypersensitivity of rats which was

induced by a heterotypic intermittent stress (HIS) protocol. EA at acupoint Zu-San-Li significantly decreased visceral hypersensitivity of rats. EA-mediated analgesic effect was completely reversed by administration of naloxone (NLX) methiodide, a peripherally restricted opioid antagonist. The mechanism underlying the effect of EA on visceral hypersensitivity of rats induced by HIS which is a rat model of IBS appears to involve the mediation of peripheral opioid system.

Innovations and breakthroughs

This is the first study to indicate that EA treatment produces an analgesic effect on visceral hyperalgesia in a rat model of IBS induced by heterotypic intermittent stress. The EA effect is mediated by endogenous opioid pathways, possibly at peripheral sites.

Applications

The present study demonstrated that EA treatment produces an analgesic effect on visceral hyperalgesia in a rat model of IBS. The EA effect is mediated by endogenous opioid pathways, possibly at peripheral sites, thus providing scientific evidence for the treatment of visceral pain in functional gastrointestinal disorders using EA.

Terminology

IBS is a common gastrointestinal disorder characterized by chronic visceral pain and bloating in association with altered bowel movements. EA, acupuncture needles are stimulated by electricity at various frequencies (1-100 Hz); this method is developed from acupuncture, an ancient form of traditional Chinese medicine. NLX methiodide, a selectively peripherally acting opioid receptor antagonist.

Peer review

This paper describes positive effects of EA on responses to bowel extension in stressed rats. It is reported that EA diminished the number of pellets produced, the subjectively scored abdominal reflexes and the power of the electromyography of the abdominal muscles in response to colon extension, in stressed rats compared to control rats. Moreover, the effect of EA was antagonized by both a central and peripheral acting opiate antagonist. The effects presented are significant, and the antagonizing effect with NLX was convincing. The results presented are important for clinicians and for the fundamental scientific community as well.

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Comparative genomic study of gastric epithelial cells co-cultured with *Helicobacter pylori*

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Abstract

AIM: To identify genes potentially involved in *Helicobacter pylori* (*H. pylori*)-induced gastric carcinogenesis.

METHODS: GES-1 cells were co-cultured with *H. pylori* strains isolated from patients with gastric carcinoma (GC, $n = 10$) or chronic gastritis (CG, $n = 10$) for *in vitro* proliferation and apoptosis assays to identify the most and least virulent strains. These two strains were *cagA*-genotyped and used for further *in vivo* carcinogenic virulence assays by infecting Mongolian gerbils for 52 wk, respectively; a broth free of *H. pylori* was lavaged as control. Genomic profiles of GES-1 cells co-cultured with the most and least virulent strains were determined by microarray analysis. The most differentially expressed genes were further verified using quantitative real-time polymerase chain reaction in GES-1

cells infected with the most and least virulent strains, and by immunohistochemistry in *H. pylori* positive CG, precancerous diseases, and GC biopsy specimens in an independent experiment.

RESULTS: GC-derived *H. pylori* strains induced a potent proliferative effect in GES-1 cells in co-culture, whereas CG-derived strains did not. The most (from a GC patient) and least (from a CG patient) virulent strains were *cagA*-positive and negative, respectively. At week 52, CG, atrophy, metaplasia, dysplasia, and GC were observed in 90.0%, 80.0%, 80.0%, 90%, and 60.0%, respectively, of the animals lavaged with the most virulent strain. However, only mild CG was observed in 90% of the animals lavaged with the least virulent strain. On microarray analysis, 800 differentially expressed genes (49 up- and 751 down-regulated), involving those associated with cell cycle regulation, cell apoptosis, cytoskeleton, immune response, and substance and energy metabolisms, were identified in cells co-cultured with the most virulent strain as compared with those co-cultured with the least virulent strain. The six most differentially expressed genes (with a betweenness centrality of 0.1-0.2) were identified among the significant differential gene profile network, including *JUN*, *KRAS*, *BRCA1*, *SMAD2*, *TRAF1*, and *HDAC6*. Quantitative real-time polymerase chain reaction analyses verified that *HDAC6* and *TRAF1* mRNA expressions were significantly more up-regulated in GES-1 cells co-cultured with the most virulent strain than in those co-cultured with the least virulent strain. Immunohistochemistry of gastric mucosal specimens from *H. pylori*-positive patients with CG, intestinal metaplasia (IM), dysplasia, and GC showed that moderately positive and strongly positive *HDAC6* expression was detected in 21.7% of CG patients, 30.0% of IM patients, 54.5% of dysplasia patients, and 77.8% of GC patients ($P < 0.001$). The up-regulation of *TRAF1* expressions was detected in 34.8%, 53.3%, 72.7%, and 88.9% specimens of CG, IM, dysplasia, and GC, respectively ($P < 0.001$).

CONCLUSION: The overexpression of *HDAC6* and *TRAF1* in GES-1 cells co-cultured with the GC-derived strain and in *H. pylori*-positive dysplasia and GC suggests that HDAC6 and TRAF1 may be involved in *H. pylori*-induced gastric carcinogenesis.

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Key words: *Helicobacter pylori*; Gastric carcinoma; Proliferation; Genomic profiles

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a pathogenic bacterium colonizing gastric mucosae, especially in the antrum. It has been accepted to be the primary cause of upper gastrointestinal disorders, such as acute and chronic gastritis, peptic ulcer disease, and gastric cancer^[1]. *H. pylori* infection is common worldwide with a prevalence of approximately 50%, especially in Eastern Asian populations, and the infection is usually a life-long event^[2]. However, over 80% of *H. pylori*-infected individuals remain asymptomatic for their whole lifetime despite the presence of chronic gastric inflammation or chronic gastritis (CG), caused by *H. pylori* infection^[3]. The subsequent outcomes of persistent *H. pylori* infection are highly variable. Approximately, 10%-20% of *H. pylori*-infected individuals are subject to peptic ulcer disease, including gastric and duodenal ulcers^[4]. Of note, individuals infected by *H. pylori* are at a higher risk of gastric carcinoma (GC) (1%-2%) and mucosa-associated lymphoid tissue (MALT) lymphoma (< 1%)^[5]. Therefore, gastric cancer may well be an infectious disease^[6]. Additionally, it has been reported that *H. pylori* infection is associated with esophageal diseases, such as gastroesophageal reflux disease, Barrett's esophagus, and esophageal carcinoma, as well as extra-gastrointestinal diseases, such as cardiovascular diseases, although controversies exist^[7,8]. Such variation in clinicopathological outcomes of *H. pylori* infection is believed to result from the variations in the virulence of different strains, genetic background of the host, and more importantly, the host-pathogen interactions.

It has been widely accepted that *H. pylori* is the major cause of GC in most (65%-80%) patients^[9]. Dietary risks, such as nitrate- and nitrite-enriched smoked or salted foods, are attributed to the *in vivo* biochemical activities of *H. pylori*^[10]. Genetic susceptibility has also been identi-

fied in 10% of GC patients^[11]. The canonical paradigm of gastric carcinogenesis has been established for more than two decades as a consecutive but dynamic progression of *H. pylori* infection, namely, CG, gastric atrophy (GA), intestinal metaplasia (IM), dysplasia, and finally GC^[12]. Thus, *H. pylori* infection plays a leading role in the pathogenesis of GC^[13].

It is likely that *H. pylori* strains themselves are highly variable in virulence to gastric mucosal epithelia, especially in terms of the carcinogenic effect. Genomic profiling analyses have identified a wide range of genetic variations among *H. pylori* strains isolated from patients with different gastric pathologies. Global gene expression profiles also vary greatly in human gastric epithelial immortalized cells infected with spiral *vs* coccoid *H. pylori*^[14]. These findings suggest that gastric epithelial cells tune in the expression of their genes, especially those associated with tumorigenesis, in response to specific *H. pylori* strains or a specific virulent factor of the strain. In addition to the core genes, strain-specific genes are thought to play an essential role in *H. pylori* propagation and pathogenesis.

How gastric epithelial cells respond to *H. pylori* clinical isolates derived from patients with different pathologies, such as GC and CG specimens, at the genome-wide level remains unknown. Therefore, this study was carried out to identify genes potentially involved in *H. pylori*-induced gastric carcinogenesis, by comparing the genomic profiles between gastric epithelial cells co-cultured with *H. pylori* strains isolated from patients with GC and those co-cultured with strains from patients with CG.

MATERIALS AND METHODS

Patients and specimens

The study protocol was approved by the Institutional Review Boards at the Third Xiangya Hospital of Central South University and Hunan Provincial Hospital, respectively. All patients gave written informed consent prior to the enrollment. A total of 350 outpatients who underwent upper gastrointestinal endoscopy at the Department of Gastroenterology, the Third Xiangya Hospital of Central South University, and the Department of Gastroenterology of Hunan Provincial Hospital were consecutively enrolled. Gastric mucosal biopsy specimens were obtained from 182 patients. Three gastric biopsy specimens, 3-5 cm to the pylorus, were collected for the rapid urease test and the histological examination. The rapid urease test was performed using a rapid urease test kit (Sanqiang Biotechnology, Sanming, China). Of the 182 patients, 113 patients were found positive for *H. pylori* infection as detected by the rapid urease test. The histological classification followed the updated Sydney system^[15]; the most serious pathology was documented as the histological diagnosis of each patient with concomitant mucosal pathologies. Thus, 23 patients were histologically diagnosed with CG, 30 with intestinal metaplasia (IM), 33 with dysplasia, and 27 with GC. In the present study, gastric specimens from CG and GC patients were used

for *H. pylori* isolation and subculture.

***H. pylori* isolation and subculture**

Gastric mucosal specimens from patients with CG and GC were ground into homogenates and inoculated onto a Columbia agar plate (Sangong Biotech, Shanghai, China) supplemented with 6% sheep blood. Plates were incubated at a mixed atmosphere of 10% CO₂, 5% O₂, and 85% N₂, at 37 °C for 72 h. *H. pylori* colonies were validated by using colony identification, Gram staining, light microscopy, and urease test (Fujian Sanqiang Biochemical Co. Ltd., Sanming, China) prior to further use. Subculture of *H. pylori* was performed as described above. Twenty *H. pylori* strains were isolated from 10 CG and 10 GC patients, and used for further experiments.

Identification of the most and least virulent strains isolated from CG and GC specimens

GES-1 cell culture and co-culture with *H. pylori*: GES-1 cell line, a human gastric epithelium immortalized cell line, was purchased from Ai-yan Biotechnology Co., Ltd., Shanghai, China. GES-1 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA), at 37 °C and in a humidified atmosphere of 5% CO₂. GES-1 cells at the exponential growth phase were harvested and seeded onto 96-well plates, at a density of 5×10^3 cells per well. *H. pylori* strains were resuspended in phosphate buffer solution (PBS) at a density of 3.0×10^8 cfu/mL using a spectrophotometer (Eppendorf, Hamburg, Germany). Following the cell cycle synchronization with 2% serum starvation, GES-1 cells were co-cultured with *H. pylori* strains in GES-1 cell culture media at a cell/bacterium ratio of 5:1, 1:1, 1:50, and 1:200, respectively. The number of bacteria was examined using a spectrometer, whilst that of cells was determined using TC10 automated cell counter (Bio-Rad Laboratories, Philadelphia, PA). A mono-culture of GES-1 cells in the absence of *H. pylori* was used as a control. The number of GES-1 cells was fixed among co-cultures as well as mono-culture. The experiments were performed in duplicate and repeated in triplicate independently.

3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide cell proliferation assay: Following 12 h, 24 h, and 48 h of co-culture, 20 µL 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide (MTT) solution (Sangong Biotech, Shanghai, China) was added into the culture medium for a further 4-h incubation at 37 °C prior to the supplementation of 150 µL dimethyl sulfoxide (Invitrogen, Carlsbad, CA). The optical density at the wavelength of 490 nm ($A_{490\text{ nm}}$) was determined using an ELISA microplate reader (Bio-Tek, Winooski, VT). The experiments were performed in duplicate and repeated in triplicate independently.

Identification of *cagA* gene in *H. pylori* strains with

real-time polymerase chain reaction: The DNA samples were isolated using a bacterial DNA extraction kit (Boehringer Ingelheim GmbH, Ingelheim, Germany). Briefly, the most and least virulent *H. pylori* strains were resuspended in GTEL buffer and pre-incubated in TESK buffer at 55 °C for 2 h. The RNA contained in the *H. pylori* strains was eliminated using 20-µg/mL RNase at 55 °C for 10 min. The DNA samples were isolated using the phenol-chloroform-isopentanol (25:24:1) extraction technique. Genotyping for *cagA* was performed using the primers of the following sequences (Sangon Biotech Co., Ltd., Shanghai, China): sense, 5'-ATGGAAAATATCATACAACCCC-3', and antisense, 5'-CATCTTCTAAATGGGAAACGCC-3'; length, 268 bp. The thermal cycling condition was as follows: pre-denatured at 96 °C for 1 min; denatured at 94 °C for 1 min and annealed at 60 °C for 1 min, 35 cycles; and extended at 94 °C for 1 min. Polymerase chain reaction (PCR) products were separated on 1% agarose gels containing 0.5 g/mL ethidium bromide and visualized by ultraviolet transillumination. *H. pylori* strain NCTC 11639 (Institute of Digestive Disease, Shanghai, China) was used as a positive control and *H. pylori* strain NCTC 12908 (Institute of Digestive Disease, Shanghai, China) as a negative control.

Cell apoptosis analysis with flow cytometry: Following 12, 24, and 48 h of co-culture, GES-1 cells were collected, washed in chilled PBS, and fixed in 70% pre-chilled (-20 °C) ethanol at 4 °C for 18 h, and resuspended at a density of 1×10^6 /mL. Fixed GES-1 cells were washed in PBS three times and stained with propidium iodide (Sigma, St Louis, MO) for 30 min prior to the analysis using an EPICS® ALTRA™ flow cytometer (Beckman Coulter, Inc., Brea, CA). The experiments were performed in duplicate and repeated in triplicate independently.

The *H. pylori* strain that exhibited the most significant cell proliferative effect on MTT assay over 24 h of co-culture (36.8% increase) was harvested from a GC specimen and deemed as the most virulent strain, while a strain from a CG specimen that exhibited the least significant cell proliferative effect on MTT assay over 24 h of co-culture (15.0% increase) was deemed the least virulent.

***In vivo* carcinogenicity assay in Mongolian gerbils**

The two representative strains, the most and the least virulent, were subsequently used to establish a *H. pylori* infection animal model^[16]. The animal care and use complied with the regulations established and approved by the Animal Research Committee at Central South University. Seven-week-old specific-pathogen-free male Mongolian gerbils ($n = 30$) were purchased from the Laboratory Animal Center, Zhejiang Provincial Institute of Medical Sciences, Hangzhou, China. Gerbils were housed in an environment constantly maintained at a temperature of 25 °C, a relative humidity of 55%, and a 12 h/12 h light/dark cycle. Animals had no access to rodent chow for 12 h and

Table 1 Primer pairs for quantitative real-time polymerase chain reaction

Gene	Primer Sequence (5' to 3')		Size of PCR product (bp)
	Sense	Anti-sense	
HDAC6	ACCGTACGAGCAGGGTA	CGAGACGTGCAGGAAAGC	155
TARF1	TCCCGTAACACCTGATTAA	ACAAC TCCCAAACCATACAC	146
GAPDH	AACGGATTGGTCGTATTGGG	TCGCTCCTGGAAAGATGGTGAT	216

PCR: Polymerase chain reaction.

drinking water for 4 h prior to the pretreatment with 0.3 mL 50% ethanol lavage per animal. *H. pylori* strains were cultured for three days using the aforementioned protocol, and resuspended in 7.5% (w/v) heat-inactivated brain heart infusion broth (Sangong Biotech, Shanghai, China) at a density of 1.0×10^9 cfu/mL using a spectrophotometer (Eppendorf). Fasted animals were lavaged with 0.5-mL suspension of the most or least virulent *H. pylori* strain ($n = 10$ for each strain) per animal three times, at a 12 h interval. Ten animals that were lavaged with the broth alone using the same protocol served as the control. Animals were allowed to resume oral intake two hours following the last lavage. Animals were sacrificed by cervical dislocation 4, 16, 28, 40 and 52 wk ($n = 2$ at each time point) following the lavage with *H. pylori* suspensions or broth. Fresh gastric mucosal specimens were collected from the gastric antrum and body for the rapid urease test, and the duplicate specimens were fixed in 4% paraformaldehyde for histological examination using hematoxylin and eosin staining.

Determination of the differentially expressed genes between GES-1 cells co-cultured with the most and least virulent strains

Total RNA extraction: The two representative *H. pylori* strains were co-cultured with GES-1 cells for 24 h, respectively, at a cell/bacterium ratio of 1:50, the optimal ratio for *H. pylori*-induced cell proliferation as determined in MTT assay. Mono-cultured GES-1 cells were used as a control. Total cellular RNA was extracted from infected and non-infected cells using Qiagen RNeasy Mini kit (Invitrogen, Carlsbad, CA) for further microarray analysis and quantitative real-time (qRT)-PCR verification. RNA concentration and purity were determined using an ultraviolet spectrometer (Eppendorf). Denaturing agarose gel electrophoresis was performed to validate the integrity of RNA samples. The experiments were performed in duplicate and repeated in triplicate independently.

Oligonucleotide microarray: The GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, United States), containing 48 000 transcript probes, was used to assess the global gene expression of GES cells in response to *H. pylori* infection. Microarray analysis was performed as instructed by the manufacturer. Chip scanning and data analysis were processed using Affymetrix Microarray Suit Software 5.0 to identify significant differential gene expression profiles. A gene with a signal ratio of more than 2.0 (up-regulated) or less than

0.5 (down-regulated) was defined to be a differentially expressed gene when co-cultured GES-1 cells were compared with control cells, or when cells co-cultured with the most virulent strain were compared with those co-cultured with the least virulent strain.

Validation of potential carcinogenesis-associated genes among the most differentially expressed genes

Quantitative real-time polymerase chain reaction in GES cells: Two carcinogenesis-associated genes that encode histone deacetylase 6 (HDAC6) and tumor necrosis factor receptor-associated factor 1 (TRAF1)^[17,18] were among the most differentially expressed genes. Due to their unknown roles in the pathogenesis of GC, the transcriptions of HDAC6 and TRAF1 were evaluated using quantitative real-time polymerase chain reaction (qRT-PCR). Primer pairs for qRT-PCR were listed in Table 1. GAPDH, as an internal control, was co-amplified with the specific genes. Briefly, total cellular RNA was extracted from GES cells infected with the most and least virulent strains as well as uninfected GES cells, and reversely transcribed into cDNA by M-MLV reverse transcriptase and random hexamer primer (Invitrogen, Carlsbad, CA). The cDNAs were amplified by 30 PCR cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 45 s, and extension at 72 °C for 30 s. As a final step, the extension was at 72 °C for 1 min. PCR products were separated on 1% agarose gels containing 0.5 g/mL ethidium bromide and visualized by ultraviolet transillumination. SYBR qPCR Mix (Invitrogen, Carlsbad, CA) was used to monitor DNA synthesis. The experiments were performed in duplicate and repeated in triplicate independently.

Immunohistochemistry in gastric biopsy specimens:

In an independent experiment, *H. pylori*-positive gastric mucosal specimens from the 113 patients were used for immunohistochemistry (IHC) to verify the expression of HDAC6 and TRAF1 in different gastric pathologies. Briefly, goat-anti-HDAC6 (1:100; Santa Cruz Biotechnology, Inc., Heidelberg, Germany) and rabbit-anti-TRAF1 (1:100; BIOSS, Beijing, China) were used as primary antibodies. Conjugated biotin was identified by using horseradish peroxidase-labeled streptavidin (1:200; Santa Cruz Biotechnology, Inc.) and visualized by using 3,3'-diaminobenzidine (1:200; Santa Cruz Biotechnology, Inc.). Overall, at least 200 cells in ten randomly selected fields were counted, and the percentages of positive cells against the total counted cells were calculated for each specimen. The IHC staining intensity was semiquantitatively

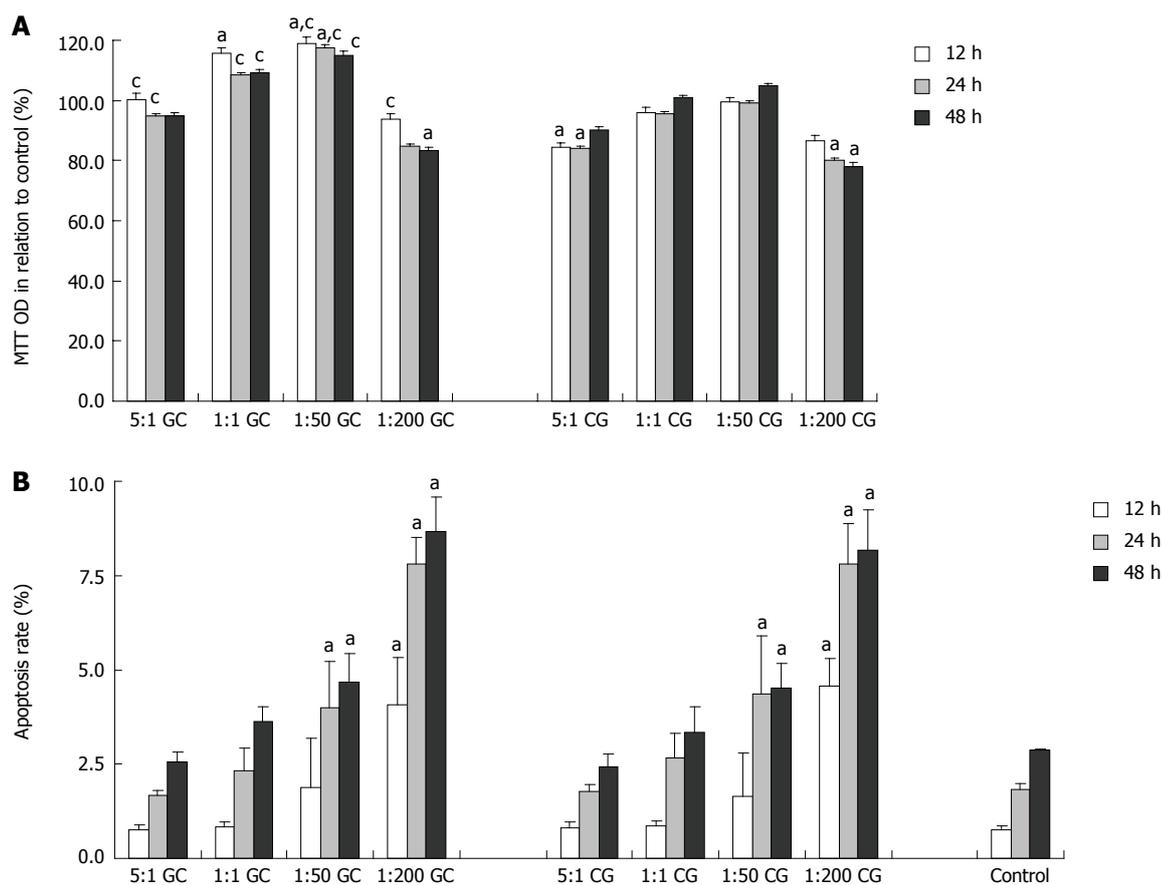


Figure 1 GES-1 cell proliferation as determined by 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide assay (A) and cell apoptosis as determined by flow cytometry (B) after co-culture with *helicobacter pylori* isolated from patients with gastric carcinoma (GC, $n = 10$) or chronic gastritis (CG, $n = 10$) for 48 h in relation to control cells. The ratio denotes the ratio of GES-1 cells vs *Helicobacter pylori* cells. ^a $P < 0.05$ vs control group; ^c $P < 0.05$ vs CG group. OD: Optical density.

determined as negative (positive cells $< 10\%$), moderately positive (10% - 50%), and strongly positive ($> 50\%$). The experiments were performed in duplicate and repeated in triplicate independently.

Statistical analysis

SPSS ver. 13.0 (SPSS Inc., Chicago, IL) was used for the statistical analysis. All numerical data were expressed as mean \pm SD, and compared by using the Student's *t*-test or analysis of variance, when appropriate. All categorical data were expressed as percentage and compared by using the χ^2 -test or Fisher's exact probability test, when appropriate. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

GC-derived *H. pylori* strains induced potent proliferation in GES-1 cells

As shown in Figure 1A, GES-1 cells exhibited an overall cell proliferative response over time to the co-culture with *H. pylori* isolated from GC. A low-concentration (5:1) GC-derived *H. pylori* infection *in vitro* had minimal effect on GES-1 cell proliferation. At a cell/*H. pylori* ratio of 1:1 or 1:50, GES-1 cells co-cultured with GC-derived *H. pylori*

strains had a significantly higher proliferation rate relative to those with CG-derived *H. pylori* strains and control cells. In contrast, CG-derived *H. pylori* co-culture at a ratio of 5:1, 1:1, and 1:50 had no significant impact on cell proliferation over time. Of note, the co-culture at a higher concentration (1:200) *H. pylori* isolated from either GC or CG compromised the proliferation capability of GES-1 cells.

GC- and CG-derived *H. pylori* strains induced comparable apoptosis in GES-1 cells

Flow cytometry analysis showed that the apoptosis rate increased over time in cells co-cultured with *H. pylori* and non-infected cells (Figure 1B). In comparison with the control cells, GES-1 cells co-cultured with GC- or CG-derived *H. pylori* strains had a significantly increased apoptotic rate at 24 and 48 h at the ratios of 1:50, and at all time points at the ratio of 1:200. However, there was no significant difference in the increase of apoptotic rate between GES-1 cells co-cultured with GC-derived *H. pylori* strains and those co-cultured with CG-derived *H. pylori* strains.

The most virulent GC-derived *H. pylori* strain induced gastric mucosal carcinogenesis in the animal model

The most virulent strain, which was derived from a GC patient, and the least virulent strain, which was derived

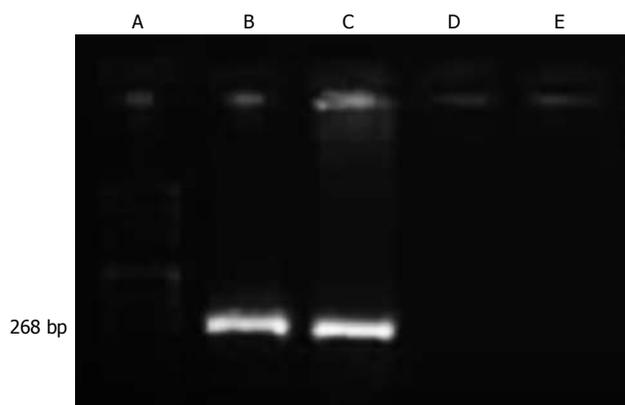


Figure 2 Identification of status of *cagA* gene in *Helicobacter pylori* strains using real-time polymerase chain reaction. A: DNA ladder; B: *Helicobacter pylori* (*H. pylori*) strain NCTC 11639 (positive control); C: The most virulent gastric carcinoma-derived strain; D: The least virulent chronic gastritis-derived strain; E: *H. pylori* strain NCTC 12908 (negative control).

from a CG patient, were genotyped to be *cagA* positive and *cagA* negative, respectively (Figure 2). *H. pylori* rapid urease testing was found positive in 60.0% (6/10), 70.0% (7/10), and 0.0% (0/10) of the animals lavaged with the most virulent strain, least virulent strain, and control lixivium, respectively. No animals, except one in the group infected with most virulent strain that died at week 3, accidentally died during the experiment. *H. pylori* infection was present in all animals lavaged with *H. pylori*, but in none of the control animals, as shown by histological examination. The animals lavaged with the most virulent strain were more prone to precancerous diseases and gastric carcinoma than those lavaged with the least virulent strain and control lixivium. At week 52, CG, atrophy, metaplasia, dysplasia, and GC were observed in 90.0% (9/10), 80.0% (8/10), 80.0% (8/10), 90% (9/10), and 60.0% (6/10), respectively, of the animals lavaged with the most virulent strain (Table 2). Precancerous lesions including gastric atrophy, intestinal metaplasia, and dysplasia were observed from the 4th week, and gastric cancer occurred as early as at week 28 (Figure 3A, Table 2). However, only mild CG was observed in 90% (9/10) of the animals lavaged with the least virulent strain (Figure 3B, Table 2). In addition, gastric ulceration was observed in 50.0% (5/10) and 40.0% (4/10), respectively, in the two groups. The gastric mucosa was within normal limits in all control animals at any time points (Figure 3C, Table 2).

Microarray analysis identified significant differential gene expression profiles in GES-1 cells co-cultured with the most versus least virulent *H. pylori* strains

Microarray analysis identified 2834 and 314 differentially expressed genes in GES-1 cells co-cultured with most or least virulent *H. pylori* strain, respectively, compared with non-infected cells (Figure 4). Furthermore, 800 differentially expressed genes (49 up- and 751 down-regulated), involving those associated with cell cycle regulation, cell apoptosis, cytoskeleton, immune response, and sub-

stance and energy metabolisms, were identified in cells co-cultured with the most virulent strain compared with those co-cultured with the least virulent strain (Table 3). Six most differentially expressed genes (with a betweenness centrality of 0.1-0.2) were identified among the significant differential gene profile network, including JUN, KRAS, BRCA1, SMAD2, TRAF1, and HDAC6 (Table 4).

Up-regulated expressions of HDAC6 and TRAF1 in GES-1 cells co-cultured with the most vs least virulent *H. pylori* strains

qRT-PCR analyses verified significant up-regulations of HDAC6 and TRAF1 mRNA expression in GES-1 cells co-cultured with the most virulent strain or least virulent *H. pylori* strain, compared with the control cells (Figure 5A). Furthermore, HDAC6 and TRAF1 mRNA expressions were more significantly up-regulated in GES-1 cells co-cultured with the most virulent strain than in those co-cultured with the least virulent strain (Figure 5B).

Progressive over-expression of HDAC6 and TRAF1 in CG, precancerous disease, and GC specimens on immunohistochemistry

The histology and immunohistochemistry of gastric mucosal specimens from *H. pylori*-positive patients with CG (Figure 6A, E and I), IM (Figure 6B, F and J), dysplasia (Figure 6C, G and K), and GC (Figure 6D, H and L) showed that moderately positive and strongly positive HDAC6 expression were detected in 5 (21.7%) of 23 CG patients, 10 (30.0%) of 33 IM patients, 18 (54.5%) of 33 dysplasia patients, and 21 (77.8%) of 27 GC patients ($P < 0.001$). Furthermore, the positive rate of HDAC6 expression was significantly higher in dysplasia and GC, compared to that in CG ($P = 0.014$; $P < 0.001$) and in IM ($P = 0.049$; $P < 0.001$). However, there was no difference in the positive rate between CG and IM, and between dysplasia and GC. Similarly, the up-regulation of TRAF1 expressions was detected in 8/23 (34.8%), 16/33 (53.3%), 24/33 (72.7%), and 24/27 (88.9%) specimens of CG, IM, dysplasia, and GC, respectively ($P < 0.001$). The positive rate of TRAF1 expression was significantly greater in dysplasia and GC vs CG ($P = 0.005$; $P < 0.001$) and in GC vs IM ($P = 0.003$), whereas there was no difference between CG and IM and between dysplasia and GC.

DISCUSSION

Gastric carcinogenesis is a pathological process of cell cycle disorder and uncontrolled growth resulting from multiple aberrant gene alterations in response to extrinsic stimulus like *H. pylori* infection. The dysregulation of the balance between cell proliferation and apoptosis plays a pivotal role in this pathogenesis^[19]. The proliferation rate of epithelial cells that are co-cultured with *H. pylori* isolated from gastric mucosal biopsy specimens is shown to be two-fold higher than that of the normal control. *H. pylori* eradication can reverse the proliferative effect on gastric mucosal epithelia though^[20]. Moreover, both *H. pylori*

Table 2 Pathological outcomes of gastric mucosae from Mongolian gerbils lavaged with the most virulent gastric carcinoma-derived strain, the least virulent chronic gastritis-derived strain, and the control broth over 52 wk

	Pathological outcomes				
	Chronic gastritis	Gastric atrophy	Intestinal metaplasia	Gastric dysplasia	Gastric carcinoma
GC (<i>n</i> = 10)	9 (90.0%) ^b	8 (80.0%) ^{b,d}	8 (80.0%) ^{b,d}	9 (90.0%) ^{b,d}	6 (60.0%) ^{b,d}
CG (<i>n</i> = 10)	9 (90.0%) ^b	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Control (<i>n</i> = 10)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)

^b*P* < 0.01 vs control group; ^d*P* < 0.01 vs CG group. GC: Gastric carcinoma; CG: Chronic gastritis.

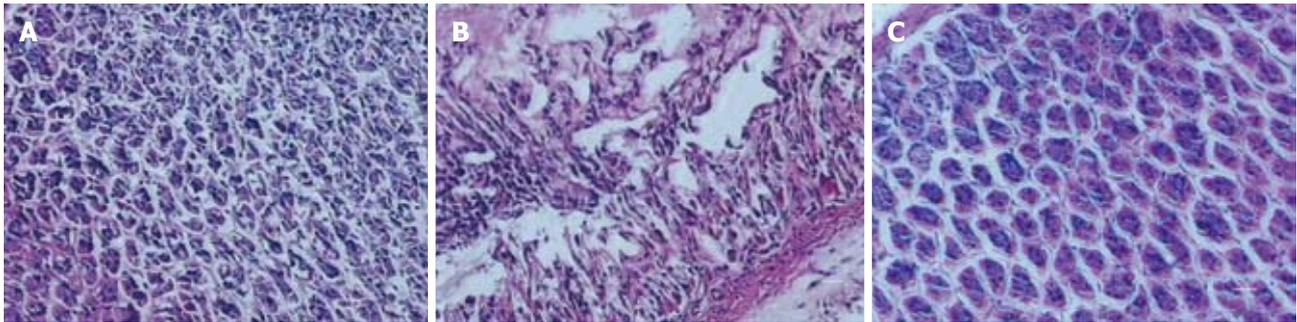


Figure 3 Representative histological microphotographs (hematoxylin and eosin staining, 200 ×, scale bar = 100 μm) of gastric mucosal specimens collected from Mongolian gerbils lavaged with the GC-derived *Helicobacter pylori* strain (A: gastric carcinoma), the CG-derived strain (B: chronic gastritis), and the control broth (C) at the 28th week.

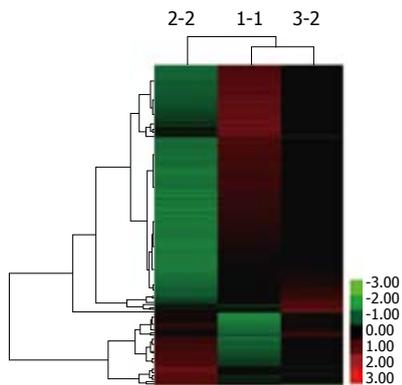


Figure 4 Cluster analysis of gene expression profiles in GES-1 cells in response to the most and least virulent *Helicobacter pylori* strains. The red, green, and black colors indicate up-regulated, down-regulated, and equivalent expression. 1-1: Uninfected GES-1 cells (control); 2-2: GES-1 cells co-cultured with the GC-derived strain; 3-2: GES-1 cells co-cultured with the CG-derived strain. GC: Gastric carcinoma; CG: Chronic gastritis.

intracellular extracts and secretions are found to directly stimulate epithelial growth. Our results indicate that the effects of *H. pylori* co-culture on GES-1 cell proliferation are *H. pylori* concentration dependent, preferably at a cell/*H. pylori* ratio of 1:50; however, the presence of high-concentration *H. pylori* suppresses GES-1 growth instead. The antiproliferative effect of high-concentration *H. pylori* has been reported in multiple cell lines. This inhibitive effect is associated with the production of massive cytotoxic factors, which cause DNA damage, induce cell apoptosis, increase the synthesis of induced nitric oxide synthase, and upregulate p53 expression^[21-23]. Of

note, GC-derived *H. pylori* strains produced a more potent proliferative effect on GES-1 cells than CG-derived *H. pylori* strains, which is consistent with the findings of Yu *et al.*^[24]. Moreover, both GC- and CG- *H. pylori* strains had a mild and comparable effect on apoptosis of GES-1 cells in a concentration-dependent but not strain-specific manner as shown by flow cytometry. These findings suggest that *H. pylori* strain-specific virulence may not be associated with gastric epithelial apoptosis, at least *in vitro*.

H. pylori strains isolated from humans are capable of colonizing the gastric mucosa of Mongolian gerbils^[16,25]. Additionally, Hirayama *et al.*^[26] reported that *H. pylori*-infected Mongolian gerbils exhibited a gastric mucosal pathology similar to human *H. pylori* infection. In the present study, infection with the most virulent GC-derived *H. pylori* strain in Mongolian gerbils resulted in a series of mucosal pathologies that manifest as chronic inflammation, atrophy, intestinal metaplasia, or dysplasia more frequently than did the infection with the CG-derived strain over 52 wk. Moreover, GC developed as early as 28 wk following the infection with the GC-derived strain. This observation may be of significant clinical implications. First, previous studies have reported that the time to develop GC is approximately 62 wk^[24] or even 72 wk after infection^[27]. We assume that less virulent or less carcinogenic strains may have been used in the previous studies, and we propose that more carcinogenic strains should be used in animal experiments related to *H. pylori*-induced carcinogenesis and prevention of *H. pylori*-induced GC. Second and more importantly, it is suggested that the *H. pylori* strains that exhibit a potent epithelial prolifera-

Table 3 Differentially expressed genes with a fold change of > 2.0 or < 0.5 in GES-1 cells co-cultured with the most virulent gastric carcinoma-derived strain vs the least virulent chronic gastritis-derived strain

Gene name (n = 185)	Fold change GC/CG	Accession number			
Cell cycle related genes					
AMN1	4.748	BG031897			
HDAC6	2.025	NM_006044			
CCNE2	0.464	AF112857			
KIF20B	0.462	NM_016195			
DOCK8	0.456	AL161725			
ASPM	0.452	AK001380			
CENPF	0.450	U30872			
NIPBL	0.432	NM_015384			
SYCP2	0.424	NM_014258			
ASPM	0.414	NM_018123			
SGOL2	0.401	N31731			
CENPE	0.398	NM_001813			
CEP70	0.382	NM_024491			
SGOL2	0.375	AW965339			
DOCK11	0.363	AI742838			
SERPINB3	0.290	BC005224			
Apoptosis-related genes					
TRAF1	2.254	NM_005658			
TIA1	0.496	AL567227			
OPA1	0.474	AB011139			
PIK3CA	0.450	NM_006218			
NUDT12	0.408	AL136592			
PEG10	0.367	BE858180			
Cytoskeleton and sports					
ADD3	0.450	AI818488			
KIAA0774	0.429	AI818409			
KIF14	0.379	AW183154			
KIF14	0.370	NM_014875			
ADD3	0.344	BE545756			
ADD3	0.316	NM_019903			
Intracellular transport					
VPS13A	0.435	AW629014			
VPS13A	0.438	AW629014			
IFT74	0.431	NM_025103			
GOLGA4	0.425	NM_002078			
ANKRD10	0.419	BE670056			
IFT80	0.414	AB037795			
FAM8A1	0.412	NM_016255			
SNX2	0.406	NM_003100			
ANKRD32	0.355	AL136560			
DNA synthesis, repair, recombination					
RAD50	0.472	NM_005732			
SFPQ	0.457	AV705803			
FAM8A1	0.412	NM_016255			
LIN9	0.338	BF697734			
DNA-binding, transcription, transcription factor					
ZNF253	5.254	NM_021047			
KLF11	3.598	AA149594			
SMARCA1	0.496	NM_003069			
GOLGB1	0.492	NM_004487			
KLF9	0.462	NM_001206			
CHD1	0.459	NM_001270			
PBX1	0.455	AL049381			
TRIP11	0.434	AF007217			
SMARCA1	0.433	NM_003069			
C8orf83	0.433	BE962119			
EPM2AIP1	0.430	BF432224			
ELL2	0.420	AI745624			
GLCCI1	0.400	AA058770			
ARID5B	0.395	BG285011			
ZNF644	0.390	NM_016620			
BAZ2B	0.365	NM_013450			
SAMD9	0.352	AA741307			
SAMD9	0.345	NM_017654			
TPR	0.346	BF110993			
TPR	0.344	AK023111			
LCORL	0.339	AI807408			
TPR	0.300	AW235355			
Cell signal and transduction					
PRKCB	0.498	M13975			
DST	0.493	NM_001723			
FARP1	0.462	BF725250			
GMFB	0.461	NM_004124			
PPM1A	0.454	AA886888			
IFT81	0.444	NM_014055			
PDE10A	0.442	AI143879			
ICK	0.428	NM_014920			
ANKRD10	0.419	BE670056			
IFT80	0.414	AB037795			
SNX2	0.406	NM_003100			
CNTLN	0.386	AA280904			
MAP2K6	0.384	NM_002758			
ANKRD32	0.355	AL136560			
ARHGAP18	0.307	BE644830			
Protein translation, synthesis, decomposition					
RHOBTB3	0.500	NM_014899			
ST13	0.490	U17714			
EEF2K	0.488	W68180			
TSHZ2	0.476	AW953679			
TMF1	0.417	AI767750			
HMMR	0.410	U29343			
CCDC88A	0.387	AB033038			
NSBP1	0.321	BC005342			
Protein-coding gene					
CXorf39	0.499	AI590719			
LOC286052	0.478	AA278233			
FLJ40113/LOC440295	0.411	AI632181			
LOC100133781	0.374	AA973100			
LOC100130360	0.346	BG231554			
LOC643401	0.300	BC039509			
Ion channel and transport					
IFT74	0.496	AI610355			
SLC5A3	0.493	AK024896			
SLC2A13	0.489	AL565362			
STEAP4	0.488	NM_024636			
TMEM56	0.469	AI004375			
EXOC5	0.466	BF509391			
CACNB2	0.465	AI040163			
SLC2A13	0.455	NM_052885			
TMEM133	0.435	AF247167			
DMXL2	0.405	AB020663			
SNX13	0.401	R75838			
SEC62	0.397	NM_153039			
ATP11C	0.382	BF475862			
TMEM106B	0.380	BF513060			
PEG10	0.367	BE858180			
SORBS2	0.222	AI659533			
Cell proliferation, angiogenesis					
ANGPTL4	2.460	NM_016109			
TPR	0.492	NM_003292			
TPR	0.492	NM_003292			
PNN	0.487	U59479			
PNN	0.428	U59479			
ROCK2	0.404	AL049383			
TTK	0.347	NM_003318			
Immune-related genes					
CFI	0.477	NM_000204			
SERPINB4	0.431	AB046400			
PIBF1	0.423	NM_006346			
Metabolize-related genes					
AK7	0.477	NM_152327			
TTC3	0.471	AI652848			
HS2ST1	0.438	NM_012262			
MANEA	0.427	AI587307			

PLA2G12A	0.416	AV714268
TTC3	0.413	D83077
PPP1CB	0.407	W67887
NUDT12	0.408	AL136592
TTC3	0.403	NM_003316
TTC3	0.403	AI885338
RNF150	0.398	AA722069
SEPP1	0.396	NM_005410
CRYZ	0.387	NM_001889
PDK4	0.374	AV707102
ABAT	0.359	AF237813
PLA2G12A	0.331	AI767751
AGXT2L1	0.326	NM_031279
CYP1B1t	0.314	NM_000104
METTL7A	0.274	NM_014033
Cell structure-related genes		
ARMCX3	0.385	AL121883
ANK3	0.225	NM_020987
Cell Adhesion-related genes		
KITLG	0.447	AI446414
PIK3CA	0.450	NM_006218
ANKRD10	0.419	BE670056
ANKRD32	0.355	AL136560
Stress-related genes		
DNAJB4	0.400	BG252490
LXN	0.294	NM_020169
DNAJB4	0.471	NM_007034
Protein regulation		
LOC727770	0.426	AI359676
TBC1D8B	0.368	AW172431
Cell differentiation		
LIFR	0.374	AA701657
RNA processing		
SR140	0.413	AU152088
Protein receptor		
LANCL1	0.412	NM_006055
Oxidation		
DIO2	0.434	AI038059
Cell growth-related genes		
ITCH	0.441	AA868238
TGFBR3	0.450	AW193698
TGFBR3	0.422	NM_003243
Cytokine receptor		
LTB	2.001	NM_002341

tive effect are highly virulent and carcinogenic, and that individuals infected with these strains are at high risk for the development of GC and should receive appropriate *H. pylori* eradication therapy.

Human whole-genome microarray analysis identifies a large number of significant differentially expressed genes in GES-1 cells co-cultured with *H. pylori* strains *vs* non-infected control cells, which clearly indicates that *H. pylori* activates and inactivates a series of gene transcription of GES-1 cells *in vitro*. Our transcriptional profiling results were generally consistent with the previous reports. Liu *et al*^[14] reported that *H. pylori* infection induced the up-regulated expression of multiple chemokines and chemokine receptors, such as IL-8 and CCL5, as well as of apoptosis-related genes, such as GADD45A. Eftang *et al*^[28] reported that *interleukin-8* was the single most up-regulated gene in whole genome profiling of *H. pylori* exposed gastric epithelial cells. MAPK and NF- κ B cellular pathways were also powerfully activated; the marked up-regulation of TP53BP2 corresponding to ASPP2 protein may interact

Table 4 Most differentially expressed genes in GES-1 cells co-cultured with the most virulent gastric carcinoma-derived strain *vs* the least virulent chronic gastritis-derived strain

Gene name	Betweenness centrality	Description	Identified or proposed function
JUN	0.201780	Jun oncogene	Cell growth and/or maintenance, signal transduction molecules, and transcription factors
KRAS	0.185944	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	Regulation of cell cycle, cell growth and/or maintenance
BRCA1	0.162070	Breast cancer 1, early onset	DNA damage checkpoint; p53/ATM signaling, and induction of apoptosis
SMAD2	0.121460	SMAD family member 2	Transcription factors
TRAF1	0.092496	Tumor necrosis factor receptor-associated factor 1	DNA damage checkpoint, p53/NF-KB signaling, and cell cycle control
HDAC6	0.092239	Histone deacetylase 6	Cell proliferation and tumor angiogenesis

with *H. pylori* CagA, and cause marked p53 suppression of apoptosis. Sohn *et al*^[29] suggested that the intracellularly translocated CagA may be involved in epithelial mesenchymal transition. However, the present study, for the first time, reports that the expression of HDAC6 and TRAF1 is up-regulated in gastric mucosal cells upon co-culture with GC-derived, *cagA*-positive *H. pylori* strain, and the expressions of these two proteins are progressively up-regulated in CG, intestinal metaplasia, dysplasia, and GC. Furthermore, such response seems to be strain-specific for many genes as the number of differentially expressed genes in cells co-cultivated with the GC-derived strain is 9-fold that in those co-cultured with the CG-derived strain. These significant differentially expressed genes involve genes that are known to be associated with tumorigenesis, among which HDAC6 and TRAF1 are the most prominent ones. The upregulation of HDAC6 and TRAF1 expressions in response to the GC-derived *H. pylori*-strain and in relation to *H. pylori* infection in patients with GC was verified by qRT-PCR in GES-1 cells *in vitro* and by immunohistochemistry in gastric specimens taken from patients with different gastric pathologies. To our best knowledge, this is the first study to identify the overexpression of HDAC6 and TRAF1 in *H. pylori*-associated GC, and to suggest a potential role of these genes in *H. pylori*-induced gastric carcinogenesis.

Histone is an important component of eukaryotic chromatin. Acetylation and deacetylation of histone are essential for the regulation and modification of gene expression^[30]. A newly-discovered mechanism of carcinogenesis is that HDAC family proteins aberrantly binding to a specific promoter region may cause cryptic transcription and inhibit normal gene transcription, initiating the malignant transformation^[31]. HDAC6 functions to modulate gene expression by removing the acetyl group from histones, which contributes to oncogenic cell trans-

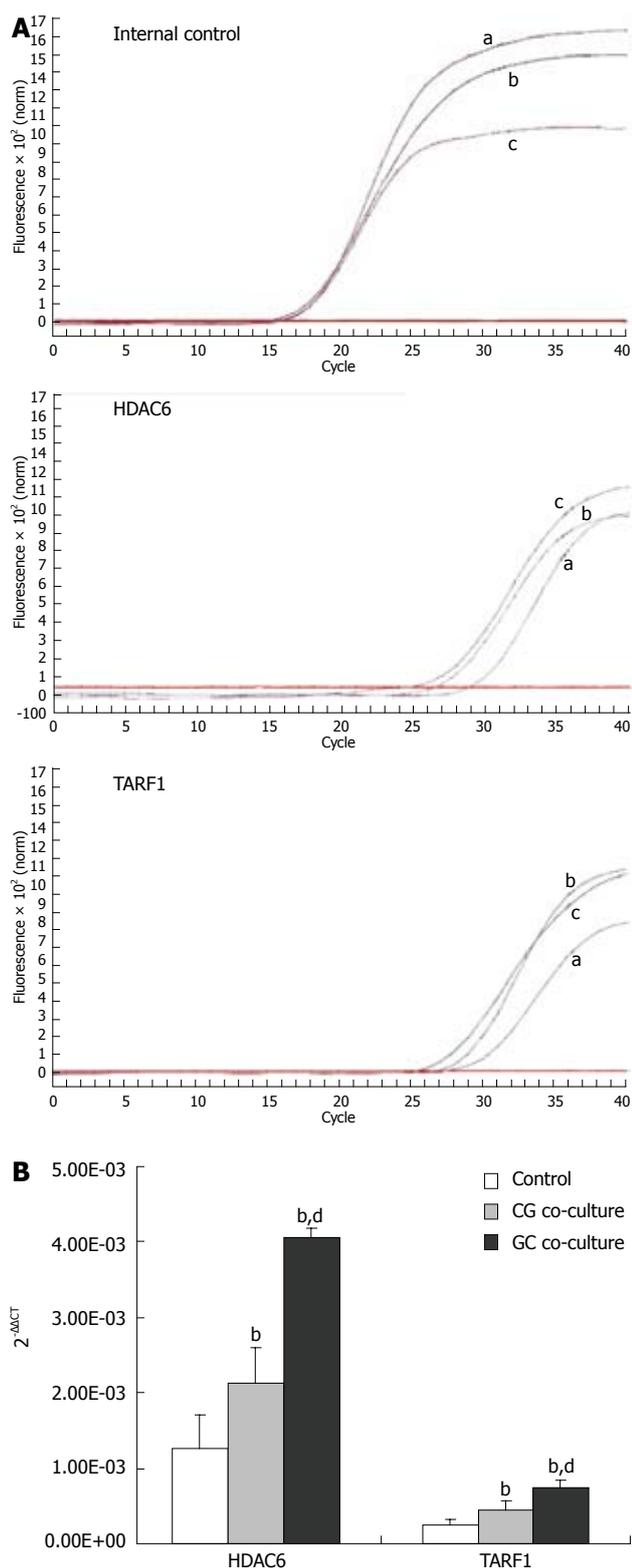


Figure 5 Verification of HDAC6 and TRAF1 up-regulation in GES-1 cells in response to the most and least virulent *Helicobacter pylori* strains as determined by quantitative real-time polymerase chain reaction analysis. A: Representative quantitation of mRNA expression of GAPDH, HDAC6 and TRAF1 in GES-1 cells (a), GES-1 cells co-cultured with the CG-derived *Helicobacter pylori* (*H. pylori*) strain (b) and GES-1 cells co-cultured with the GC-derived *H. pylori* strain (c); B: Graphic analysis showing mRNA expression of HDAC6 and TRAF1 expressions in GES-1 cells in GES-1 cells, GES-1 cells co-cultured with the CG-derived *H. pylori* strain and GES-1 cells co-cultured with the GC-derived *H. pylori* strain. ^b*P* < 0.01 vs the control; ^d*P* < 0.01 vs cells co-cultured with the CG-derived *H. pylori* strain.

formation^[32]. Aoyagi *et al*^[33] reported that HDAC6 could directly regulate HSP90 expression via deacetylation. The targeted inhibition of HDAC6 reduces the deacetylation of HSP90 but increases the acetylation simultaneously, destroying HSP90 chaperones and resulting in molecular function failure. HDAC6 synergizes with HDAC10 to regulate vascular endothelial growth factor receptors through heat shock protein mediation as well^[34]. HDAC6 has been found to be implicated in multiple malignancies, such as esophageal cancer, lung cancer, breast cancer, and oral squamous cell carcinoma^[35,36]. Zhang *et al*^[35] reported that high-HDAC6-expressing premenopausal breast cancer patients exhibited a favorable tumor-free survival and a sensitive response to endocrine therapy. HDAC is also thought to be associated with breast cancer metastasis as it de-acylates microtubules, whereas the combined use of estrogen antagonist and paclitaxel significantly suppresses the de-acylation of microtubules^[37,38]. In the present study, the expression of HDAC6 mRNA was highly up-regulated in GES-1 cells co-cultured with the GC-derived *H. pylori* strain compared with those co-cultured with the CG-derived strain and uninfected control cells. It is likely that *H. pylori* infection activates HDAC6 to dysregulate the synthesis of histones in gastric epithelial cells^[39]. However, further investigation is required to elucidate the exact mechanisms of *H. pylori* infection and the functional roles of *H. pylori*-activated HDAC6 overexpression in gastric carcinoma.

TRAF is a new member of the tumor necrosis factor (TNF) family^[40]. Seven TRAF isoforms have been reported to interact directly with cell-surface receptors and regulate cell survival/death balance^[41]. TRAF1 activates NF- κ B to gradually initiate immortalization and tumorigenesis in GC^[42]. Sughra *et al*^[43] reported that TRAF1 functions primarily to up-regulate the transcription of IKK β , an inhibitor of NF- κ B and to enhance the activity of IKK β as well. Therefore, TRAF1 activates and interacts with NF- κ B simultaneously. The overexpression of TRAF1 has been identified in nasopharyngeal carcinoma and lymphoma^[44,45]. The variation in TRAF1 expression is associated with the occurrence, metastasis, and induction of chemotherapy resistance of malignant tumors^[46]. Similar to HDAC6, TRAF1 is verified by qRT-PCR to be up-regulated in GES-1 cells co-cultured with *H. pylori* strains, especially the GC-derived strain in the present study. Again, how *H. pylori* infection up-regulates TRAF1 expression and what downstream genes are activated and/or deactivated by *H. pylori*-induced TRAF1 overexpression require further investigation.

The roles of HDAC6 and TRAF1 in *H. pylori*-associated GC remain to be elucidated although their expression is significantly up-regulated in epithelial cells stimulated with GC-derived *cagA* positive *H. pylori* strain. Our GC animal model experiment showed that the proliferative effect *in vitro* of GC-derived *cagA* positive *H. pylori* strain can contribute to the carcinogenesis of gastric mucosa *in vivo*. However, the up-regulated expression of HDAC6 and TRAF1 may not be causative of proliferative or carcinogenic effect of the GC-derived *H. pylori* strain as no

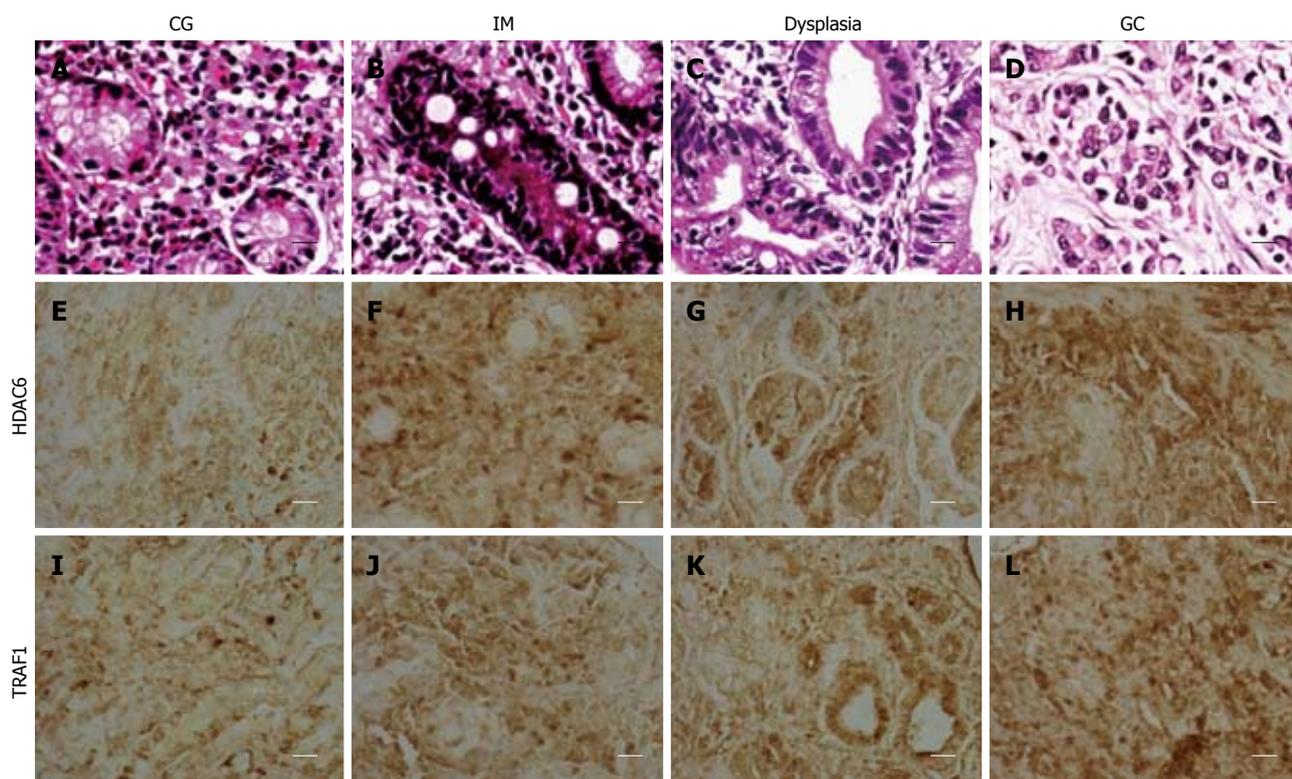


Figure 6 Histology and immunohistochemistry (400 ×, scale bar = 50 μm) of HDAC6 and TRAF1 in gastric specimens of patients with chronic gastritis (CG, *n* = 23), intestinal metaplasia (IM, *n* = 30), dysplasia (*n* = 33), and gastric carcinoma (GC, *n* = 27).

“loss-of-function” or “gain-of-function” experiment has been performed to elaborate the pathogenetic effect of HDAC6 and TRAF1 in GC. Moreover, we were unable to follow up GC patients in subsequent treatment period as they were referred to various general surgeons among multiple institutions at the patients’ own will. Therefore, the clinicopathological values of HDAC6 and TRAF1 are yet to be investigated, which are expected to be clinically useful for the prediction of prognosis and treatment resistance.

In conclusion, GC-derived *H. pylori* strains induce a more potent proliferative but comparable apoptotic effect in GES-1 cells as compared to CG-derived strains. HDAC6 and TRAF1 are identified to be up-regulated in GES-1 cells co-cultured with the GC-derived strain, which are further verified *in vivo*. These findings indicate that these two genes may be involved in *H. pylori* induced gastric carcinogenesis, although their exact roles require further investigation.

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COMMENTS

Background

Helicobacter pylori (*H. pylori*) is a pathogenic bacterium colonizing gastric mucosae. It has been accepted to be the primary cause of upper gastrointestinal

disorders, such as acute and chronic gastritis, peptic ulcer disease, and gastric cancer. However, the subsequent outcomes of persistent *H. pylori* infection are highly variable. Such variation in clinicopathological outcomes of *H. pylori* infection is believed to result from the variations in the virulence of different strains, genetic background of the host, and more importantly, the host-to-pathogen interactions. How gastric epithelial cells respond to *H. pylori* clinical isolates derived from patients with different pathologies, such as chronic gastritis (CG) and gastric cancer (GC) specimens, at the genome-wide level remains unknown. Therefore, this study was carried out to identify genes potentially involved in *H. pylori*-induced gastric carcinogenesis, by comparing the genomic profiles between gastric epithelial cells co-cultured with *H. pylori* strains isolated from patients with GC and those co-cultured with strains from patients with CG.

Research frontiers

It is likely that *H. pylori* strains themselves are highly variable in virulence to gastric mucosal epithelia, especially in terms of the carcinogenic effect. Genomic profiling analyses have identified a wide range of genetic variations among *H. pylori* strains isolated from patients with different gastric pathologies. Global gene expression profiles also vary greatly in human gastric epithelial immortalized cells infected with spiral *versus* coccoid *H. pylori*. These findings suggest that gastric epithelial cells tune in the expression of their genes, especially those associated with tumorigenesis, in response to specific *H. pylori* strains or a specific virulent factor of the strain. In addition to the core genes, strain-specific genes are thought to play an essential role in *H. pylori* propagation and pathogenesis.

Innovations and breakthroughs

This observation may be of significant clinical implications. First, previous studies have reported that the time to develop GC is approximately 62 wk or even 72 wk after infection. The authors assume that less virulent or less carcinogenic strains may have been used in the previous studies, and the authors propose that more carcinogenic strains should be used in animal experiments related to *H. pylori*-induced carcinogenesis and prevention of *H. pylori*-induced GC. Second and more importantly, it is suggested that the *H. pylori* strains that exhibit a potent epithelial proliferative effect are highly virulent and carcinogenic, and that individuals infected with these strains are at high risk for the development of GC and should receive appropriate *H. pylori* eradication therapy.

Applications

The roles of HDAC6 and TRAF1 in *H. pylori*-associated GC remain to be elucidated although their expression is significantly up-regulated in epithelial cells stimulated with GC-derived *cagA* positive *H. pylori* strain. These findings indicate that these two genes may be involved in *H. pylori* induced gastric carcinogenesis, although their exact roles require further investigation.

Peer review

The authors performed DNA microarray analysis comparing gene expression profiles between GES-1 cell lines co-cultured with highly virulent (*cagA*+) and low virulent (*cagA*-) *H. pylori* strains isolated from human gastric mucosa tissue with CG and GC tissue, respectively. These two strains showed different effect on the GES-1 cell line in cell proliferation activity but similar effect on the cell lines in apoptotic property. They identified 800 differentially expressed genes, and from six most differentially expressed genes, they selected TRAF1 and HDAC6. The authors examined HDAC1 and TRAF1 expression at the mRNA level in GES-1 cells co-cultured with the *H. pylori* strains above, and confirmed that these expressions were up-regulated in these cell lines. They also examined the expressions of these molecules immunohistochemically in surgically resected or biopsied specimens. These works are laborious and are considered to be scientifically of significant value.

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Magnetic resonance-based total liver volume and magnetic resonance-diffusion weighted imaging for staging liver fibrosis in mini-pigs

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Abstract

AIM: To determine whether and how magnetic resonance imaging (MRI)-based total liver volume (TLV) and diffusion weighted imaging (DWI) could predict liver fibrosis.

METHODS: Sixteen experimental mature mini-pigs (6 males, 10 females), weighing between 20.0 and 24.0 kg were prospectively used to model liver fibrosis induced by intraperitoneal injection of 40% CCl₄ dissolved in fat emulsion twice a week for 16 wk, and by feeding 40% CCl₄ mixed with maize flour twice daily for the subsequent 5 wk. All the survival animals underwent percutaneous liver biopsy and DWI using $b = 300, 500$ and 800 s/mm^2 followed by abdominal gadolinium-enhanced MRI at the 0, 5th, 9th, 16th and 21st weekend after beginning of the modeling. TLV was obtained on enhanced MRI, and apparent diffusion coefficient (ADC) was obtained on DWI. Hepatic tissue specimens were stained with hematoxylin and Masson's trichrome staining for staging liver fibrosis. Pathological specimens were scored using the human METAVIR classification system. Statistical analyses were performed to determine whether and how the TLV and ADC could be used to predict the stage of liver fibrosis.

RESULTS: TLV increased from stage 0 to 2 and decreased from stage 3 ($r = 0.211$; $P < 0.001$). There was a difference in TLV between stage 0-1 and 2-4 ($P = 0.03$) whereas no difference between stage 0-2 and 3-4 ($P = 0.71$). TLV could predict stage ≥ 2 [area under receiver operating characteristic curve (AUC) = 0.682]. There was a decrease in ADC values with increasing stage of fibrosis for $b = 300, 500$ and 800 s/mm^2 ($r = -0.418, -0.535$ and -0.622 , respectively; all $P < 0.001$). Differences were found between stage 0-1 and 2-4 in ADC values for $b = 300, 500$ and 800 s/mm^2 , and between stage 0-2 and 3-4 for $b = 500$ or 800 s/mm^2 (all $P < 0.05$). For predicting stage ≥ 2 and ≥ 3 , AUC was 0.803 and 0.847 for $b = 500 \text{ s/mm}^2$, and 0.848 and 0.887 for $b = 800 \text{ s/mm}^2$, respectively.

CONCLUSION: ADC for $b = 500$ or 800 s/mm^2 could be better than TLV and ADC for $b = 300 \text{ s/mm}^2$ to pre-

dict fibrosis stage ≥ 2 or ≥ 3 .

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Key words: Magnetic resonance imaging; Total liver volume; Liver fibrosis; Apparent diffusion coefficient; Stage

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INTRODUCTION

Liver fibrosis can be prevented or reversed at early stage by antifibrotic treatment or by eliminating the cause^[1,2]. If liver fibrosis could not be prevented at early stage, it would lead to end-stage liver diseases^[3]. Therefore, accurately staging liver fibrosis is important in choosing appropriate therapy to improve the prognosis of this disease. Percutaneous liver biopsy is currently considered a gold standard for assessing stage of fibrosis, but this invasive method is limited by inter- and intra-observer variability and sampling error^[4,5]. To overcome the limitations of liver biopsy, noninvasive, reproducible and reliable methods are greatly needed. Transient elastography or FibroTest (Bio-Predictive) have been proposed for the diagnosis of liver fibrosis, but their use in clinical practice is being investigated^[6].

Diffusion-weighted imaging (DWI), a type of functional magnetic resonance (MR) technique, has been developed to characterize diseased tissues. This technique allows measurement of the combined effects of micro-circulation of blood (perfusion) and molecular brownian motion of water within liver parenchyma, expressed as a whole by apparent diffusion coefficient (ADC)^[7]. Previous studies have shown that ADC values of the liver are lower in patients with cirrhosis compared with control subjects, which is thought to reflect a restriction of the motion of water molecules in fibrotic tissues^[8,9]. Nevertheless, ADC values obtained in the previous experiences for studying liver fibrosis widely varied because of the employed settings of so-called *b*-values^[8,10]. In addition, a previous study also reported that total liver volume (TLV) would change with the progress of liver fibrosis^[11]. To our knowledge, there have been no data focusing on the correlation of liver ADC value obtained by different *b* values with the stage of liver fibrosis, and the correlation between magnetic resonance imaging (MRI)-based TLV and stage of liver fibrosis^[11-14]. Thus, the purpose of this study was to prospectively investigate these correlations

and determine whether and how TLV and DWI could predict the stage of liver fibrosis.

MATERIALS AND METHODS

Animal model

Animals were used in full compliance with the National Council of Animal Care guidelines. The protocol was approved by the Committee of the Ethics of Animal Experiments of our institute.

Sixteen experimental mature mini-pigs (6 males, 10 females), weighing between 20.0 and 24.0 kg, were used in our study. Previous studies have established a standardized experimental model of liver cirrhosis in swine using CCl₄ and ethanol^[15]. According to the above-mentioned modeling method, liver fibrosis was induced by intraperitoneal injection of 40% CCl₄ dissolved in fat emulsion (0.25 mL/kg body weight) twice a week for 16 wk, and by feeding 40% CCl₄ mixed with maize flour (0.75 mL/kg body weight) twice daily for the subsequent 5 wk because of the peritoneal adhesions resulting from the intraperitoneal injection. To minimize the chemical peritonitis involving liver, we chose the left hypogastrium as the intraperitoneal injection position and the injections were stopped two days before each MR examination. In addition, because the administration of alcohol in conjunction with CCl₄ results in more stable and accelerated liver fibrogenesis in a large animal model, we used 5% alcohol-water mixture as the sole drinking water, and maize flour was taken as the staple food in the 21 wk^[16].

MRI technique

On the 0, 5th, 9th, 16th and 21st weekend after the beginning of modeling fibrosis, all mini-pigs were given general anesthesia with an injection of ketamine (15 mL/kg weight) and diazepam (0.8 mg/kg per hour) through the ear vein before the MR examination. The anterior surface of the thorax and abdomen of the animals were shaved to obtain good contact between the skin and the cardiac electrodes or respiratory triggering. We also used a belt around the abdomen to reduce the effect of respiratory motion.

The mini-pigs were examined on a 1.5 T whole body MR scanner (Signa Excite; GE Medical Systems, Milwaukee, WI). When the cardiac and respiratory signals were satisfied, each animal was positioned supinely in an 8-channel phased array body coil. After the pilot scan with axial, coronal, and sagittal images for localization, the MR protocols including SPGR T1-weighted imaging (T1WI), fast recovery fast spin echo (FRFSE) T2-weighted imaging (T2WI), and single-shot echo-planar imaging (EPI) DWI were performed. The parameters for DWI were as follows: TR = 4000 ms, TE = 49.2 ms, field of view (FOV) = 34 × 34 cm, slice thickness = 5.0 mm, slice space = 2.0 mm, matrix of 192 × 256 mm, number of excitation = 2, *b* values of 0-300, 0-500 and 0-800 s/mm², and tridirectional diffusion gradients. After DWI acquisition, 20 mL gadodiamide (Magnevist,

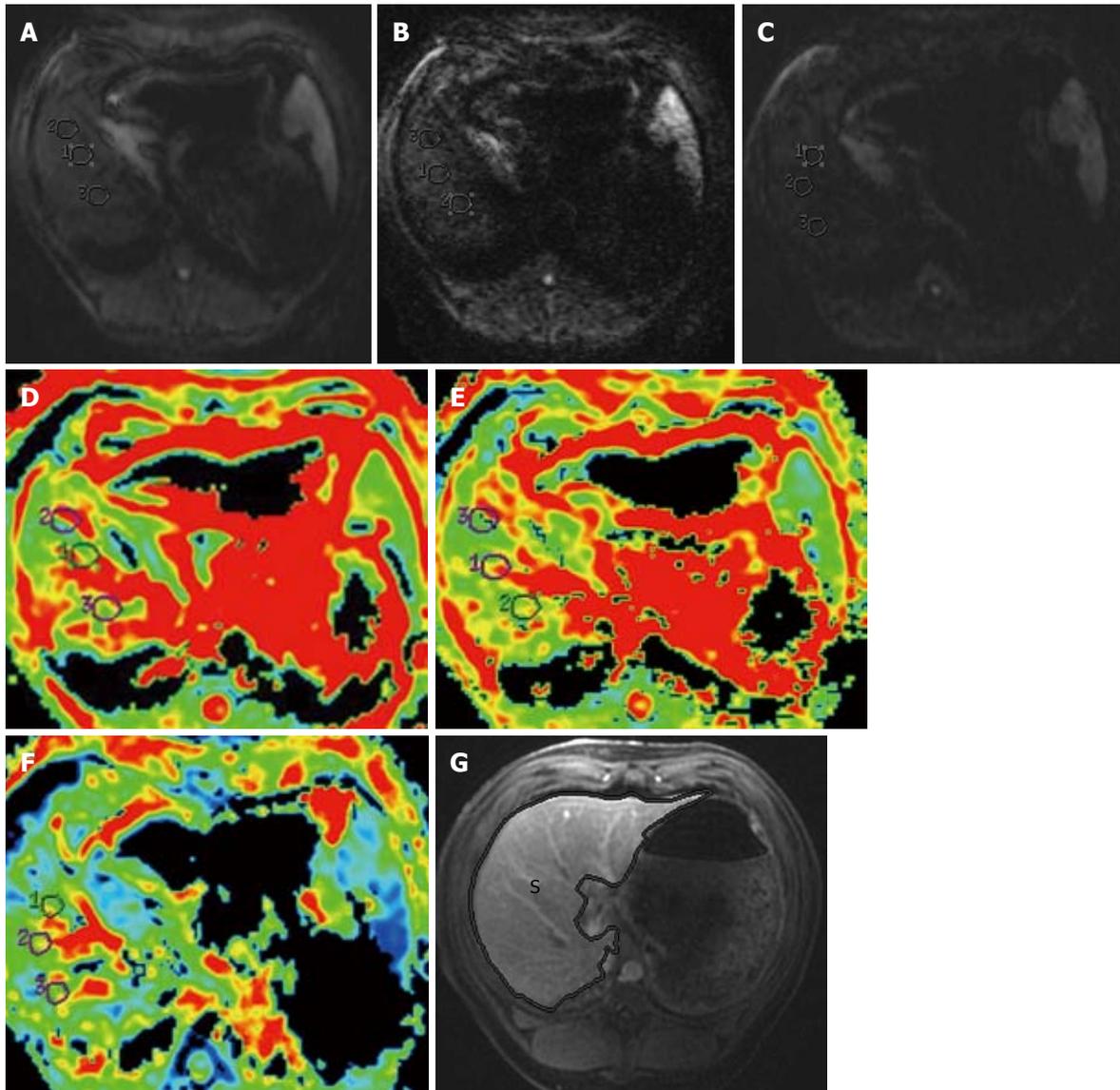


Figure 1 Magnetic resonance images. A-C: In a mini-pig with liver fibrosis stage 2, diffusion weighted images of the liver are obtained with b values of 300, 500 and 800 s/mm^2 , respectively; D-F: Corresponding apparent diffusion coefficient maps. Three regions of interest are carefully drawn on the right lobes of the liver for each b values; G: A manual tracing of the liver is shown on an axial contrast-enhanced magnetic resonance imaging, and the outline shows the liver areas (S).

Bayer Healthcare, Germany) was intravenously injected (0.2 mmol/L per kg body weight) with a pressure injector (Spectris MR Injection System; Medrad, Inc, Warrendale, PA) at a dose of 3 mL/s followed by a 20-mL saline solution flush for contrast-enhanced three-dimensional liver acquisition with volume acceleration (3D LAVA). The parameters for 3D LAVA were: TR = 3.9 ms, TE = 1.8 ms, FOV = 34 × 34 cm, slice thickness = 5.0 mm, and matrix 256 × 224 mm.

MR image analysis

The original MRI data were directly interfaced and forwarded to the workstation (GE, AW4.1, Sun Microsystems, Palo Alto, CA, United States) to obtain ADC maps at each b values. An experienced radiologist (the first author with 3 years of experience in thoracoabdominal radiology) who was blinded to the pathologic results

placed three circular regions of interest (ROIs) each with approximately 1-2 cm in diameter in the consecutive three maximal slices of the right lobe of the liver (3 ROIs per slice, 9 ROIs per mini-pig) for each b value (Figure 1A-C), avoiding areas of artifact, diaphragm and intrahepatic vasculature. The ADC value of each ROI as well as the ADC map (Figure 1D-F) was automatically generated. The ADC values of the three ROIs in each slice were then averaged to give an estimate of ADC value for this slice. Representative ADC values of the three slices were then averaged to obtain a final estimate of the liver ADC value to be used for data analysis.

In addition, TLV measurements were performed employing planimetry in all animals by the above-mentioned radiologist. Initially, liver profile was manually traced on each transverse image using a trackball. The software automatically calculated the number of pixels enclosed by

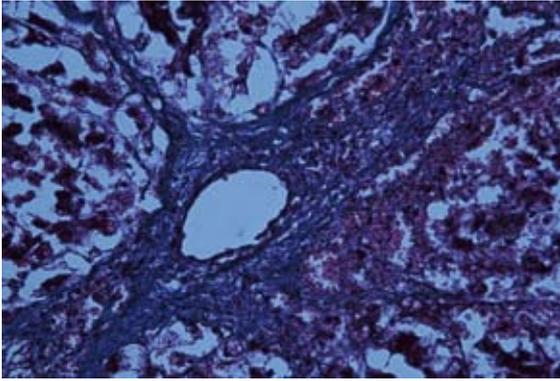


Figure 2 Photomicrograph (original magnification, $\times 400$; Masson's trichrome stains) of histologic sections from liver biopsy specimens in a mini-pig with stage 2 liver fibrosis shows liver periportal fibrosis.

the traced liver contours on each image, and provided the cross-sectional area of the liver on a slice-by-slice basis. The sum of the above areas multiplied by the section thickness provided the TLV (Figure 1G)^[17].

Reproducibility studies

To test the interobserver variability of TLV and ADC values measurement, we randomly chose the MR data of all animals on the 9th weekend for the repeated MR measurements, which were performed two weeks after the first measurement by an experienced radiological professor (the corresponding author with 14 years of experience in abdominal radiology). The precision of the two measurements on the 9th weekend was determined as the coefficient of variance (CV) on the basis of the formula: $CV (\%) = (s/X) \times 100$, where s is the SD, and X is the mean of TLV or ADC values. The resultant precision was expressed as an average %CV. When %CV was less than 10%, interobserver variability of TLV and ADC values measurements were regarded as small, and the results were reliable.

Histopathology

After the MR examination in each mini-pig, an 18-gauge ultrasound-guided core percutaneous biopsy was performed in the right liver lobe because the biopsy in the right lobe of the liver was used as the standard for staging liver fibrosis^[18]. When the mini-pigs died during the follow-up period before the 21st weekend, the dead mini-pigs underwent immediate laparotomy, and the entire liver was resected. When the mini-pigs were living on the 21st weekend, 1/3 animals were randomly sacrificed by air injection into the auricular vein shortly after the last percutaneous biopsy and underwent the laparotomy, and the entire liver was also resected. Subsequently, three thin slices were randomly cut from any lobe of the liver and diced into the usual tissue block of about 2 mm thickness for further staging liver fibrosis to confirm the stage of the fibrosis determined by percutaneous biopsy immediately prior to death. Furthermore, the final fibrosis stage of resected liver was determined by the average

of the stages across the three thin slices.

Hepatic tissue specimens obtained by biopsy and laparotomy were stained with hematoxylin and eosin (H and E) and Masson's trichrome staining for pathologic examination. Because the morphology and histology of experimental mini-pig liver are similar to that of human liver^[15], two experienced hepatopathologists (the 11th and 12th authors with 10 and 36 years of experience in hepatopathology, respectively) scored pathological specimens using the human METAVIR classification system^[19]. Any discrepancies between the two observers were settled by consensus. This scoring system has a five-point scale: stage 0, no fibrosis; stage 1, portal fibrosis; stage 2, periportal fibrosis (Figure 2); stage 3, septal fibrosis; and stage 4, cirrhosis. Additionally, we considered the weekend on which liver fibrosis was initially confirmed by pathologic examination as that the fibrosis occurred during the follow-up.

Statistical analysis

All statistical analyses were carried out with SPSS (version 17.0, SPSS, Chicago IL, United States). A $P < 0.05$ was considered to represent a significant difference. The interobserver agreement for the two pathologists with regard to the fibrosis stage, or for the percutaneous biopsy and the laparotomy was expressed by means of κ statistics. When κ values were 0-0.40, 0.41-0.60, 0.61-0.80, and 0.81-1.00, the concordance was considered as poor, moderate, good, and excellent agreement, respectively. The TLV and ADC values were of skewness. Spearman's rank correlation analyses were used to assess the correlation between TLV and fibrosis stage, or between the ADC values and fibrosis stage. TLV or ADC values were compared between patients stratified by fibrosis stages using Mann-Whitney tests together with Bonferroni correction for multiple comparisons. The cutoff value of TLV or ADC values were then determined with receiver-operating characteristic (ROC) analysis for predicting moderate liver fibrosis (stage ≥ 2) and advanced liver fibrosis (stage ≥ 3).

RESULTS

Animal model and histopathologic findings

During the follow-up period, one, two and four animals died between week 5 and 9, between week 9 and 16, and between week 16 and 21, respectively. On the 0, 5th, 9th, 16th and 21st weekend, mean body weight of the survived animals was 22 ± 1.31 kg, 24.1 ± 2.41 kg, 24 ± 2.45 kg, 25.3 ± 2.81 kg, and 23.5 ± 1.78 kg, respectively. There was no significant difference in body weight between any two weekends during the follow-up period (all $P > 0.05$), mini-pigs treated with CCl_4 did not put on weight during the whole progress of experiment. In addition, there was good agreement between fibrosis stages determined by the percutaneous biopsy and by the laparotomy (κ , 0.80; 95%CI, 0.75-0.84). Therefore, according to the human METAVIR classification system, survived mini-pigs with liver fibrosis at different stages confirmed on the follow-

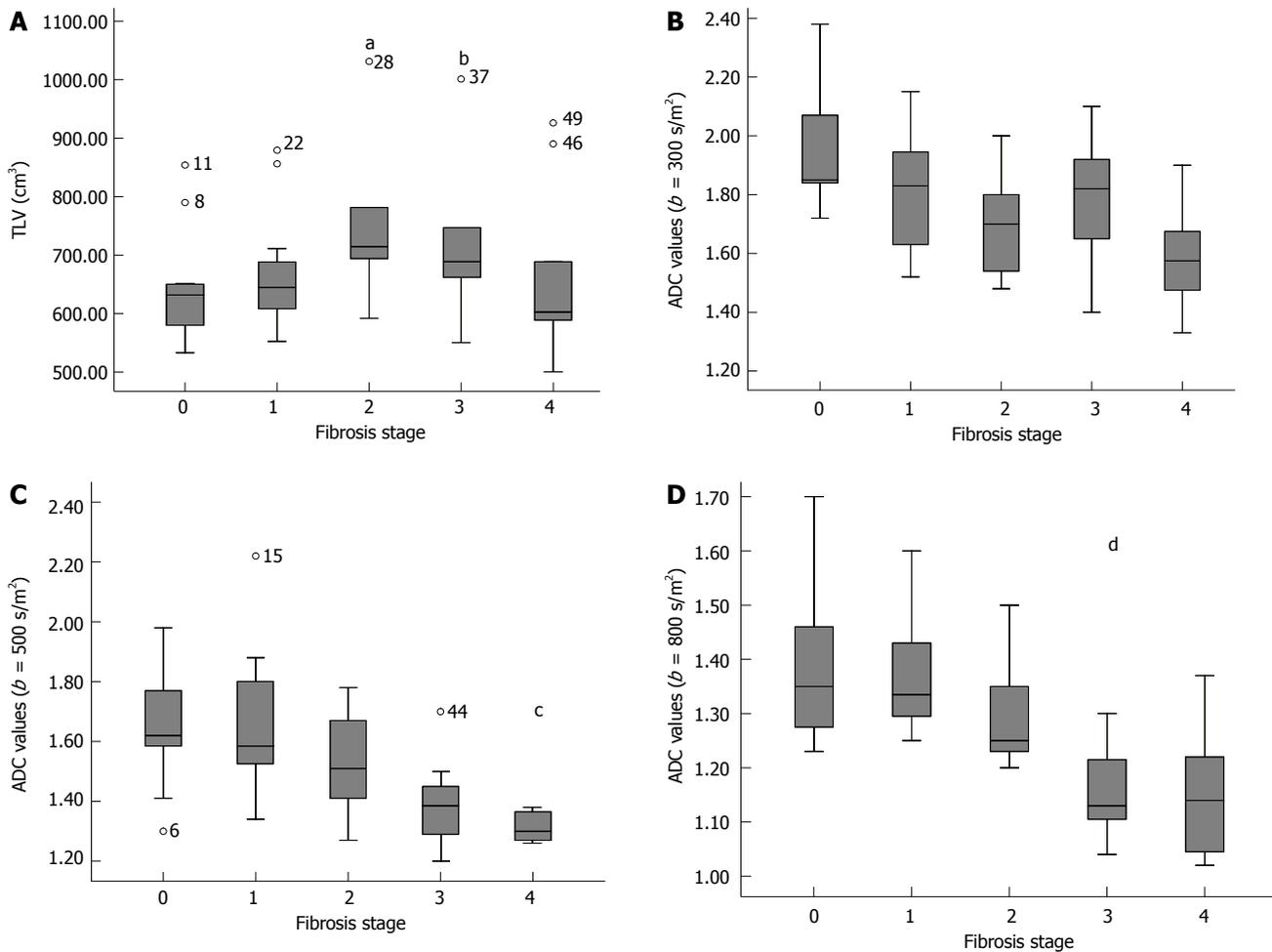


Figure 3 Box plots. A-D: Total liver volume (TLV), and apparent diffusion coefficient (ADC) values of the liver for $b = 300 \text{ s/mm}^2$, 500 s/mm^2 and 800 s/mm^2 corresponding to fibrosis stages, respectively. In A, symbols a and b indicate extreme outliers of TLV in stage 2 and 3, respectively; and symbol c in C and symbol d in D indicate extreme outliers of ADC for $b = 500 \text{ s/mm}^2$ in stage 4 and for $b = 800 \text{ s/mm}^2$ in stage 3, respectively. Horizontal bars represent medians of TLV or ADC.

Table 1 Mini-pigs with liver fibrosis at different stages confirmed on the follow-up weekends (n)					
Weekend	S0	S1	S2	S3	S4
0 (16)	16	0	0	0	0
5 (16)	3 ¹	8	5	0	0
9 (15)	0	3	7	5	0
16 (13)	0	1 ¹	1	7	4
21 (9)	0	1 ¹	1 ¹	1	6

¹The liver fibrosis at this stage in the mini-pigs is similar to this disease at the immediately preceding stage during the follow-up. S0, S1, S2, S3 and S4 represent stage 0, stage 1, stage 2, stage 3 and stage 4, respectively.

up weekends are illustrated in Table 1.

Interobserver variability of TLV and ADC measurements

In all animals on the 9th weekend, CV was 8.2% (range, 2.7%-15.6%) for TLV. CV was 9.2% (range, 5.2%-17.3%), 8.4% (range, 5.3%-15.5%) and 7.6% (range, 4.5%-13.2%) for ADC values with $b = 300, 500$ and 800 s/mm^2 , respectively. Therefore, interobserver variability of TLV and ADC values were small, and the first measurement

was used as the final results.

TLV corresponding to stages of liver fibrosis

TLV corresponding to stage of liver fibrosis is shown in Table 2. TLV tended to increase from stage 0 to 2, but decrease from stage 3 (Figure 3A) ($r = 0.211, P < 0.001$). Furthermore, significant difference could be found between stage 0 and 2 ($P < 0.001$) while TLV could not differentiate other individual stages of fibrosis (all $P > 0.05$). Additionally, there was significant difference between stage 0-1 and 2-4 ($P = 0.03$), whereas there was no difference between stage 0-2 and 3-4 ($P = 0.71$).

ADC values corresponding to stage of liver fibrosis

ADC values corresponding to stage of liver fibrosis are illustrated in Table 2. There was a decrease in liver ADC values with the increasing degree of fibrosis for $b = 300, 500$ and 800 s/mm^2 (Figure 3B-D, respectively) ($r = -0.418, -0.535$ and -0.622 , respectively; $P < 0.001$). ADC values could differentiate fibrosis stage between stage 0 and 2 for $b = 300 \text{ s/mm}^2$; between stage 0 and 4 for $b = 300, 500$ and 800 s/mm^2 ; between stage 1 and 4, stage

Table 2 Total liver volume and apparent diffusion coefficient values corresponding to the stage of liver fibrosis

Fibrosis stage (n)	ADC value ($\times 10^{-3}$ mm ² /s), mean (SD), 95%CI			TLV (cm ³), mean (SD), 95%CI
	$b = 300$ s/mm ²	$b = 500$ s/mm ²	$b = 800$ s/mm ²	
0 (16)	1.96 (0.19), 1.83-2.09	1.67 (0.21), 1.53-1.81	1.39 (0.15), 1.29-1.50	641.08 (99.22), 574.42-707.74
1 (11)	1.81 (0.19), 1.69-1.93	1.66 (0.23), 1.51-1.81	1.37 (0.11), 1.30-1.44	671.62 (106.12), 600.33-742.91
2 (13)	1.69 (0.17) ¹ , 1.57-1.81	1.52 (0.17), 1.40-1.64	1.29 (0.11), 1.22-1.37	776.57 (156.91) ¹ , 655.95-897.18
3 (13)	1.78 (0.21), 1.64-1.91	1.39 (0.13) ² , 1.30-1.47	1.18 (0.15) ² , 1.09-1.28	745.04 (155.51), 625.51-864.58
4 (10)	1.58 (0.17) ¹ , 1.44-1.73	1.35 (0.15) ² , 1.23-1.48	1.15 (0.12) ³ , 1.05-1.25	672.03 (145.33), 560.32-783.75
Grouped stages				
0-1 (27)	1.88 (0.20), 1.79-1.97	1.66 (0.22), 1.57-1.76	1.38 (0.13), 1.32-1.44	656.35 (101.46), 611.36-701.34
0-2 (40)	1.82 (0.21), 1.75-1.90	1.62 (0.21), 1.54-1.70	1.35 (0.13), 1.31-1.40	691.25 (129.80), 643.64-738.86
2-4 (36)	1.69 (0.19) ⁴ , 1.62-1.77	1.42 (0.16) ⁴ , 1.36-1.48	1.21 (0.14) ⁴ , 1.16-1.26	731.21 (153.32) ⁴ , 670.56-791.81
3-4 (23)	1.70 (0.21), 1.60-1.80	1.37 (0.14) ⁵ , 1.31-1.44	1.17 (0.13) ⁵ , 1.10-1.23	708.54 (150.77), 633.56-783.51

¹Different from stage 0, $P < 0.05$; ²Different from stage 0, and from stage 1, $P < 0.05$; ³Different from stage 0, from stage 1, and from stage 2, $P < 0.05$; ⁴Different from stage 0-1, $P < 0.05$; ⁵Different from stage 0-2, $P < 0.05$. TLV: Total liver volume; ADC: Apparent diffusion coefficient.

Table 3 Receiver operating curve analyses of total liver volume and liver apparent diffusion coefficient values for prediction of fibrosis stage ≥ 2 and ≥ 3 (%)

Cut-off	Stage differentiations	AUC	Sensitivity	Specificity
TLV 656.25 cm ³	Prediction of stage ≥ 2	0.682	74.1	73.7
ADC ($b = 800$ s/mm ²)				
1.82×10^{-3} mm ² /s	Prediction of stage ≥ 2	0.743	69.6	73.3
1.75×10^{-3} mm ² /s	Prediction of stage ≥ 3	0.646	63.6	60.0
ADC ($b = 800$ s/mm ²)				
1.51×10^{-3} mm ² /s	Prediction of stage ≥ 2	0.803	82.6	76.7
1.44×10^{-3} mm ² /s	Prediction of stage ≥ 3	0.847	78.8	80.0
ADC ($b = 800$ s/mm ²)				
1.29×10^{-3} mm ² /s	Prediction of stage ≥ 2	0.848	78.3	80.0
1.23×10^{-3} mm ² /s	Prediction of stage ≥ 3	0.887	84.8	81.2

TLV: Total liver volume; AUC: Area under the receiver operating curve; ADC: Apparent diffusion coefficient.

0 and 3, or stage 1 and 3 for $b = 500$ or 800 s/mm²; and between stage 2 and 4 for $b = 800$ s/mm² (all $P < 0.05$). There were no differences between stage 0 and 1, between stage 2 and 3, and between stage 3 and 4 for $b = 300$, 500 and 800 s/mm² (all $P > 0.05$). Additionally, there were significant differences between stage 0-1 and 2-4 for $b = 300$, 500 and 800 s/mm², and between stage 0-2 and 3-4 for $b = 500$ or 800 s/mm² (all $P < 0.05$).

ROC analyses of TLV and liver ADC for predicting stage ≥ 2 and ≥ 3

According to the generally accepted guidelines in Turkey and elsewhere^[20], the patients who had liver fibrosis stage ≥ 2 should receive treatment whereas those with liver fibrosis stage ≤ 1 should not. Therefore, we did not perform ROC analyses of TLV and liver ADC values for predicting fibrosis stage ≥ 1 . There was relatively more overlap for the TLV predicting fibrosis stage ≥ 2 and stage ≥ 3 . ROC analyses showed that TLV could predict fibrosis stage ≥ 2 with a cut-off value of 656.25 cm³, representing a small AUC. However, liver ADC values had a better sensitivity and a specificity for predicting fibrosis stage ≥ 2 and ≥ 3 (Table 3). We also found that

there was a higher diagnostic performance for $b = 500$ or 800 s/mm² compared with $b = 300$ s/mm² for prediction of fibrosis stage ≥ 2 and ≥ 3 .

DISCUSSION

Activation of hepatic stellate cells (the main collagen-producing cells) by fibrogenic cytokines is a central event in fibrosis^[21]. Other cells including portal fibroblasts and bone marrow - derived cells may also be involved in the fibrogenic process^[22-26]. In the past, liver fibrosis was considered to be irreversible, however, hepatic fibrosis is now regarded as a dynamic process with potential for regression^[27]. The accumulation of proteins in the extracellular matrix promotes the formation of scars that bridge together adjacent portal triads and central veins. Ultimately, hepatic fibrosis leads to cirrhosis, associated with nodule formation and organ contraction^[27].

To prevent progression of this disease resulting in cirrhosis, accurately staging fibrosis is of clinical importance. Some studies indicated that DWI could be a useful technique for staging liver fibrosis^[12,28], and that TLV would change with the progress of liver fibrosis^[11,14]. We initially performed a follow-up study focusing on the correlation between stage of liver fibrosis and TLV or liver ADC values by comparing the performance of different b values, and determining how TLV and DWI could predict the stage of liver fibrosis. This study was performed with a large animal model (experimental mini-pigs) because the morphology and histology of its liver were similar to that of human liver, and the liver fibrosis was induced mainly by a matured method of inferior intraperitoneal injection of 40% CCl₄ according to a previous study^[15].

In this study, we found that TLV tended to increase from stage 0 to 2, but decrease from stage 3 of liver fibrosis. Our results were almost consistent with a published study^[29]. According to Liu *et al.*^[29], liver volume (LV) in patients with liver fibrosis tended to increase with the severity of fibrosis from stage 0 to 3, but decrease in stage 4, indicating that TLV tended to increase gradu-

ally with the severity of fibrosis. The presumed pathologic mechanism for LV increase in early stage would be the ballooning of hepatocytes along with increased fibrotic component^[14]. In advanced fibrosis, LV gradually decreased because of the segmental or global liver atrophy^[30]. However, our findings were inconsistent with other published reports^[11,14]. According to Taraou *et al.*^[14], LV in patients with alcoholic liver fibrosis increased gradually with the severity of fibrosis but did not decrease in the stage of alcoholic cirrhosis. The inconsistency of our findings with this published article could be explained by the reason that liver fibrosis was induced by alcoholics. As demonstrated by Li *et al.*^[11], TLV tended to decrease gradually with the increasing degree of virus-induced liver fibrosis staged with the pathologic scoring system (Ishak scale). It is the difference in pathologic scoring system that could be used to explain the inconsistency of our findings with this published article.

In addition to TLV, liver ADC value would be another indicator to quantitatively evaluate liver fibrosis. We found negative correlations between ADC and stage of fibrosis for $b = 300, 500$ and 800 s/mm^2 , which were consistent with these previous studies^[12,31]. According to these articles^[12,31], there was a decrease in liver ADC with increasing degree of fibrosis, and moderate negative correlations could be found between ADC values and fibrosis stages. However, Boulanger *et al.*^[32] found that there was no correlation between the histological grade of fibrosis and ADC using small b values of $50\text{-}250 \text{ s/mm}^2$, and we could presume that the difference in ADC value between normal and fibrotic liver could not be found by using the small b values because the ADC value obtained by the small b values could be prone to be influenced by liver perfusion^[33].

For prediction of fibrosis stage ≥ 2 , ROC analyses in our study showed that TLV could be a predictor with an AUC of 0.682 and the sensitivity and specificity of more than 70% by using a cut-off value of 656.25 mm^3 . TLV could not be a predictor of stage ≥ 3 because there was no difference between stage 0-2 and 3-4.

In comparison with TLV, we found that ADC values obtained with $b = 500 \text{ s/mm}^2$ and 800 s/mm^2 could be better predictors to predict liver fibrosis stage ≥ 2 because of higher AUC of 0.803 and 0.848 using cut-off ADC of $1.51 \times 10^{-3} \text{ mm}^2/\text{s}$ and $1.29 \times 10^{-3} \text{ mm}^2/\text{s}$, respectively. Moreover, ADC values obtained with $b = 500 \text{ s/mm}^2$ and 800 s/mm^2 could also predict liver fibrosis stage ≥ 3 with AUC of 0.847 and 0.887 using cut-off ADC of $1.44 \times 10^{-3} \text{ mm}^2/\text{s}$ and $1.23 \times 10^{-3} \text{ mm}^2/\text{s}$, respectively. Comparing with $b = 500 \text{ s/mm}^2$ and 800 s/mm^2 , we found a relatively lower AUC of 0.743 for a liver ADC value of $1.82 \times 10^{-3} \text{ mm}^2/\text{s}$ obtained using $b = 300 \text{ s/mm}^2$ for prediction of fibrosis stage ≥ 2 . Because there was no difference in ADC value for $b = 300 \text{ s/mm}^2$ between liver fibrosis stage 0-2 and 3-4, ADC value obtained in the current setting could not be used to predict liver fibrosis stage ≥ 3 . Generally, we found that ADC

values with $b = 500$ or 800 s/mm^2 could be better than $b = 300 \text{ s/mm}^2$ for predicting stage of liver fibrosis, which were consistent with the study by Taouli *et al.*^[12]. They suggested that ADC measured on DWI could be used to best quantify liver fibrosis when the b value is 500 s/mm^2 or greater in the comparisons among b values of 0, 50, 300, 500, 700 and 1000 s/mm^2 . This may be because ADC maps acquired with longer b values are less contaminated by perfusion effects. That is, they are more truly diffusion weighted^[33]. Taken together, we concluded that liver ADC measured on DWI with $b = 500$ or 800 s/mm^2 could be recommended for predicting stage of liver fibrosis.

Compared with previous studies, the advantages of our study might be more persuasive to confirm that DWI obtained with several b values could be used to predict stage of liver fibrosis because the normal animals used in this study had been confirmed to have no fibrosis, hepatic steatosis and inflammation by biopsy. A previous clinical study suggested that healthy patients without any symptoms of liver disease could be defined as nonfibrotic, free of steatosis and inflammation, which might influence the ADC value^[34]. According to Angulo *et al.*^[35], most patients with acute liver disease and even with chronic liver cirrhosis remained asymptomatic until decompensation occurred. We could presume that the authors of the previous study might consider the patients with asymptomatic acute or chronic liver disease as healthy patients, which resulted in selection bias. Furthermore, there might be a long interval between patients undergoing liver biopsy and undergoing MRI, and the stage of liver fibrosis might change during the interval, which would potentially affect the results^[36]. Another advantage of this study was that we determined how to use TLV and ADC values to predict stage of liver fibrosis.

There were some limitations in our study. Firstly, our sample size was relatively small. Therefore, further studies involving a larger number of samples are needed to evaluate TLV and ADC values for predicting liver fibrosis stage. Secondly, our study was based on an animal experiment, but our findings could provide some useful information that TLV and ADC values could predict the stage of fibrosis, and we will conduct further studies to confirm the results.

In conclusion, TLV and ADC values might be used to predict stage of liver fibrosis. TLV could predict stage ≥ 2 with a lower diagnostic accuracy, but ADC values with $b = 500$ or 800 s/mm^2 might be good predictors for stage ≥ 2 and ≥ 3 with a higher diagnostic accuracy. This study might provide a noninvasive method for predicting stage of liver fibrosis.

COMMENTS

Background

As a noninvasive method, magnetic resonance imaging has been developed to characterize liver fibrosis. Apparent diffusion coefficient (ADC) obtained by diffusion-weighted imaging (DWI) for studying liver fibrosis widely varied because of the employed settings of so-called b -values. In addition, total liver vol-

ume (TLV) obtained by computed tomography would change with the progress of liver fibrosis. However, correlations of liver ADC value obtained by different *b* values or magnetic resonance imaging (MRI)-based TLV with the stage of liver fibrosis, and whether and how TLV and DWI could predict the stage of liver fibrosis remained unclear.

Research frontiers

ADC obtained by magnetic resonance (MR) DWI for multiple *b* values and TLV obtained by enhanced MR imaging are the hotspots for the research on assessing the stages of liver fibrosis. How the ADC and TLV would change with the increasing stage of liver fibrosis, and whether TLV and DWI could predict the stage of liver fibrosis have not been determined.

Innovations and breakthroughs

The authors utilized magnetic resonance imaging to assess the changes of apparent diffusion coefficient obtained by DWI and TLV obtained by enhanced scans with the stage of liver fibrosis, and utilized receiver-operating characteristic (ROC) curve analysis to determine whether TLV and DWI could predict the stage of liver fibrosis.

Applications

The authors found that TLV and ADC values might predict stage of liver fibrosis. ADC values with *b* = 500 or 800 s/mm² might be good predictors for stage ≥ 2 and ≥ 3 with higher diagnostic accuracy, but TLV could predict stage ≥ 2 with a lower diagnostic accuracy. Although this is an experimental study, the findings could be helpful for the relevant clinical studies.

Terminology

DWI, a method that assesses the diffusion of protons within tissue by applying motion sensitizing gradients that causes diffusing protons to lose signal. The amount of signal loss is influenced by the strength of the diffusion weighting (the diffusion sensitivity parameter or *b* value of the sequence) and the ability of protons to diffuse through tissues (the ADC).

Peer review

This interesting study determined whether MRI based liver volume and DWI are useful to stage liver fibrosis in mini-pigs. Results presented in this paper showed that TLV increased during stage 0-2, whereas it decreased from stage 3 liver fibrosis. The ADC values were decreased with increasing stage of fibrosis. Authors conducted histopathological studies in liver biopsy specimens to analyze the damage during fibrosis. Overall, these findings show that both TLV and ADC values provide an important tool to predict stage of liver fibrosis.

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Adult-to-adult living donor liver transplantation for acute liver failure in China

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Abstract

AIM: To investigate the long-term outcome of recipients and donors of adult-to-adult living-donor liver transplantation (AALDLT) for acute liver failure (ALF).

METHODS: Between January 2005 and March 2010, 170 living donor liver transplantations were performed at West China Hospital of Sichuan University. All living liver donor was voluntary and provided informed consent. Twenty ALF patients underwent AALDLT for rapid deterioration of liver function. ALF was defined based on the criteria of the American Association for the Study of Liver Diseases, including evidence of coagulation abnormality [international normalized ratio (INR) ≥ 1.5] and degree of mental alteration without pre-ex-

isting cirrhosis and with an illness of < 26 wk duration. We reviewed the clinical indications, operative procedure and prognosis of AALDLT performed on patients with ALF and corresponding living donors. The potential factors of recipient with ALF and corresponding donor outcome were respectively investigated using multivariate analysis. Survival rates after operation were analyzed using the Kaplan-Meier method. Receiver operator characteristic (ROC) curve analysis was undertaken to identify the threshold of potential risk factors.

RESULTS: The causes of ALF were hepatitis B ($n = 18$), drug-induced ($n = 1$) and indeterminate ($n = 1$). The score of the model for end-stage liver disease was 37.1 ± 8.6 , and the waiting duration of recipients was 5 ± 4 d. The graft types included right lobe ($n = 17$) and dual graft ($n = 3$). The mean graft weight was 623.3 ± 111.3 g, which corresponded to graft-to-recipient weight ratio of $0.95\% \pm 0.14\%$. The segment V or VIII hepatic vein was reconstructed in 11 right-lobe grafts. The 1-year and 3-year recipient's survival and graft survival rates were 65% (13 of 20). Postoperative results of total bilirubin, INR and creatinine showed obvious improvements in the survived patients. However, the creatinine level of the deaths was increased postoperatively and became more aggravated compared with the level of the survived recipients. Multivariate analysis showed that waiting duration was independently correlated with increased mortality ($P = 0.014$). Furthermore, ROC curve revealed the cut-off value of waiting time was 5 d ($P = 0.011$, area under the curve = 0.791) for determining the mortality. The short-term creatinine level with different recipient's waiting duration was described. The recipients with waiting duration ≥ 5 d showed the worse renal function and higher mortality than those with waiting duration < 5 d (66.7% vs 9.1%, $P = 0.017$). In addition, all donors had no residual morbidity. Furthermore, univariate analysis did not show that short assessment time induced the high morbidity ($P = 0.573$).

CONCLUSION: Timely AALDLT for patients with ALF greatly improves the recipient survival. However, further systemic review is needed to investigate the optimal treatment strategy for ALF.

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Key words: Acute liver failure; Adult-to-adult liver donor liver transplantation; Recipient; Donor; Risk factors

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INTRODUCTION

Acute liver failure (ALF) is a condition in which rapid deterioration of liver function results in hepatic encephalopathy and coagulopathy in individuals without pre-existing cirrhosis^[1]. Because ALF progresses rapidly and recovers poorly, the emergency liver transplantation is recommended to treat ALF which has a high likelihood of death^[1]. However, because the shortage of donor organ and the long waiting time for a suitable graft, the patients might deteriorate further and eventually die while waiting. Living donor liver transplantation (LDLT) is an effective option for this dilemma, which may reduce waiting time and provide more optimal timing for surgery and shorter cold ischemia time.

Adult-to-adult LDLT (AALDLT) for ALF has recently been reported, mostly in Asians^[2-4], although it possibly increases the donor's risk because of large-sized graft removed and an evaluation of donor in an urgent scenario. In addition, an optimal graft might not be obtained because of a short assessment time for donors. The recent reports have described the optimal survival rate of recipients and the minimum rate of morbidity of the donors^[2-6]. However, a small sample could not support AALDLT as a better solution for patients with ALF. Thus, the continued development of AALDLT treatment is necessary to determine treatment option for patients with ALF.

ALF caused by hepatitis B virus (HBV) infection has a lower spontaneous recovery rate than that induced by drugs^[7]. In China, the most frequent cause of ALF is HBV, thus emergency AALDLT will be particularly significant for these patients with a high mortality and limited deceased organs. In this study, we reported the long-term outcome of recipients and donors of emergency

AALDLT for ALF performed in our center.

MATERIALS AND METHODS

Patients

Between January 2005 and March 2010, 170 LDLTs were performed at West China Hospital of Sichuan University. Twenty ALF patients underwent AALDLT for rapid deterioration of liver function. ALF was defined based on the criteria of the American Association for the Study of Liver Diseases (AASLD)^[1], including evidence of coagulation abnormality [international normalized ratio (INR) ≥ 1.5] and any degree of mental alteration without pre-existing cirrhosis and with an illness of < 26 wk duration. Patients with underlying chronic diseases such as chronic hepatitis B and autoimmune hepatitis were included if their disease has only been recognized for < 26 wk. Patients with cirrhosis identified by histologic examination of the liver explants were excluded. Informed consent was obtained from the patients and their families.

Donor evaluation

The primary selection criteria for a living liver donor were: being voluntary and providing informed consent which clearly stated that living liver donation can lead to donor risk. Each donation was approved by the Ethics Committee of West China Hospital of Sichuan University. The first essentiality of donor medical evaluation included ABO blood type identity or compatibility and age < 65 or > 18 years. Donors with known medical disorder that significantly increased a perioperative risk or contraindicated donation were excluded. Liver biochemistry, hepatitis serological tests, and complete blood cell count, coagulation test, cardio-pulmonary function tests to exclude chronic liver disease or potential contraindication were routinely performed in donors. Computed tomography (CT) scan for volumetric size measurement was performed to evaluate graft size and the size of the future remnant donor liver. The donors' remnant liver volume should be greater than 30% of the total liver volume (TLV) by CT volumetry. Dual graft liver transplantation with two left hemiliver or a combination of a right hemiliver and left hemiliver was adopted when the suitability of single graft transplant was in doubt in view of donor safety [remnant liver volume (RLV) < 30% of TLV] or small-for-size graft for recipients [graft volume to recipient standard liver volume (GV/SLV) ratio < 40%]. The donor assessment was usually completed within 24-48 h to shorten the waiting time of recipients.

For minimizing the risks and complications of definitive donors, the typical preoperative invasive diagnostic procedures, including hepatic angiography, liver biopsy, and cholangiography, were abolished and the following managements were adopted: (1) hepatic angiography was substituted with CT arteriography to study the tracks and variations of the hepatic artery, but hepatic angiography will further be performed if hepatic artery was not visualized; (2) preoperative endoscopic retrograde

choledochopancreatography was routinely substituted by magnetic resonance cholangiopancreatography and intraoperative cholangiography^[8].

Donor and recipient operations

The donor and recipient operation was performed according to the previously published technique^[8]. Intraoperative liver biopsy was routinely performed to exclude donors with severe hepatic steatosis. We emphasized the following practices, including identifying hepatic incision line with intraoperative ultrasonography, hepatectomy using an ultrasonic dissector without inflow occlusion, identifying biliary duct anatomy by intraoperative cholangiography and leaving middle hepatic vein (MHV) in the donor side. Recipients' great saphenous vein or cryopreserved vessels were anastomosed between the crassitude tributaries of the graft MHV (> 5 mm in diameter) and inferior vena cava to avoid graft (segment V and VIII) congestion and to provide sufficient functioning liver mass. Weight and volume of the grafts were respectively measured using the balance and water replacement method in the back table, and graft-to-recipient weight ratio (GRWR) and GV/SLV ratio were calculated. In addition, the rate of donor RLV was calculated as follows: $[(TLV - GV)/TLV] \times 100\%$.

Postoperative treatment and follow-up

Each donor and recipient were routinely cared in the intensive care unit of liver transplantation after operation, and transferred to the regular ward when their conditions became stable. Liver biochemical tests, blood routine examination, hepatic vascular status and remnant liver volume regeneration were monitored during hospital stay and follow-up. The Clavien classification system for liver transplantation was used to respectively define postoperative recipient^[9] and donor complications^[10]. Standard immunosuppression regimen is triads of ciclosporin or tacrolimus, mycophenolate mofetil and prednisone. Lamivudine was given orally to recipients with hepatitis B once the decision to perform liver transplantation was made, and was continued throughout and after the operation. Hepatitis B immunoglobulin was used for the prevention of hepatitis B relapse. Discharge donors and recipients were regularly followed up with an endpoint of September 30, 2010.

Statistical analysis

Data were expressed as mean and standard deviations or as median and range depending on the distribution. Continuous variables of two groups were compared by the Student *t* test or the Mann-Whitney test as appropriate. Survival rates after operation were analyzed using the Kaplan-Meier method. Multivariate Cox regression further analyzed the independently related factors of mortality. Receiver operator characteristic (ROC) curve analysis was undertaken to identify the threshold of potential risk factors. Statistical significance was defined as $P < 0.05$. All statistical analysis were performed using

Table 1 The characteristics of recipients with acute liver failure

Parameters	Recipients (<i>n</i> = 20)
Preoperative	
Age (yr)	39.5 ± 7.3
Gender (male/female)	17/3
BMI (kg/m ²)	23.4 ± 3.4
SLV (cm ³)	1339.7 ± 147.5
Etiologies (<i>n</i>)	
Hepatitis B	18
Drug induced	1
Indeterminate	1
MELD scores	
Total bilirubin (μmol/L)	456.1 ± 207.3
Creatinine (μmol/L)	136.7 ± 102.1
INR	4.18 ± 3.42
Hepatorenal syndrome (<i>n</i>)	4
Waiting duration (d)	5 ± 4
Intraoperative	
Graft volume (cm ³)	618.3 ± 111.0
Graft weight(g)	623.3 ± 111.3
GV/SLV	46.2% ± 7.2%
GRWR	0.95% ± 0.14%
Graft type (<i>n</i>)	
Right lobe	17
Dual graft	3
Cold ischemia time (min)	131 ± 24
Anhepatic phase time (min)	95.3 ± 24.9
Operation time (h)	11.5 ± 3.1
Blood loss (mL)	2700 (1000-7000)
Postoperative	
Hospital stay (d)	33.7 ± 18.5
Death in hospital (<i>n</i>)	7
Follow-up (d)	425 (1-1654)

BMI: Body mass index; GRWR: Graft-to-recipient weight ratio; GV/SLV: Graft volume to recipient standard liver volume; MELD: Model for end-stage liver disease; INR: International normalized ratio.

SPSS for Windows 13.0.

RESULTS

Recipient characteristics

Patients included 17 men and 3 women, with mean age of 39.5 ± 7.3 years (range, 29-63 years). The causes of ALF were hepatitis B (*n* = 18, including 10 patients with acute hepatitis B and 8 patients with acute-on-chronic hepatitis B), drug-induced (*n* = 1) and indeterminate (*n* = 1). The parameters of the recipients are summarized in Table 1. The average score of the model for end-stage liver disease (MELD) was 37.1 ± 8.6, ranging from 24 to 55. Hepatorenal syndrome was present in four patients, but none required preoperative hemodialysis. Three patients received dual grafts transplantation with right hemiliver and left hemiliver, and their accumulated grafts weight was 800 g, 754 g and 680 g, respectively. The overall graft weight was 623.3 ± 111.3 g (range, 400-850 g) and the graft volume was 618.3 ± 111.0 cm³ (range, 400-870 cm³), which corresponded to the GRWR of 0.95% ± 0.14% (range, 0.75%-1.31%) and GV/SLV of 46.2% ± 7.2% (range, 37%-68%). The segment V or VIII hepatic vein, which was 8.5 ± 2.5 mm (range, 5-13 mm), were reconstructed in 11 right-lobe grafts. The methods

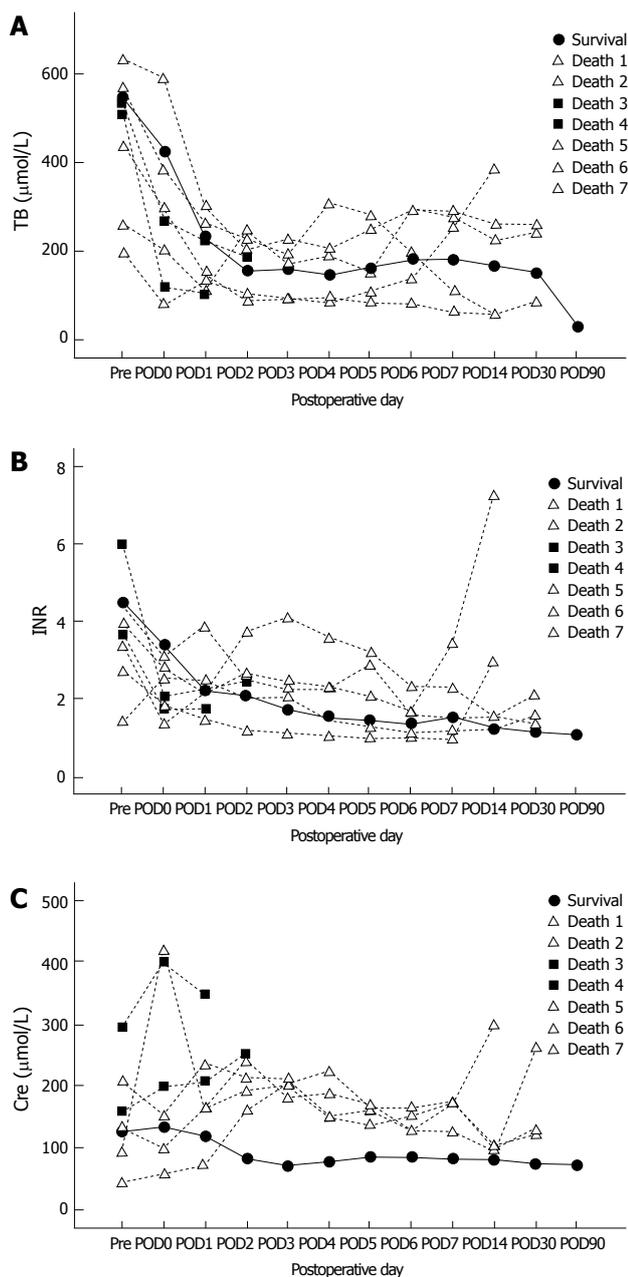


Figure 1 Hepatic and renal function change of all recipients with acute liver failure. The solid line displays the mean of values level in survived recipients ($n = 17$). The dashed line displays the values level of seven dead recipients. The triangles show the dead recipients with survival time > 7 d, and the black boxes show the dead recipients with survival time < 2 d. A: Total bilirubin(TB) tendency; B: INR: International normalized ratio tendency; C: Creatinine (Cre) tendency.

of biliary reconstruction included duct-to-duct manner without T-tube ($n = 17$) and with T-tube ($n = 1$), Roux-en-Y anastomosis ($n = 1$) and combined duct-to-duct and Roux-en-Y anastomosis ($n = 1$).

Recipient outcomes

The respiratory tube was extubated at median postoperative 10 h (range, 5-95 h) in all patients. Hospital stay was 33.7 ± 18.5 d (range, 1-84 d) and seven recipients died in hospital. Postoperative liver and renal function

Clavien grade	Complications	n
Grade I ($n = 2$)	Pleural effusion (mild)	1
	Anastomotic stoma stenosis of right hepatic vein without treatment	1
Grade II ($n = 10$)	II a	
	Pleural effusion (server) using pleurocentesis	1
	Transfusion 4 foreign blood units and pleural effusion (mild)	2
	A persistent elevated prothrombin time > 20 over 3 d	1
	Transient increase in creatinine levels ($>$ twice the pretransplantation level) for one month	1
	II b	
Bile duct stricture corrected by endoscopic therapy, and pulmonary infection	1	
Hepatic artery thrombus requiring surgery, and bile leakage requiring endoscopic procedure	1	
Postoperative bleeding requiring laparotomy	1	
Grade III ($n = 0$)	None	0
Grade IV ($n = 7$)	Renal failure and/or hepatic function failure	5
	Pulmonary infection	1
	Abdominal infection from bile leakage	1
	Total	17

are shown in Figure 1. Postoperative results of total bilirubin, INR and creatinine showed obvious improvement in survived patients. However, the creatinine level of dead cases was increased postoperatively and was more aggravated than the level of survived recipients.

A total of 17 (85%) recipients suffered from different grades of complications (Table 2). One biliary anastomotic stricture occurred, which was successfully treated by an endoscopic procedure. One patient required surgical revision for hepatic artery thrombosis and endoscopic procedure for bile leakage. One case required laparotomy for intraperitoneal hemorrhage. Seven recipients (35%) died in hospital, and the others were still alive. The 1-year and 3-year recipient survival and graft survival rates were 65%. One patient died from the acute rejection after one month. One case developed severe pulmonary mixed infection (*Burkholderia cepacia*, and *Acinetobacter baumannii*/*Acinetobacter baemolytius*) and died three weeks later. Two patients died from severe pulmonary infection (*Pseudomonas aeruginosa* and *Klebsiella pneumonia*) after one month. One case required surgical procedure for bile leakage on POD14, but died of abdominal infection on the second postoperative day 5. Two patients died from the continuing severe hepatic function failure on POD1 and POD2.

The patient 1-year and 3-year survival rates were 65%, and 13 patients were still alive (Figure 2). No significant related factors for a recipient mortality were observed using univariate analysis, including MELD scores, preoperative total bilirubin, pre-creatinine, pre-INR, GV/SLV and reconstruction of segment hepatic vein outflow as shown in Table 3 (all $P > 0.05$). And the similar operative liver and renal functions were observed in group with and without segment hepatic vein outflow (all $P > 0.05$). However, gender ($P = 0.037$), the longer duration of waiting ($P = 0.014$) and higher creatinine level on POD1 ($P = 0.021$) were associated with the mortality. Furthermore,

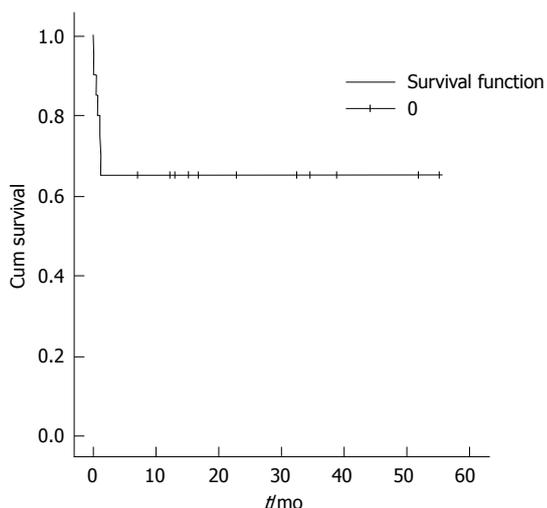


Figure 2 Survival curve of patients with acute liver failure. The patient 1-year and 3-year survival rates in the present study were 65%.

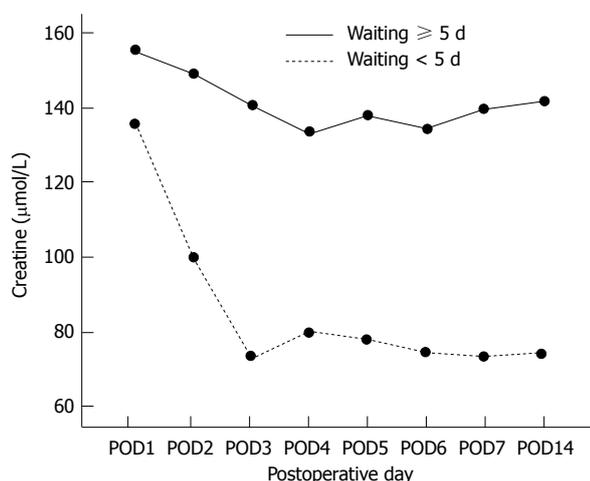


Figure 3 Short-term creatinine level change with different recipient waiting time. The recipients with waiting time ≥ 5 d (the solid line) showed a higher creatinine level than those with waiting time < 5 d (the dashed line).

Multivariate analysis showed that waiting time was only independently correlated with the increased mortality ($P = 0.016$), but the clinical significance was likely not accepted because of a low odds ratio (OR = 1.19, 95%CI = 1.033-1.385). Furthermore, ROC curve revealed the cut-off value of waiting time was 5 d ($P = 0.011$, area under the curve = 0.791) for determining the mortality. The short-term creatinine levels and recipient's waiting duration are shown in Figure 3. We found that recipients with waiting duration ≥ 5 d showed the worse renal function and higher mortality than with waiting duration < 5 d (66.7% vs 9.1%, $P = 0.017$).

Donor results

The mean age of the 23 living donors was 37.7 ± 10.2 years (range, 19-58 years). The preoperative parameters of donors are shown in Table 3. The mean donor assessment was 2.1 ± 1.3 d (range, 1-5 d). The assessment

Table 3 Univariate analysis of the mortality in the acute liver failure cases

Variables	P value	Variables	P value
Gender	0.037	Outflow reconstruction	0.924
Age	0.442	Urine volume	0.291
BMI	0.914	Blood loss	0.608
Child-Pugh scoring	0.963	Operation duration	0.536
MELD scoring	0.727	Re-warm ischemia	0.394
MELD scoring grade (> 30)	0.306	Pre_TB	0.878
Waiting duration	0.014	Pre_Cre	0.563
GV/SLV	0.396	Pre_INR	0.670
GW/BW	0.385	POD1_TB	0.622
Anhepatic phase	0.620	POD1_Cre	0.021
Cold ischemia	0.767	POD1_INR	0.930

GV/SLV: Graft volume to recipient standard liver volume; GW/BW: Graft weight to recipient body weight; MELD: Model for end-stage liver disease; BMI: Body mass index; Pre: Preoperative; POD1: One of postoperative day one; TB: Total bilirubin; INR: International normalized ratio.

time was more than 3 d in five donors, including 3 d ($n = 2$), 4 d ($n = 1$) and 5 d ($n = 2$). The assessment time was prolonged because some excluded potential donors resulted in re-evaluating another donor, whose causes included viral hepatitis, blood type incompatibility, obesity or graft size mismatch. Five donors with mild steatosis were observed by biopsy and their macrosteatosis was less than 30% (maximum 25%), and three of the five cases developed mild hepatic function impairment and recovered within one week. The RLV/TLV was $56.6\% \pm 12.1\%$ (range, 26%-79%). It was less than 30% in only one case, but the total bilirubin (60-90.8 $\mu\text{mol/L}$) was high within postoperative three days.

The complications of donors are shown in Table 4. There was no death and grade IV morbidity. Surgical morbidity was found in 11 patients (47.8%) in this series. However, the major complication only occurred in one donor, which was severe pleural effusion with pleurocentesis and classified as grade IIIa. Pleural effusion was the most frequent morbidity for living donor, and occurred in 8 cases (34.8%). Four donors developed mild hepatic function impairment, but spontaneously recovered within one week. One case with pulmonary infection had hyperbilirubinemia more than 3 wk, and the hepatic function was recovered with Transmetil treatment. The mean hospital stay was 13.4 ± 3.5 d (range, 6-22 d). The similar morbidity and postoperative hepatic function were found between donors with short (< 3 d) and with long (≥ 3 d) assessment time (all $P > 0.1$). With a median follow-up of 29 mo, all donors had no residual morbidity and resumed normal preoperative activities with normal liver function. Furthermore, univariate analysis did not show that short assessment time induced the high morbidity ($P = 0.573$) and the correlation between RLV/TLV and the morbidity ($P = 0.268$).

DISCUSSION

Initially LDLT for ALF was only performed in children.

Table 4 The characteristics of living donors

Parameters	Donors (n = 23)
Preoperative	
Age (yr)	37.7 ± 10.2
Gender (male/female)	8/15
BMI (kg/m ²)	23.8 ± 3.5
Relationship with recipient (n)	
Siblings	12
Daughter	4
Wives	5
Father	1
Uncle	1
Assessment time (d)	2.1 ± 1.3
Intraoperative	
Graft steatosis (normal/mild)	18/5
Graft type (right lobe/left lobe)	20/3
RLV/TLV (%)	56.5 ± 12.1
Operation time (h)	6.9 ± 1.4
Blood lost (mL)	665.2 ± 480.9
Postoperative	
Hospital stay (d)	13.4 ± 3.5
Peak of TB (μmol/L)	53.4 ± 22.7
Peak of INR	1.71 ± 0.36
Complications (n)	11
Grade I (n = 7)	
Pleural effusion (mild)	4
Hepatic function impairment (mild)	2
Hepatic cut-section local fluid collection	1
Grade II (n = 3)	
Pleural effusion (moderate) leading to partial compression atelectasis and hepatic function impairment (mild)	1
Pleural effusion (moderate) and pericardial effusion	1
Pulmonary infection undergone antibiotic therapy and hepatic function impairment (moderate)	1
Grade IIIa (n = 1)	
Pleural effusion (server) using pleurocentesis	1

Hepatic function impairment: Mild, hyperbilirubinemia (TB being 51-85 μmol/L for more than 3 d or 34-51 μmol/L on POD7) and/or PT prolongation (6-8 s for more than 3 d or 4-6 s on POD7); Moderate, hyperbilirubinemia (TB > 85 μmol/L for more than 3 d or exceeding 51 μmol/L on POD7) and/or PT prolongation (> 8 s for more than 3 d or exceeding 6 s on POD7). BMI: Body mass index; RLV: Remanet live volume; TLV: Total liver volume; TB: Total bilirubin; INR: International normalized ratio; PT: Prothrombin time.

Since the first report of successful AALDLT using right-lobe graft for adult ALF patients by Lo *et al.*^[11], AALDLT has been gradually accepted as an alternative treatment for adult ALF patients. Most cases came from Asian countries^[2-4,6], whereas a few cases were reported from Western countries^[5]. Experiences of AALDLT in ALF are summarized in Table 5^[2-6,12-17]. The mean waiting time was mostly less than five days and 1-year survival rate (> 70%) was satisfactory in the recipients. Zero mortality and low morbidity (< 40%) were achieved in all donors. Thus, it is rational to conclude that AALDLT is a safe treatment for patients with ALF.

This study demonstrated that AALDLT was an efficient treatment for patients with ALF. Patients who survived postoperatively (n = 13, 65%) were still alive at the postoperative 1-year and 3-year. This result was worse

than those reported in literature (> 70%), the possible explanations include worse preoperative patient condition, increased waiting time, inadequate venous draining resulting from right-lobe graft without MHV.

The MELD score was used to allocate cadaveric liver grafts among patients with end-stage liver disease^[18], and was also considered to be a useful indicator of LDLT in ALF patients^[19-21]. Several authors suggested that MELD scores greater than 25 should be considered as a relative contraindication for transplantation because of the poor outcomes^[22]. Yantorno *et al.*^[20] advocated the MELD score cut-off value for determining whether a transplantation was indicated should be 30. In our series, the mean MELD was 37.1 ± 8.6, which was higher than that reported from all other centers (Table 5). The mortality of recipients with MELD > 30 (7 of 15, 46.7%) was higher than those with MELD ≤ 30 (0 of 5), but not statistically significant (P = 0.306). The increased MELD score may account for an increased mortality (65%) in our reports. However, Matsui *et al.*^[13] considered that MELD score has little clinical significance for ALF patients who received plasma exchange.

In the present study, multivariate analysis identified waiting duration as the sole independent prognostic factor for 1-year mortality. The duration of waiting time for liver transplantation can significantly affect patient survival, especially for patients with ALF^[2,6,23]. Prolonged waiting time means increased risk of severe complications, including deterioration of hepatic or/and renal function, intracranial bleedings or sepsis^[6,24], which contributed to increased mortality in our series. The mean waiting time in this study was slightly longer than that in other centers (5 d *vs* 3.7 d). Recipients with waiting duration ≥ 5 d correlated with worse outcome. This was possibly due to the following causes, such as delayed donor evaluation or/and the hesitancy for LDLT from the patient's family. This result was possibly of defective clinical significance because of the low odds ratio of waiting duration (OR = 1.19) for a recipient mortality and the small sample size of our study. However, this data showed that timely LDLT for ALF patients greatly improved the recipient survival.

On one hand, the graft volume is positively correlated with the recipient outcome in LDLT^[16,25,26]. To meet the demand of patients with ALF, a safety margin of a smaller graft was GV/SLV > 30%-35%^[4,12,27]. Despite acceptable survival with GV/SLV < 30%^[28] was reported, small graft should be avoided if possible^[4,11]. Based on recent reports (Table 4), satisfactory survival resulted from right-lobe graft with a GV/SLV more than 30%. In this study, the mortality of recipients was not associated with GV/SLV because of the GV/SLV > 37%. On the other hand, right liver graft without middle hepatic vein may lead to venous congestion of right anterior segments^[16,29-31]. The functional graft volume relies on a perfect outflow^[32,33], thus it is necessary to reconstruct the crassitude hepatic vein outflow to avoid graft congestion. Our data can not provide evidence to answer whether a graft with or without MHV correlates to recipient mor-

Table 5 Adult-to-adult living donor liver transplantation for acute liver failure between 2000 and 2010

Study	Recipient								Donor complications				
	Cases	MELD	Waiting time (d)	Graft type			GV/SLV (%)	Mortality	Survival rate, <i>n</i> (%)	Cases	Minor	Major	Morbidity
				Right	Left	Dual							
Park <i>et al</i> ^[2]	40	36	2.5	35	0	6	ND	6	1 (85)	45	11	0	24.40%
Shi-Chun <i>et al</i> ^[3]	10	34.5 ± 2.1	3	10	0	0	56.7 (47.7-66.9)	2	2 (80)	10	4	0	40%
Ikegami <i>et al</i> ^[4,12]	44	24 ± 6	8 ± 6	12	32	10	22.8-56.8	10	1 (78.3); 3 (71.6)	44	15	0	34%
Campsen <i>et al</i> ^[5]	10	> 21	2.7	10	0	0	ND	3	1-5 (70)	10	5	0	50%
Matsui <i>et al</i> ^[13]	36 (4) ¹	22 ± 6	ND	18	16	0	46 (22-75)	4	1 (94); 5 (87)	36	1	1	5.60%
Rajekar <i>et al</i> ^[14]	15	32	ND				ND	3	4 (80)	ND	ND	ND	ND
Lee <i>et al</i> ^[15]	57	32	2.4	33	9	15	27-81	10	1-5 (82)	72	ND	ND	ND
Liu <i>et al</i> ^[16]	32	36 ± 1.8	2.5	32	0	0	52 (33-87)	4	2 (88)	32	8	2	31.30%
Nishizaki <i>et al</i> ^[17]	15	ND	5	0	15	0	36.7 (23-54)	3	1 (80)	15	ND	ND	ND

¹Four pediatric recipients were not clarified from 36 cases in this report. Major complications of donors were defined as the Clavine grade III-IV. MELD: Model for end-stage liver disease; GV/SLV: Graft volume to recipient standard liver volume; ND: Not described. The latest report was as the summarized standard if multiple reports were from the same center and author.

tality because all grafts were without middle hepatic vein. However, comparable patient outcome was achieved in our series which warrants that the reconstruction of a crassitude hepatic vein outflow is necessary.

Some scholars concerned that the expedited donor assessment may incur poor donor outcome. However, there was no mortality or reoperation among donors in our series. Although 34.8% of donors suffered from complications, all improved spontaneously or with conservative management except one pleural effusion with pleurocentesis. This morbidity was slightly higher than that reported by other centers because of the variation of defining and reporting complications^[34,35]. Postoperative complications of donors is usually underestimated even using the Clavien classification (1992 version)^[13,36]. Thus, this morbidity in our center was acceptable using the new Clavien classification. In addition, our results also indicated that transitory assessment for donors had no negative effect on the donor outcome. Similarly, the small sample size in our study is still insufficient to determine a potential correlation between expedited donor assessment and donor outcome. In our experience, a rapid evaluation process did not bring any negative effect to donors.

In summary, this study demonstrated that AALDL should be performed as early as possible in patients with ALF for a satisfactory survival rate. There are few studies with a large sample size to support AALDLT as a better treatment for patients with ALF. Thus, a systemic review and continued development of AALDLT are important to determine treatment option for patients with ALF.

COMMENTS

Background

Acute liver failure (ALF) is a condition with rapid deterioration of liver function. Because ALF progresses rapidly and recovers poorly, the emergency liver transplantation is recommended as the effective treatment for ALF which has a high likelihood of death. Adult-to-adult living-donor liver transplantation (AALDLT) would help address the shortage of available organs for patients with ALF.

Research frontiers

AALDLT for ALF has recently been mostly reported in Asia and there were

also cases reported in Western countries. The recent reports have described the optimal survival rate of recipients and the minimum rate of morbidity of the donor. However, a small sample could not support AALDLT as a better solution for patients with ALF. Thus, the continued development of AALDLT treatment is necessary to determine the treatment option for patients with ALF.

Innovations and breakthroughs

This study evaluated the outcome of recipients with ALF and living donor in detail. Based on the Clavien classification, the outcome of recipients and donors were first objectively evaluated. The authors indicated that the duration of waiting was an independent risk factor for a recipient's mortality. In addition, sample size is still small among studies of AALDLT for ALF in the world. Thus, this study presented some experience for further system reviews.

Applications

This study indicated that patients with ALF should receive AALDL as early as possible for satisfactory survival rate. And the model for end-stage liver disease score may not be related to the outcome of recipient's outcome.

Terminology

ALF is a condition in which rapid deterioration of liver function results in altered mentality and coagulopathy in individuals without preexisting cirrhosis.

Peer review

The manuscript provided information about LTx for ALF in China. This is a very interesting analysis of a series of 20 patients who underwent LDLT for ALF. From the "west-countries" point of view, these series are important as we may consider increasing our rate of LDLT for several etiologies, including ALF.

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Highlights for α -fetoprotein in determining prognosis and treatment monitoring for hepatocellular carcinoma

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Abstract

AIM: To explore the prognostic value in the monitoring of treatment efficacy of serial α -fetoprotein (AFP) in hepatocellular carcinoma (HCC) patients.

METHODS: We searched MEDLINE, EMBASE and COCHRANE LIBRARY through April 21, 2012, to find qualifying articles. Our overall search strategy included terms for HCC, AFP, treatment response, and prognosis. Literature was limited to English-language, human studies. Studies reporting cumulative survival rates were summa-

rized qualitatively. For the prognostic meta-analysis, we undertook a series of meta-analyses that summarised the Cox proportional hazard ratios (HRs) by assuming a random effects model. With regards to the correlation of AFP change with radiologic response, the categorical dichotomous variables were assessed using Poisson relative risks (RRs), which were incorporated into the random effects model meta-analysis of accuracy prediction. Between-study heterogeneity was estimated by use of the I^2 statistic. Publication bias was evaluated using the Begg funnel plot and Egger plot. Sensitivity analyses were conducted first by separating systemic treatment estimates from locoregional therapy estimates, evaluating different AFP response cut-off point effects, and exploring the impact of different study sizes.

RESULTS: Of 142 titles identified in our original search, 11 articles (12 clinical studies) met our criteria. Six studies investigated outcome in a total of 464 cases who underwent systemic treatment, and six studies investigated outcome in a total of 510 patients who received locoregional therapy. A random-effects model meta-analysis showed that AFP response was associated with an mortality HR of 0.55 (95%CI, 0.47-0.65) across HCC in overall survival (OS) and 0.50 (95%CI, 0.38-0.65) in progression-free survival. Restricting analysis to the six eligible analyses of systemic treatment, the pooled HRs were 0.64 (95%CI, 0.53-0.77) for OS. Limiting analysis to the six analyses of locoregional therapy, the pooled HRs for OS was 0.39 (95%CI, 0.29-0.53). We showed a larger pooled HR in the 50% definition studies (HR, 0.67, 95%CI, 0.55-0.83) compared with that from the 20% definition studies (HR, 0.41, 95%CI, 0.32-0.53). Restricting analysis to the four studies including over 100 patients individually, the pooled HR was 0.65 (95%CI, 0.54-0.79), with a pooled HR for OS of 0.35 (95%CI, 0.23-0.46) in the studies of less than 100 patients. As to radiological imaging, 43.1% (155/360) of the patients in the AFP response group presented with a radiological overall response, while the response rate decreased to 11.5% (36/313) in the patients from the

AFP nonresponse group. The RR of having no overall response was significantly lower in the AFP response group than the AFP nonresponse group (RR, 0.67; 95%CI, 0.61-0.75). In terms of disease control rate, 86.9% (287/330) in the AFP response group and 51.0% (153/300) in the AFP nonresponse group showed successful disease control, respectively. The RR of disease control failure, similarly, was significantly lower in the AFP response group (RR, 0.37; 95%CI, 0.23-0.58). But these findings could be overestimates because of publication and reporting bias.

CONCLUSION: HCC patients presenting with an AFP response are at decreased risk of mortality. In addition, patients with an AFP response also present with a higher overall response rate and disease control rate.

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Key words: Liver cancer; α -fetoprotein; Response; Prognosis; Monitoring

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, and as a result of the spread of hepatitis C virus and hepatitis B virus infection during the past century, its incidence will further increase in the future in both Asia and in western countries^[1,2]. Transarterial therapy, systemic agents, and radiofrequency ablation remain as mainstays of treatment in advanced HCC^[3]. Conventionally, the treatment response of HCC to systemic therapy or other nonsurgical treatment modalities is assessed by radiologic imaging using conventional criteria, such as World Health Organization (WHO) criteria or Response Evaluation Criteria in Solid Tumors^[4-6]. However, despite successful clinical correlations for other solid tumors, these radiological response-based criteria have been criticized for not adequately reflecting treatment response and tumor viability for HCC. It has constantly been observed that a small subset of HCC patients could derive benefits from treatment despite the absence of radiologic response^[7-9]. Thus, the sole use of radiologic criteria has underestimated the efficacy of novel treatment in early clinical trials and the search for better, alternative ways of assessing treatment response continues to be important.

While imaging is being explored, widespread efforts have been made to identify serological markers that predict survival and response to treatment^[10], and among which, serum α -fetoprotein (AFP) (a glycoprotein that is expressed by HCC and secreted into the serum of approximately 70% of patients with HCC) have been widely studied^[11,12]. In the half century after AFP was first described, it was extensively studied as a screening and diagnostic tool for HCC^[13-15]. Although there have been some early observational studies that have suggested that AFP trend might be useful in assessing treatment response, until recently, there has been no clinical study to validate the significance of serial AFP monitoring in association with the treatment response of HCC^[16,17]. In the past few years, several studies have investigated the role of AFP response to treatment in HCC, which mainly focused on the overall survival (OS) and progression-free survival (PFS), and validated its correlation with radiological response. However, estimates of the prognostic value of the AFP response between studies differed wildly. The aim of this analysis is to review published studies that investigated the correlation between AFP response and prognosis in HCC, and to use standard meta-analysis techniques to summarise the accuracy of AFP response in prediction of survival in HCC patients.

MATERIALS AND METHODS

Study identification

We searched MEDLINE, EMBASE, and COCHRANE LIBRARY from inception to April 21, 2012, for articles evaluating the AFP level and response to treatment on the prognostic outcome in HCC patients. Our overall search strategy included terms for HCC (e.g., hepatocellular carcinoma, liver carcinoma, liver cancer, liver malignant neoplasm), AFP (e.g., α -fetoprotein, alpha-fetoprotein, AFP), treatment response (e.g., change, decline, response), and prognosis (e.g., mortality, survival, recurrence). Literature was limited to English-language, human studies. We also searched references of included articles. Only published studies in peer-review journals were included. Data from review articles, case reports, abstracts, and letters were not included.

Study eligibility and selection

Studies were eligible if survival was analyzed in HCC cases stratified by AFP response or not. To be included in our meta-analysis, articles had to meet both of the following criteria: they reported a risk estimate [e.g., hazard ratio (HR) or relative risk (RR) relating AFP response to subsequent death using survival analysis regression models], and they reported an estimate of precision, such as a standard error or 95%CI. We also included articles that failed to report precision directly but from which we could reconstruct an estimate of precision using *P* values and other study data^[18]. Correlation of AFP change with radiologic response was desirable, but it was not a must (Table 1).

Table 1 Main characteristics and results of eligible studies

Author	Year	Country	OS		PFS		Overall response rate	Disease control rate
			Univariate analysis	Multivariate analysis	Univariate analysis	Multivariate analysis		
Chen <i>et al</i> ^[21]	2005	China	S	S	N/A	N/A	N/A	N/A
Chan <i>et al</i> ^[20]	2009	China	S	S	N/A	N/A	P	P
Riaz <i>et al</i> ^[27]	2009	United States	S	S	N/A	N/A	P	P
Vora <i>et al</i> ^[29]	2009	United States	N/A	NS	N/A	NS	P	P
Shao <i>et al</i> ^[28]	2010	China	N/A	S	N/A	S	P	P
Kim <i>et al</i> ^[23]	2011	South Korea	S	S	S	S	P	P
Yau <i>et al</i> ^[30]	2011	China	S	S	S	S	N/A	N/A
Kao <i>et al</i> ^[22]	2012	China	N/A	S	N/A	N/A	N/A	N/A
Lee <i>et al</i> ^[24]	2012/H	South Korea	S	S	S	NS	P	P
Lee <i>et al</i> ^[24]	2012/C	South Korea	S	S	S	NS	P	P
Memon <i>et al</i> ^[25]	2012	United States	S	S	N/A	N/A	C	N/A
Personeni <i>et al</i> ^[26]	2012	Italy	N/A	S	N/A	N/A	N/A	N/A

2012/H: Hepatic arterial infusional chemotherapy study; 2012/C: Concurrent chemoradiation therapy study; S: Significant relationship between α -fetoprotein (AFP) response and survival; NS: No significant relationship between AFP response and survival; N/A: Not available or not applicable; P: Directly provided by the article; C: Need to be calculated by the data provided by the article; OS: Overall survival; PFS: Progression-free survival.

Data synthesis and statistical analysis

To avoid duplicate data, we identified articles that included the same cohort of patients by reviewing inter-study similarity in the country in which the study was done, investigators in the study, source of patients, recruitment period, and inclusion criteria. Early studies published as a series of articles from the same author or institution that contained significant overlap of patient data were excluded and only the most recently published study containing the most up-to-date data was included. If several estimates were reported in the same article, we chose the most fully adjusted estimate (i.e., multivariate regression was selected over univariate regression, which was selected over unadjusted Kaplan-Meier analysis).

For the prognostic meta-analysis, we undertook a series of meta-analysis that summarised the HRs by assuming a random effects model. With regards to the correlation of AFP change with radiologic response, the categorical dichotomous variables were assessed using the RRs, which were incorporated into the random effects model meta-analysis of accuracy prediction. Between-study heterogeneity was estimated by use of the *I*² statistic; typically, values above 50% are deemed to suggest large between-study heterogeneity, values below 50% are deemed to represent low heterogeneity. Publication bias was evaluated using the Begg funnel plot and Egger plot. However, these estimates can have large uncertainty, especially in the presence of few trials, and should be interpreted with caution^[19].

Sensitivity analysis were conducted first by separating systemic treatment estimates from locoregional therapy estimates. Second, to evaluate AFP response cut-off point effects, we calculated estimates for studies whose AFP response was defined as 20% decline of the initial level *vs* those with the AFP response definition of 50% decline. Third, to explore the impact of study size, we conducted sensitivity analyses by components which included over 100 patients. Finally, we evaluated the influence of each study on the overall estimate by calculat-

ing a pooled HR, omitting each estimate 1 at a time. All analysis were conducted using Stata 12 (StataCorp, College Station, Texas).

RESULTS

Eligible studies

Thirteen studies were identified that provided survival data stratified by AFP response or not^[20-32]. Two studies were excluded from further analysis: one was excluded because it did not provide a definite AFP response cut-off point, and extraction of survival data for the AFP response cases and AFP nonresponse cases was not possible, and another article, which was a letter to editor, was also excluded^[31,32]. Hence a total of 11 articles remained eligible for pooling risk estimates, reporting on 974 patients, of whom 463 had a positive AFP response. The main characteristics and results of eligible studies evaluating AFP response in HCC patients were summarised in Table 1.

Description of studies

Of the 11 eligible articles, all were based on retrospective analysis of survival data with quite heterogeneity. Sample sizes ranged from 42 to 149 patients with a median of 62 patients. Six eligible articles assessed survival in the systemic treatment setting, with data from a total of 464 patients available for pooling (sample size range, 42 to 107 patients)^[20,21,26,28-30]. In the locoregional therapy setting, five articles were eligible, including 6 individual clinical studies, resulting in a total of 510 patients available for pooling (sample size range, 51 to 149 patients)^[22-25,27]. In one of these studies, patients were treated in the locoregional setting either by hepatic artery infusion chemotherapy, or concurrent chemoradiation therapy^[24]. In this study, it was possible to assess estimators of survival in the two patient cohorts separately, and each cohort was therefore considered separately for pooling purposes. In the systemic treatment studies, all patients received systemic regimens

Table 2 Characteristics and demographic information of eligible studies

Author	Year	Treatment	HCC stage	AFP change level (%)	No. of patients		
					Study size (n = 974)	M/F (776/198)	AFP response (n = 463)
Chen <i>et al</i> ^[221]	2005	Sys/thalidomide	I - III	50	42	33/9	10 (23.8%)
Chan <i>et al</i> ^[220]	2009	Sys/doxorubicin or PIAF	I - III	20	117	104/13	47 (40.2%)
Riaz <i>et al</i> ^[227]	2009	Loc/chemoembolization or radioembolization	I - IV	50	125	91/34	81 (64.8%)
Vora <i>et al</i> ^[229]	2009	Sys/five systemic regimens	I - III	50	107	78/29	18 (16.8%)
Shao <i>et al</i> ^[228]	2010	Sys/Antiangiogenic agents	I - II	20	72	65/7	12 (16.7%)
Kim <i>et al</i> ^[223]	2011	Loc/chemoradiotherapy	III-IV	50	149	127/22	101 (67.8%)
Yau <i>et al</i> ^[230]	2011	Sys/sorafenib	II - IV	20	41	36/5	9 (21.9%)
Kao <i>et al</i> ^[222]	2012	Loc/radiofrequency ablation	I - III	20	58	34/24	46 (79.3%)
Lee <i>et al</i> ^[224]	2012/H	Loc/HAIC	III-IV	20	60	49/11	25 (41.7%)
Lee <i>et al</i> ^[224]	2012/C	Loc/CCRT	III-IV	20	67	55/12	52 (77.6%)
Memon <i>et al</i> ^[225]	2012	Loc/transarterialtherapy	I - III	50	51	30/21	30 (58.8%)
Personeni <i>et al</i> ^[226]	2012	Sys/sorafenib	I - III	20	85	74/11	32 (37.6%)

Sys: System treatment; Loc: Locoregional therapy; HAIC: Hepatic artery infusion chemotherapy; CCRT: Concurrent chemoradiation therapy. 2012/H: HAIC study of Lee *et al*; 2012/C: CCRT study of Lee *et al*; M/F: Male/female. HCC: Hepatocellular carcinoma; AFP: α -fetoprotein.

Table 3 Results of survival analyses related to α -fetoprotein response

Author	Year	Overall survival			Progression-free survival		
		HR	95%CI	P value	HR	95%CI	P value
Chen <i>et al</i> ^[221]	2005	0.24 ²	0.10-0.61	0.003	-	-	-
Chan <i>et al</i> ^[220]	2009	0.41 ²	0.27-0.63	0.0001	-	-	-
Riaz <i>et al</i> ^[227]	2009	0.37 ^{1,2}	0.13-1.01 ¹	0.0002	-	-	-
Vora <i>et al</i> ^[229]	2009	0.95 ²	0.73-1.23 ¹	0.88	0.48 ²	0.15-1.45 ¹	0.09
Shao <i>et al</i> ^[228]	2010	0.36 ²	0.15-0.83	0.017	0.31 ²	0.14-0.67	0.003
Kim <i>et al</i> ^[223]	2011	0.43 ²	0.29-0.65	0.001	0.48 ²	0.33-0.70	0.001
Yau <i>et al</i> ^[230]	2011	0.3 ²	0.09-1.02	0.05	0.31 ²	0.13-0.76	0.01
Kao <i>et al</i> ^[222]	2012	0.18 ^{1,2}	0.02-1.67 ¹	0.023	-	-	-
Lee <i>et al</i> ^[224]	2012/H	0.43 ²	0.23-0.81	0.009	0.67 ²	0.35-1.27	0.22
Lee <i>et al</i> ^[224]	2012/C	0.33 ²	0.15-0.75	0.008	0.97 ²	0.42-2.25	0.97
Memon <i>et al</i> ^[225]	2012	0.14 ²	0.02-0.83	0.03	-	-	-
Personeni <i>et al</i> ^[226]	2012	0.52 ²	0.31-0.85	0.009	-	-	-

¹Calculated result from data presented in article; ²Multivariate result; -: Not performed; 2012/H: Hepatic arterial infusional chemotherapy study; 2012/C: Concurrent chemoradiation therapy study; HR: Hazard ratio; AFP: α -fetoprotein.

(sorafenib, thalidomide, doxorubicin, *etc.*). In the locoregional therapy studies, chemotherapy and radiotherapy were used. Of all the included analysis, five defined AFP response as a 50% decline from the initial level^[21,23,25,27,29], and another seven proposed a definition of 20% decline^[20,22,24,26,28,30]. Across the included studies that reported the number of participants with AFP response, the overall prevalence of AFP response was 47% (range, 17%-79%), with a median sample size of 38 (range, 9 to 101). Follow-up time varied widely across studies, with a median of 35.3 mo (range, 4 to 100 mo) (Table 2).

With regards to radiologic response, the evaluation criteria were based on the WHO criteria. Complete response (CR) was defined as the complete disappearance of all known lesions on radiologic grounds. Partial response (PR) was defined as a decrease of 50% or more in the product of two perpendicular diameters of the largest tumor nodule for at least 4 wk without the appearance of new lesions or progression of lesions. Sta-

ble disease (SD) was defined as a less than 50% decrease or not more than a 25% increase in the product of two perpendicular diameters of the largest tumor nodule. Progressive disease was defined as more than a 25% increase in the product of two perpendicular diameters for the largest tumor nodule or one of the measurable lesions or as the appearance of new lesions. In view of these indices, the overall response rate referred to the total rate of CR and PR, whereas the disease control rate was defined as the total rate of CR, PR and SD.

Relationship between AFP response and OS in HCC

Of the 12 clinical studies eligible for pooling of OS data, 9 provided estimates of the HR associated with AFP response and its 95%CI^[20,21,23-26,28,30]. In the remaining studies, these data points were calculated from data presented in the articles^[22,27,29]. Table 3 details the results of survival analyses performed in eligible studies^[18], and Figure 1 displays a plot of HRs and associated 95%CIs for OS from each study. AFP response was associated with a decrease in all-cause mortality following HCC, with a pooled HR for OS across all studies of 0.55 (95%CI, 0.47-0.65), and with significant evidence of heterogeneity between the contributing studies ($I^2 = 65\%$, $P = 0.001$). Evidence of significant publication bias was observed according to the funnel plot of lnHRs, with smaller studies showing significant effects^[18].

Because the investigators' approaches to adjustment for confounding factors varied widely by study and type of treatment, we conducted a sensitivity analysis to confirm robustness. Restricting analysis to the six eligible analysis in which patients received systemic treatment, the pooled HR was 0.64 (95%CI, 0.53-0.77) ($I^2 = 77\%$, $P = 0.001$) for OS^[20,21,26,28-30]. Limiting analysis to the six analysis in which patients under went locoregional therapy, the pooled HR for OS was 0.39 (95%CI, 0.29-0.53) ($I^2 = 0\%$, $P = 0.83$)^[22-25,27] (Figure 1A).

To assess the effect of cut-off point of AFP response, HRs were pooled from particular size studies that defined AFP response as either 50% decline of initial level^[21,23,25,27,29] or

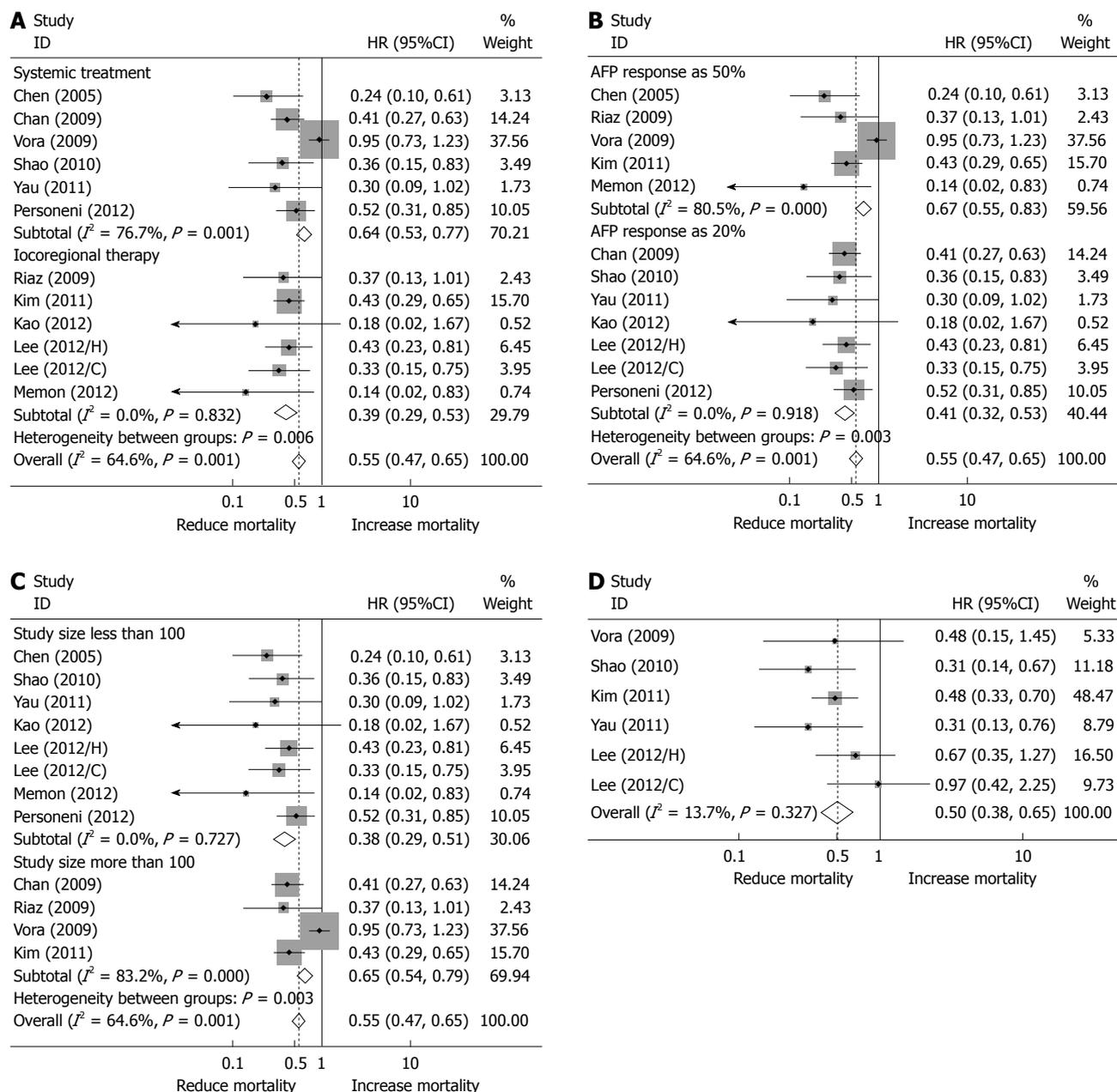


Figure 1 Forest plots representing hazard ratios of overall survival and progression free survival in hepatocellular carcinoma patients associated with α -fetoprotein response. A: Subgroup analysis according to systemic treatment and locoregional therapy; B: Subgroup analysis according to α -fetoprotein (AFP) response definition of 20% decline of its initial level and 50% decline; C: Subgroup analysis according to study size larger than 100 patients and less than 100 patients; D: Hazard ratios (HRs) of progression free survival in hepatocellular carcinoma patients associated with AFP response.

20% decline, respectively^[20,22,24,26,28,30]. This demonstrated a larger pooled HR in the 50% definition studies (HR, 0.67, 95%CI, 0.55-0.83) compared with that from the 20% definition studies (HR, 0.41, 95%CI, 0.32-0.53). In the 50% group, there was significant evidence of study heterogeneity ($I^2 = 81\%$, $P < 0.001$) (Figure 1B). This observed increased HR of mortality in the 50% definition studies contradicts our supposition that greater AFP response in the patient would predict better overall survival.

Restricting analysis to the four studies including over 100 patients individually, the pooled HR was 0.65 (95%CI, 0.54-0.79), again with evidence of study heterogeneity ($I^2 = 83\%$, $P = 0.003$)^[21,22,24-26,28,30]. The remaining eight studies

included less than 100 patients individually, and the pooled HR for OS was 0.35 (95%CI, 0.23-0.46), with no evidence of heterogeneity ($I^2 = 0$, $P < 0.001$)^[20,23,27,29] (Figure 1C).

Finally, we excluded individual study estimates 1 at a time to examine the influence of each study on the overall HR. It turned out that heterogeneity was mainly from the study by the Vora *et al*^[29] study, that only the omission of this study appreciably changed the pooled HR, with the HR decreasing to 0.40 (95%CI, 0.33-0.49) and eliminating the heterogeneity across all studies ($I^2 = 0$, $P = 0.91$).

Relationship between AFP response and PFS in HCC

Only 6 of the 12 eligible analyses presented data evalu-

Table 4 Results of overall response rate and disease control rate analysis

Author	Year	Overall response rate (%)			Disease control rate (%)		
		AFP responders	AFP nonresponders	RR ¹	AFP responders	AFP nonresponders	RR ¹
Chen <i>et al</i> ^[21]	2005	-	-	-	-	-	-
Chan <i>et al</i> ^[20]	2009	46.8	7.1	0.57	91.5	41.4	0.15
Riaz <i>et al</i> ^[27]	2009	43.5	31.6	0.61	91.3	68.4	0.22
Vora <i>et al</i> ^[29]	2009	25.0	10.0	0.83	93.8	56.7	0.14
Shao <i>et al</i> ^[28]	2010	33.0	8.0	0.73	83.0	35.0	0.26
Kim <i>et al</i> ^[23]	2011	44.5	12.5	0.63	87.1	58.3	0.50
Yau <i>et al</i> ^[30]	2011	-	-	-	-	-	-
Kao <i>et al</i> ^[22]	2012	-	-	-	-	-	-
Lee <i>et al</i> ^[24]	2012/H	36.0	8.6	0.70	64.0	45.7	0.66
Lee <i>et al</i> ^[24]	2012/C	36.5	20.0	0.79	80.8	66.7	0.58
Memon <i>et al</i> ^[25]	2012	36.7	7.7	0.69	-	-	-
Personeni <i>et al</i> ^[26]	2012	-	-	-	-	-	-

¹Calculated result from data presented in article; 2012/H: Hepatic arterial infusional chemotherapy study; 2012/C: Concurrent chemoradiation therapy study; -: Not performed; AFP: α -fetoprotein; RR: Relative risk.

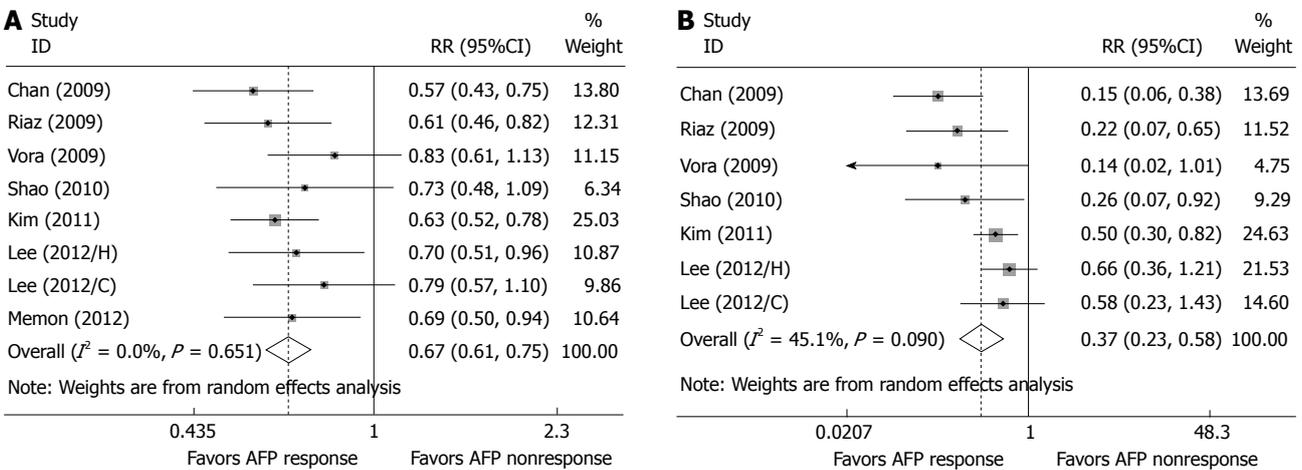


Figure 2 Forest plots representing the correlation between α -fetoprotein response and radiological response. A: Risks of no radiological response; B: Risks of disease control failure. AFP: α -fetoprotein; RR: Relative risk.

able for assessment of PFS, and the pooled HR was 0.50 (95%CI, 0.38-0.65), with no obvious heterogeneity ($I^2 = 13\%$, $P = 0.33$)^[23,24,28-30]. This result should, however, be interpreted with caution because of the small number of contributing studies (Figure 1D).

AFP response in association with radiological response

Of the 12 eligible clinical studies, 8 provided data evaluable for assessment of radiological overall response rate^[20,23-25,27-29] and 7 reported on disease control rate^[20,23,24,27-29]. A radiological response summary is presented in Table 4. Overall, 43.1% (155/360) of the patients in the AFP response group presented the radiological overall response, while the response rate declined to 11.5% (36/313) in the patients from the AFP nonresponse group. The RR of having no overall response rate (Figure 2A) was significantly lower in the AFP response group than the AFP nonresponse group (RR, 0.67; 95%CI, 0.61-0.75) with no heterogeneity ($P = 0.65$, $I^2 = 0\%$). In terms of disease control rate, 86.9% (287/330) in the AFP response group and 51.0% (153/300) in the AFP nonresponse group

showed successful disease control, respectively. The RR of disease control failure, similarly, was significantly lower in the AFP response group (RR, 0.37; 95%CI, 0.23-0.58) (Figure 2B). No significant heterogeneity of studies was found on these parameters ($P = 0.09$, $I^2 = 45\%$).

DISCUSSION

Treatment response in HCC patients has been heterogeneous, with some patients showing impressive treatment effects, but others showing limited or no evidence of response^[33,34]. The hypothesis that AFP response to treatment is a determinant of prognosis in HCC is an attractive mechanism for explaining any inter-individual variation in clinical outcome^[55]. For patients with elevated AFP at baseline, the AFP trend was shown to be static or rising during the course of the disease, and a number of patients with undetectable AFP at baseline were found to have detectable and rising trends of AFP value upon disease progression^[17,36]. For patients who underwent partial hepatectomy, the AFP level fell rapidly and remark-

ably after removal of tumors, but this rose at the time of recurrence^[37,38]. These findings suggested that AFP response could be potentially useful in predicting survival and the efficacy of treatment.

The present systematic review of the literature and meta-analysis was done to assess the impact of AFP response on HCC prognosis. Twelve eligible studies were identified that investigated the relationship between AFP response and prognosis in HCC. Strengths of the study included a comprehensive, systematic review of the literature by a multidisciplinary team including specialists in cancer, hepatology, and epidemiologic methods, thereby avoiding selection bias on the basis of study quality.

Using these studies, pooled estimates of outcome of HCCs expressing AFP were derived. Although our results showed that estimates of the significance of AFP response varied substantially between studies, these results support the notion that in the HCC patients who underwent systemic treatment and locoregional therapy, AFP response is predictive for better overall survival, with a pooled HR of 0.55 (95%CI, 0.47-0.65). In the HCC patients, AFP response had prognostic significance, whether it was defined by 50% decline of its initial level or 20% decline. This latter result, however, contradicted our supposition that greater AFP response should be associated with better survival, which was probably due to the individual differences from different clinical trials, and the small number of contributing studies. AFP response also seems to predict better PFS, with a pooled HR of 0.50 (95%CI, 0.38-0.65). However, this result should be interpreted with caution considering the small number of contributing studies. Our findings suggest that AFP response has a strong correlation with radiological response. When compared with the AFP nonresponse group, there were significant trends toward higher overall response rates (RR, 0.67; 95%CI, 0.61-0.75) and disease control rates (RR, 0.37; 95%CI, 0.23-0.58) in the AFP response group.

Although our results showed that HCCs expressing AFP response to treatment seem to be associated with a better prognosis, one caveat to this conclusion is publication bias, which is a major concern in all forms of meta-analysis, as smaller studies showing no statistically significant effects are more likely to remain unpublished^[39,40]. Indeed, it is not unusual for small, early studies to report a positive relationship or large effect that subsequent much larger studies fail to replicate. In the present study, there was some evidence for publication bias in the AFP response, and the risks calculated in our meta-analysis could be overestimates as a result of publication and reporting bias^[41]. Furthermore, advanced tumors were over-represented in the studies, given that they constituted approximately 64% of all HCC.

Heterogeneity between studies may represent a further potential source of bias in our analysis. The importance of heterogeneity between studies on summary estimates of HRs was carefully assessed. Although no attempt was made in our meta-analysis to quality-score reports^[42,43], it is clear that the design of some studies is not optimal.

The larger study by Vora *et al*^[29] showed less of an effect of AFP response on prognosis, probably due to the great heterogeneity produced by the HCC patients selected from the different clinical trials. Through sensitivity analysis, we found that this study was the main source of the heterogeneity in this meta-analysis, and after the exclusion of this study, the HR estimate decreased to 0.40 (95%CI, 0.33-0.49), as did heterogeneity ($I^2 = 0$, $P = 0.91$).

Here we describe substantial inconsistency of results on the effectiveness of AFP response to predict survival of HCC. The main factors we identified as responsible for these findings are: long periods of patient recruitment, which leads to heterogeneity within a study because treatment of HCC has evolved over the course of the study; selection bias of patients because of different treatment clinical trials included, suggesting that AFP might be sensitive to some kinds of therapies but not to others; and the absence of a uniform definition of positive AFP response, leading to different results when using different cut-off points. Moreover, variability in the length of follow-up used to detect the events of interest also hampers comparability between studies since risk of survival is time-dependent. Inconsistency in the inclusion of clinical and pathological factors predicting survival mortality as covariables in multivariate analysis could be an additional contributing factor^[41].

Notwithstanding this, the studies pooled in this meta-analysis provide data on 974 HCC cases and support the opinion that AFP response to treatment is a determinant of prognosis in HCC. However, multicenter prospective studies that are better designed, with assessors blinded to the clinical data, and homogeneous HCC cohorts analyzed prospectively, are required to unequivocally assess the precise prognostic effect of AFP response in the HCC. In addition, most studies have empirically defined a 50% drop or 20% drop as an AFP response, while it has to be validated critically in future prospective series.

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COMMENTS

Background

Conventionally, the treatment response of hepatocellular carcinoma (HCC) to systemic therapy or other nonsurgical treatment modalities is assessed by radiologic imaging using conventional criteria, such as World Health Organization criteria or Response Evaluation Criteria in Solid Tumors. However, despite successful clinical correlations for other solid tumors, these radiological response-based criteria have been criticized for not adequately reflecting treatment response and tumor viability for HCC.

Research frontiers

In the half century after α -fetoprotein (AFP) was first described, it was extensively studied as a screening and diagnostic tool for HCC. Although there have been some early observational studies that suggested that AFP trend might be

useful in assessing treatment response, until recently, there has been no clinical study to validate the significance of serial AFP monitoring in association with treatment response of HCC.

Innovations and breakthroughs

This is the first attempt to systemically review the prognostic value of AFP response world wide. A total of 11 articles and 974 cases of HCC patients were collected from the international literature and evaluated for clinical and biochemical features such as AFP response. This study results revealed that AFP response was associated with a decreased mortality across HCC in overall survival and progression-free survival, respectively. As to radiological imaging, when compared with the AFP nonresponse patients, there were significant trends toward higher overall response rates and disease control rates in the AFP response patients.

Applications

The study results suggest that the AFP is a potentially monitoring serum index and the AFP response to treatment could be used for monitoring treatment efficacy and determining the prognosis of HCC patients. In addition, the results demonstrate that AFP could also make up for the drawbacks of imaging and is able to aid radiological tools in the monitoring of prognosis and treatment.

Terminology

AFP, which is a glycoprotein that is secreted into the serum of approximately 70% of patients with HCC, has potential values in monitoring of prognosis and treatment for HCC. AFP response, that AFP level would reduce during treatment of HCC patients, was defined as 20% or 50% decline of the initial level by most studies.

Peer review

The authors provide results of a meta-analysis, with this manuscript, on level changes of circulating AFP in HCC patients receiving local and systemic therapies, and gave some reasonable comments and proposals for further validation of the results from literature. The analysis is of importance for prognosis assessment and evaluation of different therapies. A multi-center study is needed for further confirmation.

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Single nucleotide polymorphisms in the *CDH17* gene of colorectal carcinoma

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Abstract

AIM: To investigate the relationship between c.343A>G and c.2216A>C polymorphism sites in the *CDH17* gene and colorectal carcinoma.

METHODS: Ninety-three non-consanguineous colorectal carcinoma patients admitted to the Department of Oncology at the First Affiliated Hospital of Zhengzhou University were included in this study. Ninety-three peripheral venous blood samples, of approximately one milliliter from each patient, were collected between

December 2009 and August 2010. The genomic DNA of these peripheral venous blood samples were extracted and purified using a Fermentas Genomic DNA Purification Kit (Fermentas, CA) according to the manufacturer's protocol. The single nucleotide polymorphisms (SNPs) of the liver-intestine cadherin (*CDH17*) gene c.343A>G and c.2216A>C were determined by the polymerase chain reaction-single strand conformation polymorphism method (PCR-SSCP) in 93 peripheral venous blood samples from patients suffering with colorectal carcinoma. Typical samples that showed different migration bands in SSCP were confirmed by sequencing. Directed DNA sequencing was used to check the correctness of the genotype results from the PCR-SSCP method.

RESULTS: There was a significant association between the c.2216 A>C SNPs of the *CDH17* gene and the tumor-node-metastasis (TNM) grade, as well as with lymph node status, in 93 peripheral venous blood samples from colorectal carcinoma patients. The genotype frequencies of A/C, A/A, and C/C were 12.90%, 33.33% and 53.76%, respectively. There was a significant correlation between lymph node metastasis, TNM grade, and the genotype distribution ($P < 0.05$). The C/C genotype raised the risk of lymph node metastasis and the TNM grade. There was a significant difference in the TNM grade and lymph node metastasis between the A/A and C/C genotypes ($P = 0.003$ and $P = 0.013$, respectively). Patients with colorectal carcinoma carrying the C allele tended to have a higher risk of lymph node metastasis and have a higher TNM grade. The difference between the TNM grades, as well as the lymph node metastasis of the two alleles, was statistically significant ($P < 0.01$).

CONCLUSION: The SNPs of the *CDH17* gene c.2216 A>C might be clinically important in the prognosis of colorectal carcinoma.

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Key words: Single nucleotide polymorphisms; Liver-

intestine cadherin; Colorectal carcinoma; Polymerase chain reaction-single strand conformation polymorphism method

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INTRODUCTION

Colorectal carcinoma (CRC) has traditionally been one of the four cancers with the highest mortality worldwide, but the incidence was relatively low historically in Asian populations. However, the prevalence of colorectal neoplasms has increased by 2- to 4-fold in some developed Asian countries, including China, Japan, South Korea and Singapore in the past few decades, due to changes in dietary habits and lifestyle, as well as certain genetic factors of Asian populations^[1]. The etiology of CRC is multifactor, involving hereditary causes, environmental factors, and somatogenic changes occurring during tumor progression. At present, the assured mechanism of the occurrence and development of colorectal carcinoma is still unknown. While several aspects of colorectal carcinoma have been investigated, CRCs can be parsimoniously subdivided into two major groups defined by the genetic pathways involved^[2]. Its correlation with single nucleotide polymorphisms (SNPs) remains largely unknown.

SNPs are the most common type of genomic sequence variation^[3], and are thought to be associated with population diversity, susceptibility to disease, and individual response to drug treatments. Many SNPs are silent, with no direct effect on the gene products but, by virtue of the linkage disequilibrium existing across the human genome, they can still be used as genetic markers to locate adjacent functional variants that contribute to diseases. Foreseeable common SNP discoveries may not permit the identification of the small subset of patients that contain most cancers^[4]. SNPs may also have functional consequences if they directly affect the coding or regulatory (usually promoter) regions of a gene. There have been cumulative studies on the associations between cancer risk and SNPs in selected candidate genes; to date, numerous SNPs associated with susceptibility to both cancer types have been identified, but their effect on disease risk may differ among populations^[5], and such information may shed light on the molecular and genetic basis of the polygenic nature of cancer. These SNPs may be used as surrogate biomarkers of the genetic background of CRC patients, to predict therapeutic response and prognosis^[6].

The *CDH17* gene is a member of the cadherin superfamily; genes encoding calcium-dependent, membrane-associated glycoproteins. The human *CDH17* gene located on chromosome 8q22.1 has eighteen exons and encodes liver-intestine cadherin (LI-cadherin) protein (also known as cadherin-17). LI-cadherin is a structurally unique member of the cadherin superfamily^[7]. In contrast to classic cadherins, such as E- and N-cadherin, the extracellular domain of LI-cadherin consists of seven, instead of the usual five, structurally defined cadherin repeats. Its cytoplasmic domain is also small, comprising only 20 amino acids without a β -catenin binding region, and exhibits no homology with the corresponding region of classic cadherins, which consists of 150-160 amino acids. LI-cadherin is also known to possess biological functions distinct from classic cadherins. The adhesive function of LI-cadherin is independent of any interaction with cytoplasmic components, such as catenins or the actin cytoskeleton, although in E-cadherin this function crucially depends on the formation of a cadherin-catenin complex and anchorage to the actin cytoskeleton^[8,9]. The mechanism responsible for the regulation and function of this cadherin has been elucidated. Expression of LI-cadherin has been reported in gastric adenocarcinoma of the intestinal type and colorectal carcinoma^[10,11]. In colorectal carcinoma, LI-cadherin expression has been reported in well-differentiated carcinoma, but not in poorly-differentiated carcinoma. It has been found that reduced expression of LI-cadherin is closely associated with tumor progression and lymph node metastasis of human colorectal carcinoma^[12]. However, the detailed clinicopathologic significance of LI-cadherin has not been elucidated.

In this study, through a PubMed, Embase, Google Scholar, CBMdisc and CNKI SNPs search, we found that for the *CDH17* gene c.343A>G and c.2216A>C, the SNPs gene phenotype is A/G and the gene frequency phenotype is about 50%. We detected the SNPs of the *CDH17* gene c.343A>G and c.2216A>C by polymerase chain reaction-single strand conformation polymorphism method (PCR-SSCP), and directed sequencing in colorectal carcinoma patients. We then analyzed the distribution frequencies of the genotypes and alleles of the two polymorphism sites. To evaluate the role of *CDH17* gene polymorphisms in colorectal tumor aggressiveness, the associations between genotype and clinicopathologic parameters were also analyzed.

MATERIALS AND METHODS

Patients, blood sample source and storage

Ninety-three patients undergoing curative surgery for colorectal cancer at the First Affiliated Hospital of Zhengzhou University were included in this study after giving their informed consent. Peripheral blood samples were collected in ethylenediaminetetraacetic acid-containing tubes between December 2009 and August 2010 from these patients. All samples were immediately placed in refrigeratory conditions and stored at 4 °C until they could

Table 1 Primer sequences and fragment sizes of polymerase chain reaction products

Gene	SNPs site (refSNP)	Primer sequence(5'-3')	Fragment sizes of PCR products (bp)
<i>CDH17</i>	c.343A>G (rs2243518)	Forward primer: CCAACATGGTTTCCTTTTCCTC Reverse primer: GTTCIGCCTTACTGAGCCTTCG	159
	c.2216A>C (rs1051624)	Forward primer: AATCCAGGGCTGGAAGTTGTA Reverse primer: TACTAGCCTGAGTTGCCTATA	198

SNPs: Single nucleotide polymorphisms; PCR: Polymerase chain reaction.

be further processed.

DNA isolation

Genomic DNA was extracted using a Fermentas Genomic DNA Purification Kit (Fermentas, CA) according to the manufacturer's protocol. The amount of isolated DNA was determined spectrophotometrically.

Primer synthesis

Primer sequences for the *CDH17* gene c.343A>G and c.2216A>C were synthesized by Shanghai Sangon Biological Engineering Technology and Services Co. Ltd (Table 1).

PCR-SSCP analysis and sequencing of the *CDH17* gene c.343A>G and c.2216A>C

The fragments encompassing the *CDH17* gene c.343A>G and c.2216A>C were amplified by PCR. The twenty microlitre PCR amplification reaction system contained < 1 µg template DNA, 10 µL of 2 × Taq PCR Master Mix [Tiangen Biotech (Beijing) Co., Ltd.], 0.4 µL of forward and reverse primers (10 µmol/L), and 3.2 µL of double distilled water. PCR reaction conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing for 30 s and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min, with preservation being carried out at 4 °C. After 2.0% agarose gel electrophoresis (containing Evans blue dye), PCR products were observed by a gel imaging instrument. Fragment sizes of PCR products were 159 bp and 198 bp, respectively, and indicated with DL100 DNA Marker.

The amplified products were denatured in 95% formamide at 95 °C for 10 min, and analyzed on 12% polyacrylamide gels at 160 V for 15 min and 30 V for 6-8 h in 1 × Tris-Borate-EDTA buffer at normal body temperature. A modified silver staining method was applied for visualization. Briefly, the gels were soaked in 10% ethanol and 0.5% acetic acid for 10 min, then transferred to 0.2% AgNO₃ solution for 10 min and washed with distilled water three times. The staining reaction was developed with the use of 3% NaOH and 0.25% formaldehyde, and the gels were fixed in 10% ethanol and 0.5% acetic acid. Staining reactions were promoted by gentle shaking.

The detection of SSCP analysis was performed. Samples showing an altered mobility pattern were then subjected to sequencing analysis by Shanghai Sangon Biological Engineering Technology and Services Co. Ltd.

Statistical analysis

Statistical analysis was performed using SPSS for Windows version 17.0. Differences in allele and genotype frequencies were evaluated by the χ^2 or Fisher exact test. We used logistic regression models to calculate χ^2 tests for genotypic and allelic association and odds ratios (OR) with their 95%CI. Association between each of the SNPs and clinicopathological parameters was assessed using the χ^2 test. To study correlation between clinical parameters and genotypes of the *CDH17* gene c.343A>G and c.2216A>C polymorphisms in multivariate mode we used binary logistic regression, with each parameter as a dependent variable, and genotypes of the two studied SNPs together with other clinical parameters as independent variables. Differences in ordered categorical data were evaluated for significance with the rank sum test; the mean ranks were used to express risk degree. The Holm-Bonferroni method was used for multiple comparisons. A *P* value of ≤ 0.05 was considered statistically significant.

RESULTS

Clinicopathologic parameters in colorectal carcinomas

The characteristics of 93 patients with colorectal carcinoma are given in Tables 2 and 3. Of the 93 patients, 49% (46/93) were female and 51% (47/93) were male. The mean age of the female patients (the mean \pm SD deviation was 56.41 \pm 14.91 years) did not differ significantly from that of the males (the mean \pm SD deviation was 58.19 \pm 11.60 years).

PCR products of *CDH17*

The PCR-SSCP analysis generated two fragments as expected; 159bp for c.343A>G, and 198bp for c.2216A>C (Figure 1).

The correlation between the genotype of the *CDH17* gene c.343A>G and clinicopathologic parameters in colorectal carcinomas

The genotype of the *CDH17* gene c.343A>G: The genotype frequencies of the *CDH17* gene c.343A>G A/A, A/G, and G/G were 27.96% (26/93), 47.31% (44/93), and 24.73% (23/93), respectively. The frequencies of the A allele and G allele were 51.61% and 48.39%, respectively.

The correlation between the genotype of the *CDH17* gene c.343A>G and clinicopathologic parameters: There were no significant differences in gender, age, tu-

Table 2 The correlation between the genotype of *CDH17* gene c.343A>G and clinicopathologic parameters in colorectal carcinomas *n* (%)

Clinicopathologic parameters	<i>n</i>	Genotype <i>CDH17</i> c.343A>G			χ^2	<i>P</i> value
		A/A	A/G	G/G		
Gender						
Male	47	10 (21.3)	25 (53.2)	12 (25.5)	2.236	0.327
Female	46	16 (34.8)	19 (41.3)	11 (23.9)		
Age (yr)						
< 50	29	10 (34.5)	15 (51.7)	4 (13.8)	2.854	0.240
≥ 50	64	16 (25)	29 (45.3)	19 (29.7)		
Tumor location						
Colon	32	8 (25.0)	16 (50.0)	8 (25.0)	0.229	0.892
Rectum	61	18 (29.5)	28 (45.9)	15 (24.6)		
Tumor size (cm)						
< 5	68	18 (26.5)	34 (50.0)	16 (23.5)	0.734	0.693
≥ 5	25	8 (32.0)	10 (40.0)	7 (28.0)		
Macroscopic appearance						
Protrude type	31	8 (25.8)	17 (54.8)	6 (19.4)	1.948	0.745 ¹
Ulcer type	46	12 (26.1)	21 (45.7)	13 (28.3)		
Infiltrating type	16	6 (37.5)	6 (37.5)	4 (25.0)		
Colloid type	0	0 (0.0)	0 (0.0)	0 (0.0)		
Histologic type						
Adenocarcinoma	71	18 (25.4)	34 (47.9)	19 (26.8)	2.179	0.703 ¹
Adenocarcinoma and mucinous carcinoma	9	4 (44.4)	3 (33.3)	2 (22.2)		
Mucinous carcinoma	13	4 (30.8)	7 (53.8)	2 (15.4)		
Undifferentiated carcinoma	0	0 (0.0)	0 (0.0)	0 (0.0)		
Adenosquamous carcinoma	0	0 (0.0)	0 (0.0)	0 (0.0)		
Squamous cell carcinoma	0	0 (0.0)	0 (0.0)	0 (0.0)		
Invasion depth						
Submucosa	2	0 (0.0)	2 (100.0)	0 (0.0)	0.696	0.706 ²
Muscular layer	34	12 (35.3)	15 (44.1)	7 (20.6)		
Serous coat	48	12 (25)	21 (43.8)	15 (31.3)		
Other organs	9	2 (22.2)	6 (66.7)	1 (11.1)		
Differentiation degree						
Well-differentiated	4	0 (0.0)	2 (50.0)	2 (50.0)	2.212	0.331 ²
Moderately-differentiated	63	17 (27.0)	33 (52.4)	13 (20.6)		
Poorly-differentiated	26	9 (34.6)	9 (34.6)	8 (30.8)		
Lymph node metastasis						
No	59	16 (27.1)	35 (59.3)	8 (13.6)	13.105	0.001 ³
Yes	34	10 (29.4)	9 (26.5)	15 (44.1)		
TNM grade						
I	26	8 (30.8)	16 (61.5)	2 (7.7)	10.344	0.006 ^{2,3}
II	31	7 (22.6)	18 (58.1)	6 (19.4)		
III	33	9 (27.3)	9 (27.3)	15 (45.5)		
IV	3	2 (66.7)	1 (33.3)	0 (0.0)		
Hematogenous metastasis						
No	90	24 (26.7)	43 (47.8)	23 (25.6)	2.556	0.279 ¹
Yes	3	2 (66.7)	1 (33.3)	0 (0.0)		
Recurrence						
No	91	24 (26.4)	44 (48.4)	23 (25.3)	5.267	0.072 ¹
Yes	2	2 (100.0)	0 (0.0)	0 (0.0)		

¹The Fisher's Exact Test; ²The Kruskal-Wallis Test; ³Statistically significant.

mor location, tumor size, macroscopic appearance, histologic type, invasion depth, differentiation, hematogenous metastasis, or recurrence among the three genotypes of the *CDH17* gene c.343A>G ($P > 0.05$). However, there was a significant correlation between lymph node metastasis and tumor-node-metastasis (TNM) grade, and its

Table 3 The correlation between the genotype of *CDH17* gene c.2216A>C and clinicopathologic parameters in colorectal carcinomas *n* (%)

Clinicopathologic parameters	<i>n</i>	Genotype <i>CDH17</i> c.2216A>C			χ^2	<i>P</i> value
		A/A	A/C	C/C		
Gender						
Male	47	20 (42.6)	5 (10.6)	22 (46.8)	3.656	0.161
Female	46	11 (23.9)	7 (15.2)	28 (60.9)		
Age (yr)						
< 50	29	6 (20.7)	5 (17.2)	18 (62.1)	3.176	0.204
≥ 50	64	25 (39.1)	7 (10.9)	32 (50.0)		
Tumor location						
Colon	32	10 (31.3)	6 (18.8)	16 (50.0)	1.485	0.476
Rectum	61	21 (34.4)	6 (9.8)	34 (55.7)		
Tumor size (cm)						
< 5	68	25 (36.8)	5 (7.4)	38 (55.9)	7.144	0.028 ³
≥ 5	25	6 (24.0)	7 (28.0)	12 (48.0)		
Macroscopic appearance						
Protrude type	31	11 (35.5)	4 (12.9)	16 (51.6)	1.025	0.906 ¹
Ulcer type	46	16 (34.8)	5 (10.9)	25 (54.3)		
Infiltrating type	16	4 (25.0)	3 (18.8)	9 (56.3)		
Colloid type	0	0 (0.0)	0 (0.0)	0 (0.0)		
Histologic type						
Adenocarcinoma	71	23 (32.4)	10 (14.1)	38 (53.5)	1.684	0.794 ¹
Adenocarcinoma and mucinous carcinoma	9	4 (44.4)	0 (0.0)	5 (55.6)		
Mucinous carcinoma	13	4 (30.8)	2 (15.4)	7 (53.8)		
Undifferentiated carcinoma	0	0 (0.0)	0 (0.0)	0 (0.0)		
Adenosquamous carcinoma	0	0 (0.0)	0 (0.0)	0 (0.0)		
Squamous cell carcinoma	0	0 (0.0)	0 (0.0)	0 (0.0)		
Invasion depth						
Submucosa	2	2 (100.0)	0 (0.0)	0 (0.0)	1.610	0.447 ²
Muscular layer	34	12 (35.3)	4 (11.8)	18 (52.9)		
Serous coat	48	14 (29.2)	5 (10.4)	29 (60.4)		
Other organs	9	3 (33.3)	3 (33.3)	3 (33.3)		
Differentiation degree						
Well-differentiated	4	0 (0.0)	0 (0.0)	4 (100.0)	0.090	0.956 ²
Moderately-differentiated	63	24 (38.1)	9 (14.3)	30 (47.6)		
Poorly-differentiated	26	7 (26.9)	3 (11.5)	16 (61.5)		
Lymph node metastasis						
No	59	25 (42.4)	10 (16.9)	24 (40.7)	11.143	0.004 ³
Yes	34	6 (17.6)	2 (5.9)	26 (76.5)		
TNM grade						
I	26	12 (46.2)	4 (15.4)	10 (38.5)	6.710	0.035 ^{2,3}
II	31	12 (38.7)	5 (16.1)	14 (45.2)		
III	33	6 (18.2)	2 (6.1)	25 (75.8)		
IV	3	1 (33.3)	1 (33.3)	1 (33.3)		
Hematogenous metastasis						
No	90	30 (33.3)	11 (12.2)	49 (54.4)	1.243	0.537 ¹
Yes	3	1 (33.3)	1 (33.3)	1 (33.3)		
Recurrence						
No	91	29 (31.9)	12 (13.2)	50 (54.9)	4.088	0.130 ¹
Yes	2	2 (100.0)	0 (0.0)	0 (0.0)		

¹The Fisher's Exact Test; ²The Kruskal-Wallis Test; ³Statistically significant.

genotype frequencies ($P < 0.05$) (Table 1).

The sequencing analysis: The detection of SSCP analysis was performed. Samples showing an altered mobility pattern were then subjected to sequencing analysis by Shanghai Sangon Biological Engineering Technology and

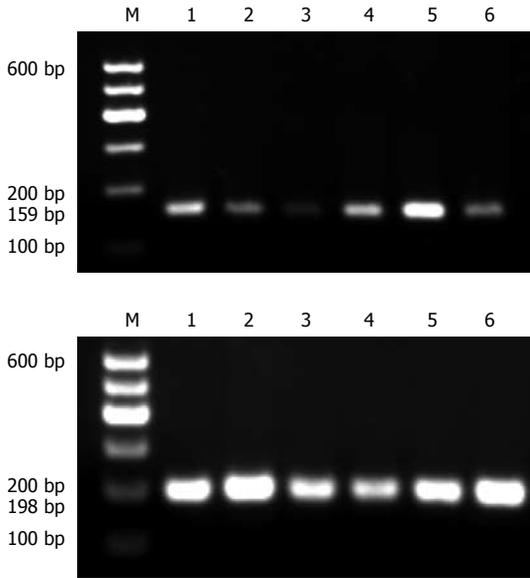


Figure 1 Polymerase chain reaction amplification of *CDH17* gene c.343A>G (the upper) and c.2216A>C (the lower) from genomic DNA of colorectal carcinoma. Lane M: Marker; Lane 1-6: Samples.

Genotype	Lymph node metastasis		OR (95%CI)
	No	Yes	
A/G	35 (59.3)	9 (26.5)	1.000 (reference)
A/A	16 (27.1)	10 (29.4)	2.431 (0.828-7.139)
G/G	8 (13.6)	15 (44.1)	7.292 (2.360-22.532)
Allele frequency			
A	67 (56.8)	29 (42.6)	1.000 (reference)
G	51 (43.2)	39 (57.4)	1.767 (0.967-3.229)

OR: Odds ratios.

Services Co. Ltd (Figure 2).

The correlation between the genotype of the *CDH17* gene c.343A>G and lymph node metastasis: The results of logistic regression analysis are shown in Table 4, and indicate that the G/G genotype of the *CDH17* gene c.343A>G raised the risk of lymph node metastasis (OR of genotype G/G, genotype A/A to genotype A/G were 7.292 and 2.431, respectively). The results of multiple comparisons by the Holm-Bonferroni method ($\alpha' = 0.017$) were as follows: *P* value was 0.102 when genotype A/A was compared with genotype A/G; *P* value was 0.062 when genotype A/A was compared with genotype G/G; *P* value was 0.000 when genotype A/G was compared with genotype G/G. This indicates that there was a significant difference in lymph node metastasis between the A/G and G/G genotypes.

The *CDH17* gene c.343A>G site allele G raised the risk of lymph node metastasis (OR of allele G to allele

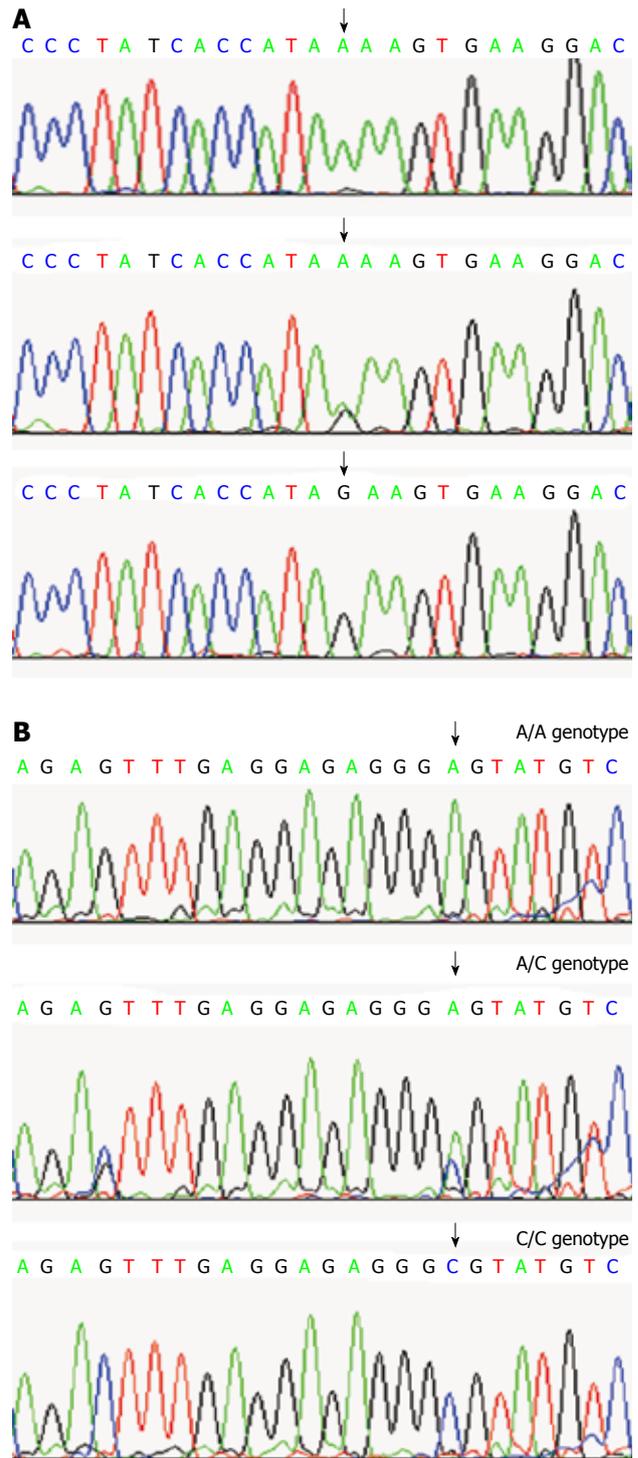


Figure 2 The sequence results of *CDH17* gene c.343A>G (A) and *CDH17* gene c.2216A>C (B). A: The upper homozygous genotype (A/A); The middle heterozygous genotype (A/G); The lower homozygous genotype (G/G); Arrow head: Point mutations of *CDH17* gene c.343A>G; B: The upper homozygous genotype (A/A); The middle heterozygous genotype (A/C); The lower homozygous genotype (C/C); Arrow head: Point mutations of *CDH17* gene c.2216A>C.

A was 1.767) (Table 4). However, a χ^2 test showed that the difference between the lymph node metastasis of the two alleles was not statistically significant ($\chi^2 = 3.450$; *df* = 1; *P* = 0.063).

Table 5 The correlation between the genotype and allele frequency of *CDH17* gene c.343A>G and tumor-node-metastasis grade *n* (%)

	TNM grade				Mean rank
	I	II	III	IV	
Genotype					
A/G	16 (61.5)	18 (58.1)	9 (27.3)	1 (33.3)	39.32
A/A	8 (30.8)	7 (22.6)	9 (27.3)	2 (66.7)	48.15
G/G	2 (7.7)	6 (19.4)	15 (45.5)	0 (0.0)	60.39
Allele frequency					
A	32 (61.5)	32 (51.6)	27 (40.9)	5 (83.3)	87.71
G	20 (38.5)	30 (48.4)	39 (59.1)	1 (16.7)	99.68

TNM: Tumor-node-metastasis.

The correlation between the genotype of the *CDH17* gene c.343A>G and TNM grade: The results of the Kruskal-Wallis test are shown in Table 5. It revealed that the *CDH17* gene c.343A>G genotype G/G raised the risk of TNM grade (the mean ranks of A/G, A/A and G/G genotype were 39.32, 48.15 and 60.39, respectively). The results of multiple comparisons by the Holm-Bonferroni method ($\alpha' = 0.017$) were as follows: *P* value was 0.197 when genotype A/A was compared with genotype A/G; *P* value was 0.127 when genotype A/A was compared with genotype G/G; *P* value was 0.001 when genotype A/G was compared with genotype G/G. This suggests that there was a significant difference in TNM grade between the A/G and G/G genotypes.

The correlation between the genotype of *CDH17* gene c.2216A>C and clinicopathologic parameters in colorectal carcinomas

The genotype of the *CDH17* gene c.2216A>C: The genotype frequencies of the *CDH17* gene c.2216A>C A/C, A/A, and C/C were 12.90% (12/93), 33.33% (31/93), and 53.76% (50/93), respectively. The frequencies of the A allele and C allele were 39.78% and 60.22%, respectively.

The allele G raised the risk of TNM grade (the mean ranks of allele A and allele G were 87.71 and 99.68, respectively) (Table 5). However, the Kruskal-Wallis test showed that the difference between the TNM grade of the two alleles was not statistically significant ($H = 2.561$; $df = 1$; $P = 0.110$).

The correlation between the genotype of the *CDH17* gene c.2216A>C and clinicopathologic parameters: There was no significant difference in gender, age, tumor location, macroscopic appearance, histologic type, invasion depth, differentiation, hematogenous metastasis, or recurrence among the three genotypes of the *CDH17* gene c.2216A>C ($P > 0.05$). However, there was a significant correlation between tumor size, lymph node metastasis, and TNM grade, and its genotype frequencies ($P < 0.05$) (Table 3).

The correlation between the genotype of the *CDH17* gene c.2216A>C and tumor size: The results of the χ^2

Table 6 The correlation between the genotype and allele frequency of *CDH17* gene c.2216A>C and lymph node metastasis *n* (%)

	Lymph node metastasis		OR (95%CI)
	No	Yes	
Genotype			
A/C	10 (16.9)	2 (5.9)	1.000 (reference)
A/A	25 (42.4)	6 (17.6)	1.200 (0.206-6.977)
C/C	24 (40.7)	26 (76.5)	5.417 (1.076-27.272)
Allele frequency			
A	60 (50.8)	14 (20.6)	1.000 (reference)
C	58 (49.2)	54 (79.4)	3.990 (2.002-7.953)

OD: Odds ratio.

test indicate that there was a significant correlation between tumor size and the genotype of the *CDH17* gene c.2216A>C. However, the results of multiple comparisons by the Holm-Bonferroni method ($\alpha' = 0.017$) suggest that there was no significant difference in tumor size between any of its genotypes (*P* value was 0.018 when genotype A/A was compared with genotype A/C; *P* value was 0.625 when genotype A/A was compared with genotype C/C; *P* value was 0.027 when genotype A/C was compared with genotype C/C).

The correlation between the genotype of the *CDH17* gene c.2216A>C and lymph node metastasis: The results of logistic regression analysis are shown in Table 6, and indicate that the C/C genotype of the *CDH17* gene c.2216A>C raised the risk of lymph node metastasis (OR of genotype C/C, genotype A/A to genotype A/C were 5.417 and 1.200, respectively). The results of multiple comparisons by the Holm-Bonferroni method ($\alpha' = 0.017$) were as follows: *P* value was 0.067 when genotype A/A was compared with genotype A/C; *P* value was 0.003 when genotype A/A was compared with genotype C/C; *P* value was 0.027 when genotype A/C was compared with genotype C/C. This indicates that there was a significant difference in lymph node metastasis between the A/A and C/C genotypes.

CDH17 gene c.2216A>C site allele C raised the risk of lymph node metastasis (OR of allele C to allele A was 3.990) (Table 6). The χ^2 test showed that the difference between the lymph node metastasis of the two alleles was statistically significant ($\chi^2 = 16.488$; $df = 1$; $P = 0.000$).

The correlation between the genotype of the *CDH17* gene c.2216A>C and TNM grade: The results of the Kruskal-Wallis test are shown in Table 7, and reveal that the *CDH17* gene c.2216A>C genotype C/C raised the risk of TNM grade (the mean ranks of the A/A, A/C, and C/C genotype were 38.77, 42.00 and 53.30, respectively). The results of multiple comparisons by the Holm-Bonferroni method ($\alpha' = 0.017$) were as follows: *P* value was 0.718 when genotype A/A was compared with genotype A/C; *P* value was 0.013 when genotype A/A was compared with genotype C/C; *P* value was 0.168 when genotype A/C was compared with genotype C/C. This

Table 7 The correlation between the genotype and allele frequency of *CDH17* gene c.2216A>C and tumor-node-metastasis grade *n* (%)

	TNM grade				Mean rank
	I	II	III	IV	
Genotype					
A/C	4 (15.4)	5 (16.1)	2 (6.1)	1 (33.3)	42
A/A	12 (46.2)	12 (38.7)	6 (18.2)	1 (33.3)	38.77
C/C	10 (38.5)	14 (45.2)	25 (75.8)	1 (33.3)	53.3
Allele frequency					
A	28 (53.8)	29 (46.8)	14 (21.2)	3 (50.0)	78.09
C	24 (46.2)	33 (53.2)	52 (78.8)	3 (50.0)	103.68

TNM: Tumor-node-metastasis.

suggests that there was a significant difference in TNM grade between the A/A and C/C genotypes.

The Kruskal-Wallis test showed that allele C raised the risk of TNM grade (the mean ranks of allele A and allele C were 78.09 and 103.68, respectively) (Table 7). The difference between the TNM grade of the two alleles was statistically significant ($H = 11.225$; $df = 1$; $P = 0.001$).

DISCUSSION

CDH17 is a marker for the diagnosis of adenocarcinomas of the digestive system

Cancer is a complex disease where genetic mutations, rearrangement, deletions, or gene polymorphisms may affect not only cancer development, but also cancer progression and, as a result, could influence cancer phenotypes^[13,14]. The *CDH17* gene is located on chromosome 8q22.1 and encodes LI-cadherin. LI-cadherin, belonging to a subclass of the 7D-cadherin superfamily, is present in the fetal liver and gastrointestinal tract during embryogenesis, but the gene becomes silenced in healthy adult liver and stomach tissues. It functions as a peptide transporter and cell adhesion molecule to maintain tissue integrity in epithelia. However, recent findings have reported aberrant expression of LI-cadherin in major gastrointestinal malignancies, including hepatocellular carcinoma (HCC), stomach and colorectal cancers, and its clinical association with tumor metastasis and advanced tumor stages^[15,16]. LI-cadherin manifests distinct and unique roles in tumorigenesis originating from different organs. In the epithelium of the healthy human stomach, the level of LI-cadherin expression is negligible, whereas an overexpression of this molecule is observed frequently in gastric carcinoma^[17-19]. When the transcriptome of gastric cancer was studied by serial analysis of gene expression, LI-cadherin was found associating with an intestinal type of gastric cancer^[20]. In addition, high tumoral LI-cadherin levels tend to correlate with advanced stages of gastric cancer, and are associated with a poor prognosis and lymph node metastasis^[21,22]. That silencing LI-cadherin has positive actions in the processes of LoVo cell invasion and metastasis, and the interactions among matrix metalloproteinase (MMP)-2, MMP-9, and

LI-cadherin, participates in the multiple steps of invasion and metastasis in LoVo colorectal cancer cells^[23].

This observation was further supported by a study using high density DNA microarrays to identify differentially-expressed genes in advanced stages of gastric cancer, in which an upregulation of *CDH17* was found among genes associated with cell adhesion, cell cycle, cellular motility, and DNA synthesis^[24]. Unlike normal gastric tissues, LI-cadherin is found scattered throughout the healthy human pancreas. Less LI-cadherin is expressed in poorly-differentiated tumor tissues of ductal adenocarcinoma of the pancreas, and this low level of LI-cadherin is correlated with advanced stages of the cancer. When LI-cadherin is used as a prognostic biomarker, the survival time is longer for those patients with LI-cadherin-positive tumors when compared with those having LI-cadherin-negative tumors^[25]. In the midst of identifying new target for HCC detection and treatment, LI-cadherin is likely an attractive candidate^[26]. The elevated level of LI-cadherin in tumors is correlated to high serum alpha-fetoprotein level, tumor invasion, and advanced stage tumor, associating with poor prognosis, of HCC patients. For primary colorectal carcinoma, the expression of LI-cadherin is diminished in tumor tissues. Clinically, this low level of LI-cadherin is correlated to dedifferentiation of tumors, tumor invasion, and late tumor stages. However, significant differences in the survival of cancer patients have not been found, irrespective of whether the expressions of LI-cadherin are low or high. In addition, a negligible level of LI-cadherin has been documented in several colorectal cancer cell lines when compared with those in normal colonic mucosal cells^[27]. This observation is further supported by a separate study showing a reduced expression of LI-cadherin in cancer tissues and such expression was correlated with dedifferentiation of tumors and poor survival of patients^[28]. But Gröne *et al.*^[29] study showed *CDH17* protein and gene expression analyses do not support application of molecular classifiers for prediction of clinical outcome in current routine diagnostic as a basis for patient-orientated therapy in stage Union for International Cancer Control II colon cancer. Further studies are needed to develop prognosis signatures applicable in patient care. Despite these findings, LI-cadherin was found present in colorectal adenocarcinoma in a separate study^[30].

The SNPs in c.343A>C and c.2216A>C polymorphism sites of *CDH17* gene

In this study we investigated the SNPs in c.343A>C and c.2216A>C polymorphism sites of *CDH17* gene and the associations between genotype of them and clinicopathologic parameters were also analyzed to evaluate the role of SNPs in colorectal tumor aggressiveness. The possibility that different genetic polymorphisms in the *CDH17* gene may regulate, at least in part, LI-cadherin expression and/or activity, is an attractive hypothesis that may help to identify patients who are susceptible to

colorectal carcinoma.

There have been many studies on the associations between cancer risk and SNPs. But the associations between *CDH17* gene SNPs in c.343A>C and c.2216A>C polymorphism sites and colorectal carcinoma risk have not been reported.

The missense substitution (c.343A>G) mapped

In our study, the first missense substitution (c.343A>G) mapped to the amino acid position 115 and was responsible for the amino acid change of 115 Glu with Lys. The genotype frequencies of A/A, A/G and G/G were 27.96%, 47.31% and 24.73%, respectively. The frequencies of the A allele and G allele were 51.61% and 48.39%, respectively. According to the SNP database (rs2243518), the frequency of the G allele in our population is lower than that in Europeans (84.2%). In comparison, its reported frequency in Sub-Saharan African and Asians is 95.8% and 56.7%, respectively. There were no significant difference in gender, age, tumor location, tumor size, macroscopic appearance, histologic type, invasion depth, differentiation, hematogenous metastasis, and recurrence among the genotype distribution ($P > 0.05$). But we found a significant correlation between the genotype distribution and lymph node metastasis and TNM grade ($P < 0.05$). The results of logistic regression analysis indicate that the G/G genotype raised risk of lymph node metastasis (OR of genotype G/G, genotype A/A to genotype A/G were 7.292 and 2.431, respectively). The results of multiple comparison by Holm-Bonferroni ($\alpha' = 0.017$) indicate that there was a significant difference in lymph node metastasis between the A/G and G/G genotypes ($P = 0.000$). And patients with colorectal carcinoma carrying the G allele tended to have a higher risk of lymph node metastasis (OR of allele G to allele A was 1.767). But χ^2 test showed that the difference between the lymph node metastasis of two alleles was not statistically significant ($\chi^2 = 3.450$; $df = 1$; $P = 0.063$). In addition, the results of Kruskal-Wallis revealed that the genotype G/G raised risk of TNM grade (the mean ranks of A/G, A/A and G/G genotype were 39.32, 48.15 and 60.39, respectively). And the results of multiple comparison by Bonferroni ($\alpha' = 0.017$) suggest that there was a significant difference in TNM grade between the A/G and G/G genotypes ($P = 0.001$). Patients with colorectal carcinoma carrying the G allele tended to have a higher TNM grade (the mean ranks of allele A and allele G were 87.71 and 99.68, respectively). But the Kruskal-Wallis test showed that the difference between the TNM grade of two alleles was not statistically significant ($H = 2.561$; $df = 1$; $P = 0.110$).

SNP (c.2216A>C) mapped to the amino acid

The second SNP we investigate here (c.2216A>C) mapped to the amino acid position 739 and was responsible for the amino acid change of 739 Ala with Glu. The genotype frequencies of A/C, A/A and C/C were

12.90%, 33.33% and 53.76%, respectively. The frequencies of the A allele and C allele were 39.78% and 60.22%, respectively. According to the SNP database (rs1051624), the frequency of the C allele in our population is higher than that in Europeans (58.3%). In comparison, its reported frequency in African-American and Asians is 23.9% and 58.3%, respectively. There were no significant difference in gender, age, tumor location, macroscopic appearance, histologic type, invasion depth, differentiation, hematogenous metastasis, and recurrence among the genotype distribution ($P > 0.05$). But there were a significant correlation between tumor size, lymph node metastasis and TNM grade and the genotype distribution ($P < 0.05$). Firstly, the results of χ^2 test indicate that there was a significant correlation between tumor size and the genotype distribution. But the results of multiple comparison by Holm-Bonferroni ($\alpha' = 0.017$) suggest that there were no significant difference in tumor size between any two genotypes ($P > 0.017$). But Kwak *et al.*^[31] study showed that reduced expression of liver intestine-cadherin had a significant correlation with tumoral dedifferentiation and short overall survival in this series. In addition, early and frequent loss of liver intestine-cadherin expression might be a more sensitive indicator than E-cadherin to predict more aggressive tumoral behavior. Secondly, the results of logistic regression analysis indicate that the C/C genotype raised risk of lymph node metastasis (OR of genotype C/C, genotype A/A to genotype A/C were 5.417 and 1.200, respectively). And the results of multiple comparison by Holm-Bonferroni ($\alpha' = 0.017$) indicate that there was a significant difference in lymph node metastasis between the A/A and C/C genotype ($P = 0.003$). And patients with colorectal carcinoma carrying the C allele tended to have a higher risk of lymph node metastasis (OR of allele C to allele A was 3.990). The χ^2 test showed that the difference between the lymph node metastasis of two alleles was statistically significant ($\chi^2 = 16.488$; $df = 1$; $P = 0.000$). Lastly, the results of Kruskal-Wallis test revealed that genotype C/C raised risk of TNM grade (the mean ranks of A/A, A/C and C/C genotype were 38.77, 42.00 and 53.30, respectively). And the results of multiple comparison by Holm-Bonferroni ($\alpha' = 0.017$) suggest that there was a significant difference in TNM grade between the A/A and C/C genotypes ($P = 0.013$). Patients with colorectal carcinoma carrying the C allele tended to have a higher TNM grade (the mean ranks of allele A and allele C were 78.09 and 103.68, respectively). And the difference between the TNM grade of two alleles was statistically significant ($H = 11.225$; $df = 1$; $P = 0.001$). SNPs of the *CDH17* gene c.2216A>C and lymph node metastasis deserve further consideration in future studies as a clinical indicator during presurgical evaluation. And we found here patients with colorectal carcinoma carrying the C allele tended to have a higher TNM grade. This suggests that this SNPs would be a good predictor of lymph node status and TNM grade prior to surgical intervention and thereby provides an indicator for deciding

whether chemotherapy should be given or not.

***CDH17* mRNA is alternatively spliced to produce two variant transcripts**

It has been reported^[32] that *CDH17* mRNA is alternatively spliced to produce at least two variant transcripts, one of which was with exon 7 skipping and another with both exon 6 and exon 7 skipped. The consequences of both splicing patterns would introduce premature translational stop codon in the reading frame, thereby producing either nonfunctional protein or potentially dominant negative protein. And *CDH17* aberrant splicing was highly associated with tumor dissemination and shorter survival of HCC patients. It has been showed that aberrant mRNA splicing of the *CDH17* gene in liver tissues was triggered by the specific constellation of two *CDH17* single nucleotide polymorphisms (651T and IVS6 + 35G). And the functional T-G haplotype of *CDH17* (651C>T and IVS6 + 35A>G) is a genetic susceptibility factor for the development of HCC in a Chinese population^[33]. *CDH17* becomes an attractive target for HCC therapy^[16]. And it has been proposed that LI-cadherin is a useful immunohistochemical marker for diagnosis of adenocarcinomas of the digestive system^[25] and *CDH17* may be an oncogene up-regulating invasive feature of gastric cancer cells and could be a hopeful target for the control of gastric cancer progression^[34]. Moreover, it has been revealed that LI-cadherin participates in the multiple steps of invasion and metastasis in LoVo colorectal cancer cells^[35,36]. In our study we investigated the SNPs in c.343A>C and c.2216A>C polymorphism sites of *CDH17* gene. The c.343A>G polymorphism site mapped to the amino acid position 115 and was responsible for the amino acid change of 115 Glu with Lys. The c.2216A>C polymorphism site mapped to the amino acid position 739 and was responsible for the amino acid change of 739 Ala with Glu. We found that patients with colorectal carcinoma carrying the C allele of c.2216A>C tended to have a higher risk of lymph node metastasis and a higher TNM grade. The reason is possible that the SNPs of the c.2216A>C cause the amino acid change and result in the LI-cadherin expression and/or activity changes. The polymorphism of c.343A>G also causes the amino acid change. But there is no significant difference between c.343A>G SNPs and TNM grade as well as lymph node metastasis between the A and G alleles. It is possibly because that the *CDH17* mRNA is spliced with the polymorphism site skipped (exon 6).

A significant association was found between c.2216A>C SNPs of *CDH17* gene (rs1051624) and TNM grade as well as with lymph node status

In summary, in a Chinese Han colorectal carcinoma patient population of Henan province, a significant association was found between c.2216A>C SNPs of *CDH17* gene (rs1051624) and TNM grade as well as with lymph node status. With logistic regression analysis a correlation

between TNM grades as well as with lymph node metastasis and C/C genotype of *CDH17* gene c.2216A>C was found. And patients with colorectal carcinoma carrying the C allele tended to have a higher risk of lymph node metastasis and a higher TNM grade, suggesting that this polymorphism might be clinically important in prognosis. Validation and functional studies are needed to assess the significance of these associations in clinical practice.

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COMMENTS

Background

Colorectal carcinoma (CRC) has been the fourth place of common cancer incidence rate in the China. The etiology of CRC is multifactorial, involving hereditary causes, environmental factors, and somatogenic changes occurring during tumor progression. The single nucleotide polymorphisms (SNPs), as the third generation of heredity markers, are the most common type of genomic sequence variations, which are thought to be associated with population diversity, susceptibility to diseases, and individual response to drug treatments. It has become one of the hot spots in research. There have been cumulative studies on the associations between disease risk and SNPs in selected candidate genes. *CDH17* gene, also known as cadherin-17 and liver-intestine cadherin (LI-cadherin), is a member of the cadherin superfamily, genes encoding calcium-dependent, membrane-associated glycoproteins. The human *CDH17* gene located on chromosome 8q22.1 has eighteen exons and encodes LI-cadherin protein. There has been found that reduced expression of LI-cadherin protein is closely associated with tumor progression and lymph node metastasis of human colorectal carcinoma.

Research frontiers

At present, the research of relation between *CDH17* gene SNPs and diseases are relatively less at home and abroad. In this study, authors detected the genotype of the *CDH17* gene c.343A>G and c.2216A>C SNPs sites by the polymerase chain reaction-single strand conformation polymorphism and directed DNA sequencing in ninety-three colorectal carcinoma patients. The objective of this study is to analyze the phenotype and distribution frequencies of genotype and allele of the *CDH17* gene c.343A>G and c.2216A>C polymorphism sites and to evaluate the correlation between the two polymorphism sites of *CDH17* gene and tumor occurrence and development as well as clinicopathologic parameters of colorectal carcinoma.

Innovations and breakthroughs

There were no significant difference in gender, age, tumor location, tumor size, macroscopic appearance, histologic type, invasion depth, differentiation, hematogenous metastasis, and recurrence among the genotypes of *CDH17* gene c.343A>G. But there were a significant correlation between lymph node metastasis and Tumor-node-metastasis (TNM) grade and the genotype frequencies of it. Colorectal carcinoma patients carrying the G/G genotype have a higher risk of lymph node metastasis and a higher TNM grade compared with those carrying A/G genotype. There were no significant difference in gender, age, tumor location, tumor size, macroscopic appearance, histologic type, invasion

depth, differentiation, hematogenous metastasis, and recurrence among the genotypes of *CDH17* gene c.2216A>C. But there were a significant correlation between lymph node metastasis and TNM grade and the genotype frequencies of it. The C/C genotype and C allele of this SNPs site might be a risk factor for a higher risk of lymph node metastasis and a higher TNM grade of colorectal carcinoma patient.

Applications

The SNPs of *CDH17* gene c.2216 A>C might be clinically important in prognosis of colorectal carcinoma.

Peer review

The study analyzed polymorphisms of *CDH17* gene in blood samples collected from 93 colorectal carcinoma patients and found a strong association between c.2216A>C and TNM grade of the cancer, suggesting a potential value of using this gene as a prognostic marker of colorectal carcinoma. This is an interesting study.

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Notch2 regulates matrix metalloproteinase 9 via PI3K/AKT signaling in human gastric carcinoma cell MKN-45

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Abstract

AIM: To clarify the role of activated Notch2 in the invasiveness of gastric cancer.

METHODS: To investigate the invasiveness of silencing *Notch2* gene expression, we established a Notch2

small interfering RNA (siRNA) transfected cell line using the MKN-45 gastric cancer cell line. After the successful transfection confirmed by real-time reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting, migration and invasion assays were employed to evaluate the aggressiveness of the gastric cancer. RT-PCR and Western blottings were employed to confirm the down-regulation of Notch2 and to evaluate the expression of epithelial mesenchymal transition-related gene matrix metalloproteinase 9 (MMP9), Akt, p-Akt. To confirm the relationship between PI3K-Akt and MMP9, the PI3K inhibitor LY294002 was used to treat MKN-45 cells.

RESULTS: Notch2 expression was dramatically decreased after Notch2 siRNA transfection ($100.00\% \pm 9.74\%$ vs $11.61\% \pm 3.85\%$, $P < 0.01$ by qRT-PCR). There was also a marked reduction of Notch target gene *Hes1* ($100.00\% \pm 4.74\%$ vs $61.61\% \pm 3.58\%$, $P < 0.05$) at the mRNA, indicating an inhibition of Notch signaling. Inhibition of Notch signaling was also confirmed by the marked reduction of Notch2 intracellular domain at the protein levels ($100.00\% \pm 9.74\%$ vs $65.61\% \pm 7.58\%$, $P < 0.05$). Down-regulation of Notch2 by siRNA enhanced tumor cell invasion ($100.00\% \pm 21.64\%$ vs $162.22\% \pm 16.84\%$, $P < 0.05$) and expression of MMP9 (1.56 fold, $P < 0.05$), and activated the pro-MMP9 protein to its active form (1.48 fold, $P < 0.05$). There was no significant difference in the protein levels of Akt between the two groups ($100.00\% \pm 10.87\%$ vs $96.61\% \pm 7.33\%$, $P > 0.05$), while down-regulation of Notch2 elevated p-Akt expression ($100.00\% \pm 9.87\%$ vs $154.61\% \pm 13.10\%$, $P < 0.05$). Furthermore, p-Akt and MMP9 was down-regulated in response to the inhibitor LY294002 (p-Akt $100.00\% \pm 8.87\%$ vs $58.27\% \pm 5.01\%$, $P < 0.05$; MMP9 $100.00\% \pm 9.17\%$ vs $50.03\% \pm 4.88\%$, $P < 0.05$).

CONCLUSION: Notch2 may negatively regulate cell invasion by inhibiting the PI3K-Akt signaling pathway

in gastric cancer.

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Key words: Notch2; Stomach; Cancer; Invasion; Epithelial mesenchymal transition; Matrix metalloproteinase 9; RNA interference

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INTRODUCTION

Gastric carcinoma is one of the most common malignancies and one of the most important causes of the cancer-related death worldwide^[1]. Most of the current available therapeutic methods for advanced gastric cancer, such as chemotherapy and chemoembolization, are less optimal, thus the prognosis of gastric cancer is rather poor. This is largely attributed to a lack of complete understanding of the exact cause and mechanisms for this malignancy. Hence, identification of critical molecular pathways of gastric cancer development would greatly facilitate the development of more effective therapies.

Notch signaling pathway is involved in several cellular processes, such as proliferation, differentiation, apoptosis, cell fate decision, and maintenance of stem cells^[2-5]. It also plays an important role in the control of tumorigenesis. Activated Notch receptor can be oncogenic or tumor suppressive depending on the tumor type and cellular context^[6]. However, the mechanisms by which Notch signaling activates or suppresses tumorigenesis remain unclear. Recently, activation of Notch signaling pathway has been found to stimulate tumorigenesis *via* regulating epithelial mesenchymal transition (EMT)^[7].

EMT is a unique process by which epithelial cells undergo remarkable morphologic changes characterized by a transition from epithelial cobblestone phenotype to mesenchymal phenotype leading to increased motility and invasion^[4]. During the acquisition of EMT characteristics, epithelial cells lose cell-cell junction, which is associated with actin cytoskeleton reorganization, expression of proteins that promote cell-cell contact, and appearance of the expression of mesenchymal markers.

Recently, Notch signaling pathway was found to be involved in the acquisition of EMT in both physiological and pathological processes^[8]. However, how Notch signaling regulates EMT is largely unknown.

The Notch pathway includes a conserved family of transmembrane receptors (Notch1-4) that interact with a number of specific ligands (DLL1, DLL3, DLL4, Jagged1 and Jagged2) to regulate cell fate. Notch signaling initiates following the binding of the Notch ligands to the Notch receptors, causing an enzymatic cleavage of Notch receptors by γ -secretase to release the intracellular domain of the Notch receptor (NICD). NICD is the active form of Notch receptors which can translocate into the nucleus, where it assembles a large transcriptional activation complex that interacts with the conserved transcription factor CSL [CBF-1, Su (H) and Lag-2], and then activates the transcription of CSL-dependent downstream targets^[9]. Many target genes of Notch signaling have been identified in various cell contexts, but the Hairy/Enhancer of Split (Hes) family of basic helix-loop-helix (bHLH) proteins are believed to be the direct Notch targets, including Hes1 and Hey1. Among the Notch signaling genes, Notch2 appears to function as a biological antagonist for Notch1 in many cancers, such as breast cancer^[10], colorectal cancer^[11], malignant mesothelioma^[12], multiple myeloma^[13], and embryonal brain tumors^[14]. Although major advances have been made in the understanding of the opposite effects of Notch2 and Notch1 in cancer development, the exact molecular mechanisms underlying a biological interaction between Notch1 and Notch2 remain unclear, and few studies have been done on the possible relationship between Notch1 and Notch2 in gastric cancer.

Notch2 signal pathway plays a potential oncogenic role in several malignancies, such as hematologic malignancies including multiple myeloma^[15], B cell chronic lymphocytic leukemia, and B cell and T cell acute lymphoblastic leukemia^[16,17], and solid tumors including glioblastoma^[18], and colon cancer^[19]. It also plays a tumor suppressive role in some solid tumors, such as breast cancer^[20], and small cell lung cancer^[21,22]. In gastric cancer, Notch2 has been proved to be overexpressed by Sun *et al.*^[23] and He^[24].

In this study, we aimed to address whether Notch2 is also involved in control of gastric cancer progression and investigate the effects of Notch2 signaling on gastric cancer aggressiveness.

MATERIALS AND METHODS

Cell culture and transfections

Human gastric cancer cell line MKN-45 (Cell Collection of the Chinese Academy of Sciences, Shanghai, China) was cultured in RPMI 1640 (HyClone Laboratories Inc., Logan, United States) supplemented with 10% fetal bovine serum (HyClone Laboratories Inc., Logan, United States), 100 U/mL penicillin and streptomycin, in a 5% CO₂ atmosphere at 37 °C.

Small interfering RNA knockdown of Notch2

MKN-45 cells were transfected with small interfering RNA (siRNA) against Notch2 and scrambled siRNA (Santa Cruz Biotechnology, CA, United States) constructs using the commercial transfection reagent (Santa Cruz Biotechnology, CA, United States) according to the manufacturer's instructions. Following transfection, cells were incubated at 37 °C in a CO₂ incubator for 48 h before being harvested for the assays described below.

Real-time quantitative reverse transcription-polymerase chain reaction analysis for gene expression

Total RNA was isolated by the RNAiso plus reagent (TaKaRa Biotechnology Co., Dalian, China) and then reverse transcribed into complementary DNA (cDNA) using the Primescript™ reverse transcription Master Mix (TaKaRa Biotechnology Co., Dalian, China) according to manufacturer's instructions. Reverse transcription reaction was performed at 37 °C for 15 min followed by 85 °C for 5 s. The primers used in the polymerase chain reaction (PCR) reactions are described in Table 1. One mL of reverse transcription reaction product was used for quantitative reverse transcription-PCR (qPCR) reaction in a total volume of 20 µL. The qPCR cycles were as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. PCR amplifications were undertaken with the Applied Biosystems 7500/7500 Fast Real-Time PCR Software (Applied Biosystems, CA, United States) using the SYBR® Premix Ex Taq™ II (TaKaRa Biotechnology Co., Dalian, China). Data were analyzed according to the comparative Ct method and were normalized to glyceraldehyde-3-phosphate dehydrogenase expression in each sample. All qPCR assays were performed in triplicate.

Protein extraction and Western blotting analysis

Total protein was extracted from the treated cells using RIPA lysis buffer (Beyotime Biotechnology, Haimen, China) supplemented with 1 mmol/L phenylmethanesulfonyl fluoride. The protein concentration was measured by BCA protein assay system (Beyotime Biotechnology, Haimen, China). Total proteins (40-50 µg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes. The blots were blocked with 5% skim milk in Tris buffered saline containing 0.1% Tween-20 (TBST) for 2 h at room temperature, incubated with primary antibodies against N2ICD (Abcam Inc., Cambridge, United Kingdom) (1:1000), Hes1 (Abcam Inc., Cambridge, United Kingdom) (1:500), matrix metalloproteinase 9 (MMP9) (Abcam Inc., Cambridge, United Kingdom) (1:1000), Akt (Cell Signaling Technology, Inc., Danvers, MA, United States) (1:1000), and p-Akt (Cell Signaling Technology, Inc., Danvers, MA, United States) (1:1000). β-actin (Zhongshan Golden Bridge Biotech, Beijing, China) (1:10 000) was used as a loading control. The membranes were reacted with respective primary antibodies overnight at 4 °C. After being washed in TBST

Table 1 Primer sequences used for the real-time polymerase chain reaction analysis

Primer	Sense (5'-3')	Anti-sense (5'-3')
Notch2	CCTGGGCTATACT- GGGAGCTACTG	ACACCTGATACCCCTGGGA- CAC
MMP9	ACGCACGACGTCCTC- CAGTA	CCACCTGGTTCAACTCACTCC
Hes1	AGCGGGCGCAGATGAC	CGTTTCATGCCTCGCTGAA
GAPDH	GCACCGTCAAGGCT- GAGAAC	TGGTGAAGACGCCAGTGA

MMP9: Matrix metalloproteinase 9; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

for three times, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Zhongshan Golden Bridge Biotech, Beijing, China) (1:10 000) for 1 h at room temperature. The protein bands were detected using the Super Signal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., Rockford, IL, United States) and imaged using a VersaDoc Imaging System (Bio-Rad Laboratories Co., Ltd. Hercules, CA, United States). Densitometric analysis was performed using Quantity One Software v4.62 (Bio-Rad Laboratories Co., Ltd. Hercules, CA, United States) and the results were presented as the mean of three independent experiments.

Migration and invasion assays in vitro

The effects of Notch2 siRNA on the migratory and invasive abilities of MKN-45 cells were assayed in 24-well plates using relevant kits (BD Biosciences, United States). Approximately 3×10^4 cells were seeded for the 12 h migration assay, and 3×10^5 cells for the 24 h invasion assay and the invasive activity of the Notch-2 siRNA-transfected MKN-45 cells was tested using BD Falcon™ Cell culture inserts coated with BD Matrigel™ Basement Membrane Matrix (BD Biosciences, United States). Briefly, transfected MKN-45 cells were resuspended in serum-free medium and seeded into the upper chamber of the assay system. The bottom wells of the system were filled with complete growth medium. After 12 and 24 h incubation, the migrated and invaded cells were washed twice with ice-cold PBS and then fixed with 4% paraformaldehyde for 15 min and stained with methyl violet (0.01% v/v) for 30 min. The numbers of migrated or invaded cells were then counted from 5 random fields under 200 and 400 magnification.

MMP9 activity assay

The culture media from Notch2 siRNA- and scrambled siRNA-transfected MKN-45 cells grown in 6-well plates were collected, spun at $12\ 000 \times g$ for 10 min at 4 °C to remove cell debris, and the supernatant collected for MMP9 assay using a commercial enzyme-linked immunosorbent assay kits (RD Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions.

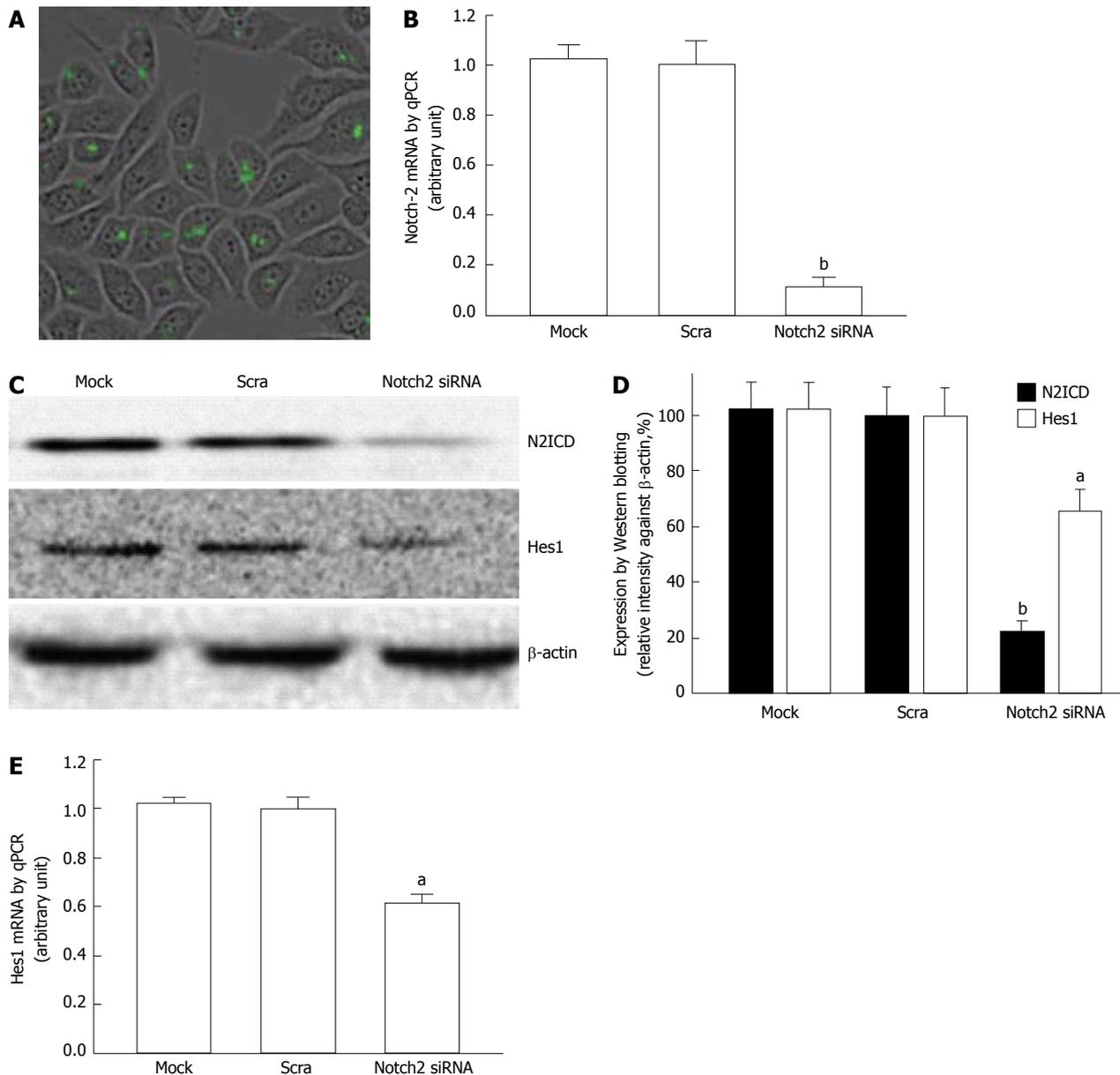


Figure 1 Verification of successful transfection and knockdown of Notch2. ^a $P < 0.05$, ^b $P < 0.01$ vs the Mock or Scra groups. Scra: Scrambled small interfering RNA (siRNA); qPCR: Quantitative reverse transcription polymerase chain reaction.

Statistical analysis

Data analysis was performed using SPSS19.0 (IBM, Armonk, New York, United States) and displayed using Sigma Plot10.0 (Systat Software Inc, San Jose, CA, United States). Comparison of the differences between the groups was performed using a one-way analysis of variance followed by the Bonferroni correction. All data were expressed as the mean \pm SD. A P value of < 0.05 was considered statistically significant.

RESULTS

Knockdown of Notch2 enhanced the migration and invasion of MKN-45 cells

After successful transfection of Notch2 siRNA into MKN-45 cells (Figure 1A) and a marked knockdown of Notch2

($> 90\%$) at mRNA (Figure 1B) and protein (Figure 1C and D) levels, there was a marked reduction of Notch target gene Hes1 at the mRNA (Figure 1E), indicating an inhibition of Notch signaling. Inhibition of Notch signaling was also confirmed by the marked reduction of Notch2 intracellular domain (N2ICD) at the mRNA (Figure 1D) and protein (Figure 1C) levels.

In the cells with confirmed knockdown of Notch2, the ability of cells to migrate and invade was evaluated as described in the "Materials and Methods". MKN-45 cells with Notch2 knockdown showed an increased cell migration (Figure 2B and E) compared with the cells transfected with scrambled siRNA (Scra, Figure 2A and E). Similarly, MKN-45 cells with Notch2 knockdown showed an increased cell invasion (Figure 2D and F) compared with the cells transfected with scrambled siRNA (Scra,

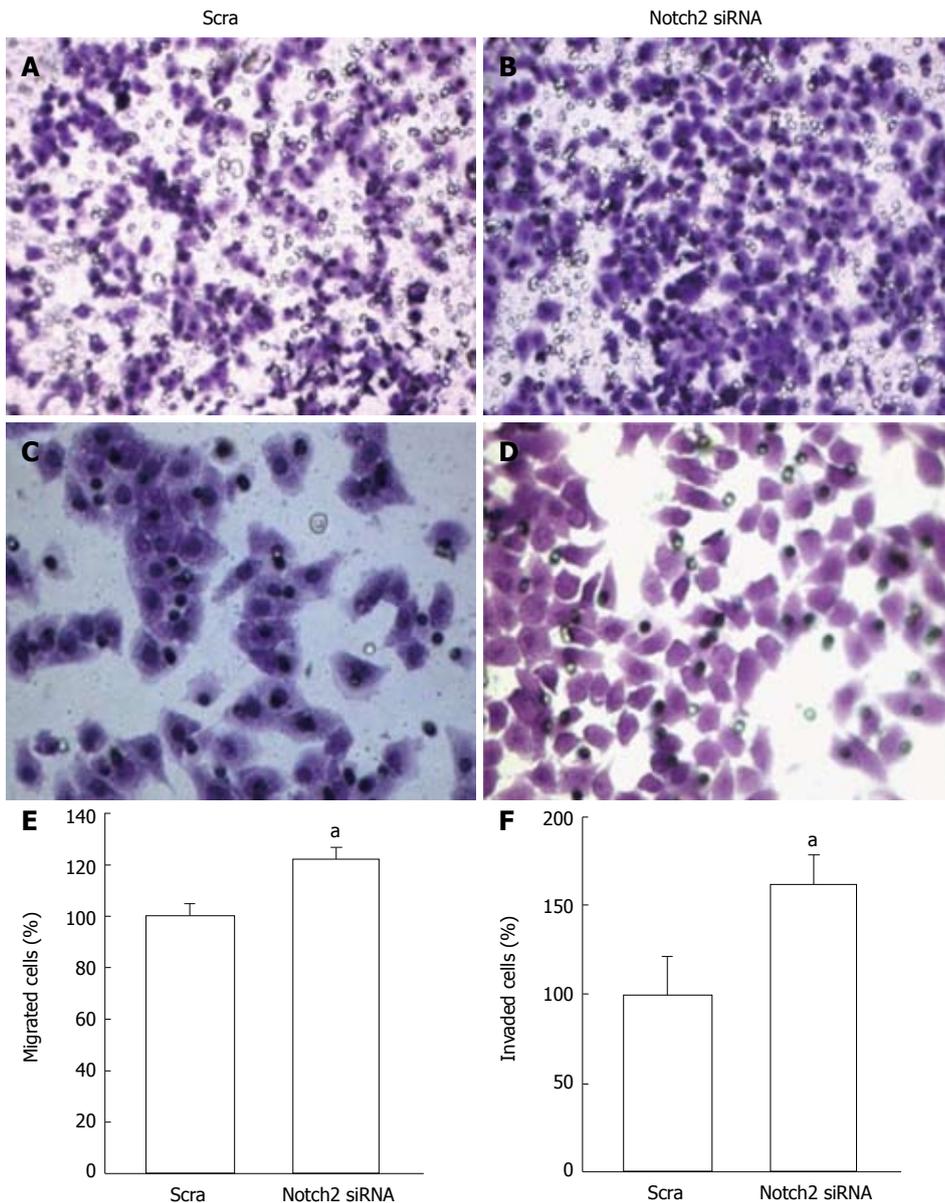


Figure 2 Knockdown of Notch2 led to an increased migration and invasion of MKN-45 cells. Cells were transfected with scrambled small interfering RNA (siRNA) (Scra, A, C) or Notch2 siRNA (B, D) for 48 h, and the effect of the migration (A, B, E) and invasion (C, D, F) were assayed as described in "Materials and Methods". The number of migrated cells or invaded cells were quantitated (E, F). ^a*P* < 0.05 vs the Scra groups. A, B, ×200; C, D, ×400.

Figure 2C and F).

Knockdown of Notch2 enhanced the expression and activity of MMP9 in MKN-45 cells

Tumor metastasis occurs by a series of steps, including cell invasion, degradation of basement membranes, and the stromal extracellular matrix, ultimately leading to tumor cell invasion and metastasis. The MMPs are a family of related enzymes that degrade extracellular matrix, which are considered to be important factors in facilitating tumor invasion and metastasis. Among these MMPs, MMP9 is considered an important factor involved in the degradation of basement membrane collagen in facilitating invasion and metastases in gastric cancer. Knockdown of Notch2 in MKN-45 cells markedly enhanced the expression of MMP9 at mRNA (Figure 3A) and protein (Figure 3B and C) levels. Additionally, knockdown of Notch2 led to a 1.48-fold increase in MMP9 activity (Figure 3D).

Effect of Notch2 knockdown on the expression of PI3K/Akt pathway in MKN-45 cells

In order to elucidate the mechanisms of Notch2 mediated alteration in MMP9, we measured the expression of PI3K downstream target Akt in the MKN-45 cells transfected with or without Notch2 siRNA. Knockdown of Notch2 by siRNA increased the Akt phosphorylation (Figure 4A and B). Blocking the PI3K/Akt pathway by PI3K inhibitor LY294002 resulted in a reduced expression of MMP9 (Figure 4C and D).

DISCUSSION

Aberrant expression of Notch pathway has been found in a variety of human cancers, including cancers of breast, brain, cervix, lung, colon, head and neck, kidney, bone marrow, lymph nodes and stomach^[25-27]. Abnormal Notch signaling is also linked to EMT. Notch signaling is known to suppress apoptosis and promote cell

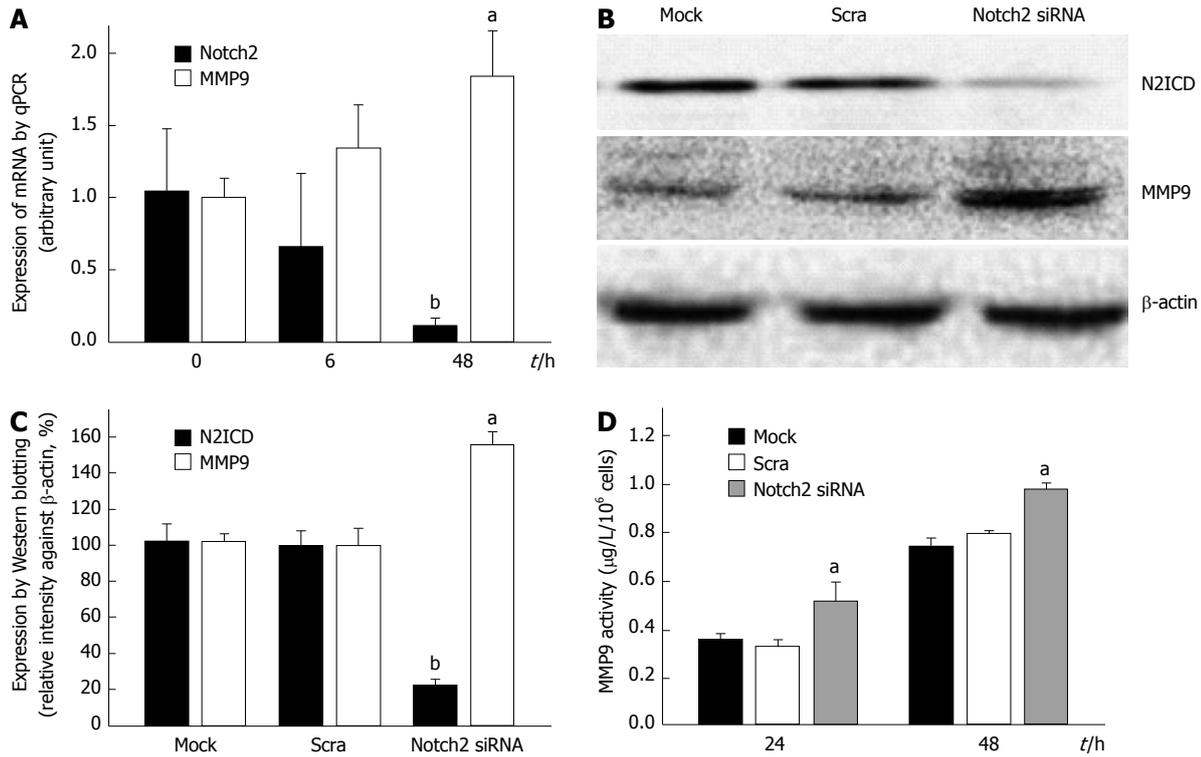


Figure 3 Knockdown of Notch2 enhanced the expression and activity of matrix metalloproteinase 9. The small interfering RNA (siRNA) mediated knockdown of Notch2 (A) and Notch2 intracellular domain (N2ICD) (B, C) was associated with a marked increase in the expression (A, B, C) and activity (D) of matrix metalloproteinase 9 (MMP9). ^a*P* < 0.05, ^b*P* < 0.01 vs the blank control groups. qPCR: Quantitative reverse transcription polymerase chain reaction.

proliferation through a growth factor-mediated survival pathway^[28-30]. However, the precise role and mechanism of Notch for tumor invasion remains unclear. In this study, we found that siRNA mediated down-regulation of Notch2 in gastric cancer cells could (1) increase tumor cell invasion; (2) enhance MMP9 expression and its activities; and (3) promote the phosphorylation of PI3K pathway as demonstrated by increased p-Akt level.

Tumor metastasis occurs *via* a series of steps, including cell invasion, degradation of basement membranes and the stromal extracellular matrix, ultimately leading to tumor cell invasion and metastasis. The MMPs are a family of related enzymes that degrade extracellular matrix, which are considered to be important factors in facilitating tumor invasion and metastasis^[31-33]. Among these MMPs, MMP9 has been considered an important factor involved in the degradation of basement membrane collagen in facilitating invasion and metastases in gastric cancer^[34,35]. MMP9 is a downstream target for PI3K/Akt pathway, which is an important signaling pathway in controlling cell proliferation^[36,37]. In physiological circumstances, MMP9 plays an important role in tissue remodeling associated with various physiological and pathological processes such as morphogenesis, angiogenesis, tissue repair, cirrhosis, arthritis, and metastasis^[38]. MMP9 is required for maintaining normal tissue structure and epithelial integrity. Under pathological conditions, particularly in various cancers, abnormal expression and activity of MMP9 have been reported^[34,39-42]. Abnormal function of MMP9 is linked to tumor cell migration, invasion and metastasis^[35,39]. The

role of MMP9 in the development of gastric cancer has been reported and the expression of metalloproteinase-9 or its inhibitor is related to a more aggressive phenotype of gastric cancer or correlated with lymph node metastasis in advanced gastric carcinoma^[35,43], but how MMP9 is regulated in gastric cancer is unclear.

Based on our study, we propose that physiological cellular level of Notch2 may be required for the maintenance of normal MMP9 function. Reduced Notch2 may enhance the proliferative and invasive potential of cancer cells, likely through activation of PI3K/Akt pathway and ensuing increase in MMP9 activities. In this perspective, Notch2 appears to function as a tumor suppressor gene in gastric cancer.

Here, we showed that the facilitation of MMP9 expression by down-regulation of Notch2 may be mediated by the up-regulation of p-Akt. Thus, these results suggest that up-regulation of Notch2 could potentiate the anti-tumor and anti-metastasis activities partly through the up-regulation of MMP expression. Because we observed that down-regulation of Notch2 promoted MMP9 expression, we tested the effects of Notch2 on the invasion of MKN-45 cells. We found that down-regulation of Notch2 promoted the invasion of MKN-45 cells. These results were consistent with MMP9 data, showing that down-regulation of Notch2 could promote cancer cell invasion partly through up-regulation of MMP9. On the basis of our results, we propose a hypothetical pathway by which Notch2 may inhibit invasion of MKN-45 cells, partly through PI3K-Akt signaling pathway.

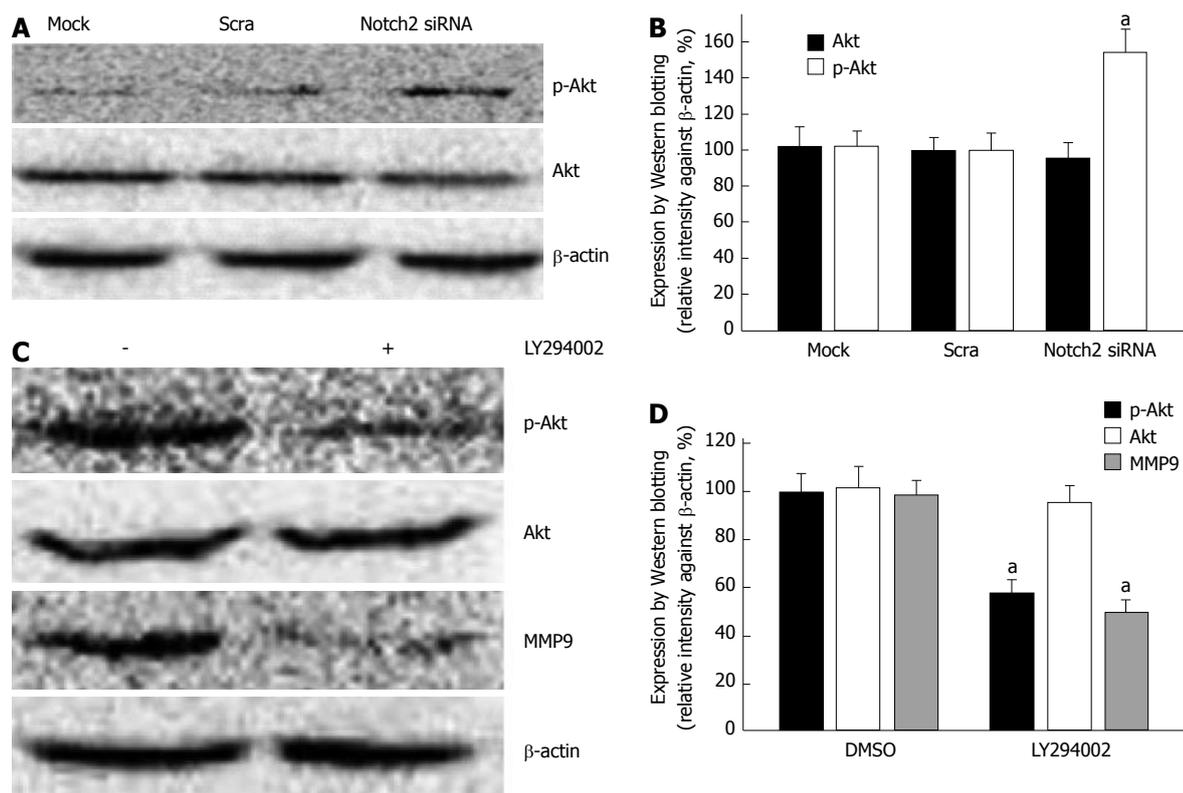


Figure 4 Knockdown of Notch2 enhanced the expression of matrix metalloproteinase 9 via increased phosphorylation of p-Akt in MKN-45 cells. A, B: Knockdown of Notch2 led to an increased phosphorylation of Akt (p-Akt); C, D: Blockade of PI3K/Akt pathway by LY294002 (20 μmol/L) abolished the effect of Notch small interfering RNA (siRNA) in Akt phosphorylation and matrix metalloproteinase 9 (MMP9). ^a*P* < 0.05 vs the all control groups. DMSO: Dimethyl sulfoxide.

Notch has been reported to cross-talk with other major cell growth and apoptotic regulatory pathways, including the PI3K-Akt pathway^[44]. Hyperactivation of PI3K/Akt pathway has previously been observed in human gastric cancer^[45]. It has recently been shown that activation of Notch1 enhanced the survival of melanoma cells^[29] and leukemia cells *via* activation of the PI3/Akt pathway^[46]. In our study, down-regulation of Notch2 by siRNA led to the activation of PI3K/Akt pathway, which is associated with an increased expression and function of MMP9, suggesting that Notch2 can regulate MMP9 *via* PI3K/Akt pathway and increased Akt phosphorylation. Interestingly, we also observed that inactivation of Akt by LY294002 eliminated Akt phosphorylation and MMP9 expression. These results suggest that Notch2 can induce Akt signaling.

In summary, the role of Notch2 in malignancies is uncertain. Although the overexpression of Notch2 has been confirmed, Notch2 appeared to function as a tumor suppressor gene in gastric cancer in this study. Further studies are warranted before Notch inhibitor based therapeutic approaches are employed in the treatment of advanced gastric cancer.

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COMMENTS

Background

Notch is one of the most important signaling pathways involved in cell fate determination. Activation of the Notch pathway requires the binding of a membrane-bound ligand to the Notch receptor in the adjacent cell which induces proteolytic cleavages and the activation of the receptor. A unique feature of the Notch signaling is that the processes such as modification, endocytosis or recycling of the ligand, have been reported to play critical roles in Notch signaling, however, the underlying molecular mechanism appears context-dependent and often controversial.

Research frontiers

There are four Notch receptors (Notch 1-4) and five ligands [Jagged 1, Jagged 2, delta-like ligand-1, -3 and -4 (DLL1, DLL3 and DLL4)] in mammals. Recently, it is reported to be involved in tumorigenesis as oncogenes or tumor suppressors, and proposed as prognostic factors or anti-cancer targets in aggressive or advanced cancers. This study was undertaken to investigate whether Notch2 is also involved in the control of gastric cancer progression, and the effects of Notch2 signaling in gastric cancer aggressiveness.

Innovations and breakthroughs

Abnormal Notch signaling has been reported in many human solid tumors. This is the first study to characterize the role of Notch signaling in gastric cancer aggressiveness. The findings indicated that Notch2 may negatively regulate the cell invasion of human gastric carcinoma.

Terminology

Four Notch receptors (Notch 1-4) and five ligands (Jagged 1, Jagged 2, DLL1, DLL3, and DLL4) are found in mammals. Ligand-receptor interaction between two neighboring cells is involved in developmental, physiologic and pathologic processes.

Peer review

The Notch signaling pathway plays a crucial role in the maintenance and the development of several tissues. Ectopic expression of Notch has been found in a variety of human cancers. In this work, the authors indicate that Notch2 could negatively regulate the cell invasion of human gastric carcinoma. By this way, the authors described Notch2 as a tumor suppressor gene in gastric cancers. At the same time, the authors detected an increased expression and activity of matrix metalloproteinase 9, arguing that such increase could be related to the enhanced migration and invasiveness. The results are clear and support the authors' hypothesis.

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Impact of intestinal ischemia/reperfusion and lymph drainage on distant organs in rats

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Abstract

AIM: To investigate the impact of intestinal ischemia/reperfusion (I/R) injury and lymph drainage on distant organs in rats.

METHODS: Thirty-two Sprague-Dawley male rats, weighing 280-320 g, were randomly divided into blank, sham, I/R, and ischemia/reperfusion and drainage (I/R + D) groups ($n = 8$). All rats were subjected to 60 min ischemia by clamping the superior mesenteric artery, followed by 120 min reperfusion. The rats in the I/R + D group received intestinal lymph drainage for 180 min. In the sham group, the abdominal cavity was opened for 180 min, but the rats received no treatment. The blank group served as a normal and untreated control. A chromogenic limulus assay kit was used for quantitative

detection of serum endotoxin. The serum concentrations of tumor necrosis factor- α (TNF- α), interleukin (IL)-6, IL-1 β , soluble cell adhesion molecules (sICAM-1), and high mobility group protein box 1 (HMGB1) were determined with an enzyme-linked immunosorbent assay kit. Histological evaluations of the intestine, liver, kidney, and lung were performed by hematoxylin and eosin staining and immunohistochemistry. HMGB1 protein expression was assayed by western blot analysis.

RESULTS: The serum levels of endotoxin and HMGB1 in the I/R and I/R + D groups were significantly higher than those in the sham group (endotoxin, I/R and I/R + D vs sham: 0.033 ± 0.004 EU/mL, 0.024 ± 0.003 EU/mL vs 0.017 ± 0.009 EU/mL, respectively, $P < 0.05$; HMGB1, I/R and I/R + D vs sham: 5.473 ± 0.963 EU/mL, 4.906 ± 0.552 EU/mL vs 0.476 ± 0.406 EU/mL, respectively, $P < 0.05$). In addition, endotoxin and HMGB1 were significantly lower in the I/R + D group compared to the I/R group ($P < 0.05$). The serum inflammatory factors IL-6, IL-1 β , and sICAM-1 in the I/R and I/R + D groups were significantly higher than those in the sham group (IL-6, I/R and I/R + D vs sham: 41.773 ± 9.753 pg/mL, 19.204 ± 4.136 pg/mL vs 11.566 ± 2.973 pg/mL, respectively, $P < 0.05$; IL-1 β , I/R and I/R + D vs sham: 144.646 ± 29.378 pg/mL, 65.829 ± 10.888 pg/mL vs 38.178 ± 7.157 pg/mL, respectively, $P < 0.05$; sICAM-1, I/R and I/R + D vs sham: 97.360 ± 12.714 ng/mL, 48.401 ± 6.547 ng/mL vs 33.073 ± 5.957 ng/mL, respectively; $P < 0.05$). The serum TNF- α in the I/R group were significantly higher than in the sham group (45.863 ± 11.553 pg/mL vs 18.863 ± 6.679 pg/mL, respectively, $P < 0.05$). These factors were significantly lower in the I/R + D group compared to the I/R group ($P < 0.05$). The HMGB1 immunohistochemical staining results showed no staining or apparent injury in the blank group, and slight staining at the top of the microvillus was detected in the sham group. In the I/R group, both the top of villi and the basement membrane were stained for HMGB1 in most areas, and injury in the I/R + D group was less than that in the I/R group. HMGB1 expression in the liver, kidney,

and lung of rats in the I/R + D group was significantly lower than the rats in the I/R group ($P < 0.05$).

CONCLUSION: Lymph drainage could block the “gut-lymph” pathway, improve intestinal barrier function, and attenuate distant organ injury incurred by intestinal I/R.

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Key words: Intestinal ischemia/reperfusion; Lymph drainage; Distant organ injury; High mobility group protein box 1; Endotoxin; Cytokines

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INTRODUCTION

The gut is an important functional organ for the immune and endocrine systems, as well as its role as a protective barrier. Intestinal ischemia/reperfusion (I/R) injury is the “motor” of systemic inflammatory response syndrome (SIRS), acute respiratory distress syndrome (ARDS), and multiple organ dysfunction syndrome (MODS)^[1-3]. Severe trauma, acute necrotizing pancreatitis, major surgery, extensive burns, and other stresses are all associated with intestinal barrier dysfunction. Although extensive investigative efforts have focused on clarifying the pathogenesis of SIRS, ARDS and MODS induced by intestinal I/R, the specific mechanism still remains controversial^[4].

Recently, numerous studies have shown that the transport of inflammatory mediators occurs through the intestinal lymphatics in trauma-hemorrhage shock (T/HS). Deitch *et al.*^[5] demonstrated that toxic gut-derived substances enter the mesenteric lymph to cause lung injury. In addition, ligating the lymph duct in a variety of species after hemorrhagic shock can prevent distant organ injury^[6]. Damle *et al.*^[7], Watkins *et al.*^[8] and Jordan *et al.*^[9] have shown that lymph is the key link between T/HS and MODS. The production and release of inflammatory factors through the “gut-lymph” pathway to the circulatory system can cause acute lung injury (ALI) and a systemic inflammation state^[10-13]. Our recent work, together with findings by other investigators, suggests that thoracic/mesenteric lymphatic duct ligation prior to intestinal I/R injury protects the lung from injury and modulates the serum levels of endotoxin, D-lactate, diamine oxidase, and cytokines^[14,15]. However, the composition of lymph that is responsible for distant organ injury remains unknown.

Some experiments have demonstrated that T/HS lymph

is sterile and does not contain measurable levels of endotoxin^[16], but does contain some biologically active non-microbial protein and lipid species^[17,18]. Recent studies proposed that mesenteric lymph-induced distant organ injury in intestinal I/R was directly mediated by gut-derived endogenous ligands, one of which is the high mobility group protein box 1 (HMGB1). In intestinal I/R injury, intestinal epithelium cells and macrophages synthesize and release toll-like receptor 4 (TLR4) endogenous ligands, which can be recognized by and combine with TLR4^[19]. HMGB1 is also the key factor of inflammation of aseptic injury (including T/HS and liver I/R injury)^[20,21]. In liver I/R injury, HMGB1 can directly combine with TLR2 or TLR4 and cause inflammation^[22]. The binding of HMGB1 and TLR4 may also be the most important trigger of the inflammatory response to I/R injury in the heart, kidney, brain, lung, and other organs^[23-26]. Recent studies suggest that HMGB1 acts as a late mediator of lethal sepsis and an early mediator of inflammation and necrosis following I/R injury^[19,27,28].

This study is a continuation of our previous study. We hypothesized that the HMGB1-TLR4 combination plays an important role in the distant organ injury caused by intestinal I/R injury. The purpose of this study was to determine the impact of intestinal I/R injury and lymph drainage on the intestine and distant organs in rats, and to clarify whether HMGB1 in the intestinal mesenteric lymph after I/R mediates distant organ injury.

MATERIALS AND METHODS

Animals

Thirty-two male Sprague-Dawley rats that were specific pathogen-free grade and weighed 280-320 g were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. The rats were housed under barrier-sustained conditions at a temperature of 25 °C with 12 h light/dark cycles, and had free access to water and food for five days prior to the operation. The rats were randomly divided into four groups: blank, sham, ischemia/reperfusion and drainage (I/R + D), and I/R ($n = 8$ for each group). All rats were maintained in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals. The research protocols were approved by the Academic Committee of Peking Union Medical College and Chinese Academy of Medical Sciences.

Intestinal I/R and lymph drainage

Prior to the operation, all rats were fasted overnight, but were allowed access to water *ad libitum*. The rats were anesthetized with an intraperitoneal injection of 1% sodium pentobarbital (50 mg/kg). A midline incision was performed to bluntly separate the superior mesenteric artery (SMA) and intestinal lymphatic trunk. In the I/R and I/R + D groups, the SMA was occluded for 60 min with an artery clamp, followed by reperfusion for 120 min. In the I/R + D group, a small incision was made on the proximal end of the intestinal lymphatic trunk. A catheter (Jinan Medical Silicone Tube Plant, China)

Table 1 Serum levels of endotoxin and high mobility group protein box 1 in the ischemia/reperfusion and ischemia/reperfusion + drainage groups compared to respective controls (mean \pm SD, $n = 8$)

Groups	Endotoxin (EU/mL)	HMGB1 (ng/mL)
Blank	0.014 \pm 0.005	0.277 \pm 0.292
Sham	0.017 \pm 0.009 ^a	0.476 \pm 0.406
I/R + D	0.024 \pm 0.003 ^{a,c,e}	4.906 \pm 0.552 ^{a,c}
I/R	0.033 \pm 0.004 ^{a,c}	5.473 \pm 0.963 ^{a,c}

^a $P < 0.05$ vs the blank group; ^c $P < 0.05$ vs the sham group; ^e $P < 0.05$ vs the I/R group. I/R: Ischemia/reperfusion; I/R + D: Ischemia/reperfusion + drainage; HMGB1: High mobility group protein box 1.

was inserted into the incision obliquely 3-5 mm toward the distal end. A small amount of medical adhesive (Beijing Fuaille Science and Technology Development Co. Ltd, China) was used on the serosa adjacent to the right kidney to fix the catheter. Outflow of lymph from the catheter was collected with a sterile test-tube (Nunc, Denmark) for 180 min. In the sham group, the abdominal cavity was opened for 180 min but the rats received no treatment. The blank group served as a normal and untreated control. The lymph (0.6-1.2 mL per rat) was collected for 180 min.

Specimen collection

After the operation, the catheter was removed. Blood was then extracted from the inferior vena cava and centrifuged at 3000 g for 15 min at 4 $^{\circ}C$; the serum was separated and stored at -80 $^{\circ}C$ for further analysis. After the rats were fully exsanguinated, a 3 cm proximal section of the jejunum and 3 cm distal section of the ileum were excised, rinsed in ice-cold normal saline, and dried on filter paper. The liver, kidney, and lung were stored at -80 $^{\circ}C$.

Sample analysis

Measurement of endotoxin: A chromogenic limulus assay kit (Yi Hua Medical Technology Co. Ltd, Shanghai, China) was used for the quantitative detection of serum endotoxin, and the assay was performed according to the manufacturer's directions.

Cytokines and HMGB1 assay: The serum concentrations of tumor necrosis factor- α (TNF- α), interleukin (IL)-6, IL-1 β , soluble cell adhesion molecules (sICAM-1), and HMGB1 were determined using enzyme-linked immunosorbent assay kits (Sun Biomedical Technology Co., Ltd., Beijing, China) according to the kit protocols.

Hematoxylin and eosin staining of rats tissue slices: Samples of the intestine, liver, kidney, and lung were fixed in 10% formalin solution and sectioned (4 mm) after dehydration, cleaning, and paraffin embedding. The sections were flattened, mounted, and heated on blank glass slides. Histological evaluations were performed by hematoxylin and eosin staining and pathological examination.

HMGB1 immunohistochemistry: The slices of intes-

tine, liver, kidney, and lung embedded in paraffin were used for histological examination. A mouse anti-HMGB1 primary antibody (Beijing Biosynthesis Biotechnology Co., Ltd., China) and biotinylated secondary antibody (Beijing Biosynthesis Biotechnology Co., Ltd., China) were used for immunohistochemical staining. Brownish-yellow stained areas were recognized as regions with positive antigen expression.

HMGB1 protein expression-Western blotting analysis:

Total protein extract was prepared, and samples were separated using sodium dodecyl sulfate polyacrylamide gels. Proteins were then transferred to nitrocellulose membranes overnight at room temperature and blocked for 8 h with 5% bovine serum albumin. The membranes were then incubated overnight in anti-HMGB1 primary antibody (1 μ g/mL, ABCAM Ltd, Cambridge, United Kingdom) diluted in blocking solution (1:500, Beijing Biosynthesis Biotechnology Co., Ltd., China). Membranes were washed in Tris-buffered saline with Tween and incubated in horseradish peroxidase-conjugated mouse secondary antibodies in 5% milk (1:3000, Santa Cruz Inc., United States) for 1 h at room temperature. Protein bands were visualized by chemiluminescence.

Statistical analysis

Quantitative data were presented as mean \pm SD. Statistical software SPSS 17.0 (SPSS, Inc., Chicago, IL, United States) was used to test the homogeneity of variance. Multiple comparisons were performed with one-way analysis of variance followed by a least-significant difference test. Statistical significance was set at $P < 0.05$.

RESULTS

Serum levels of HMGB1, endotoxin and inflammatory factors

The serum levels of HMGB1 and endotoxin in the I/R and I/R + D groups were significantly higher than those in the blank and sham groups ($P < 0.05$). In addition, the level of endotoxin in the I/R group was higher than that in the I/R + D group ($P < 0.05$). The level of HMGB1 in the I/R group was slightly higher than that in the I/R + D group, but the difference was not significant ($P > 0.05$) (Table 1).

The levels of inflammatory cytokines IL-6, IL-1 β and sICAM-1 in the I/R and I/R + D groups and TNF- α in the I/R group were remarkably higher than those in the blank and sham groups ($P < 0.05$). The levels of inflammatory factors in the I/R + D group were markedly lower compared to those in the I/R group ($P < 0.05$). There was no significant difference in cytokine levels between the blank and sham groups ($P > 0.05$) (Table 2).

Intestinal, liver, kidney and lung HMGB1 immunohistochemistry

Intestinal morphology showed little change in the sham group compared to the blank group. In contrast, the jeju-

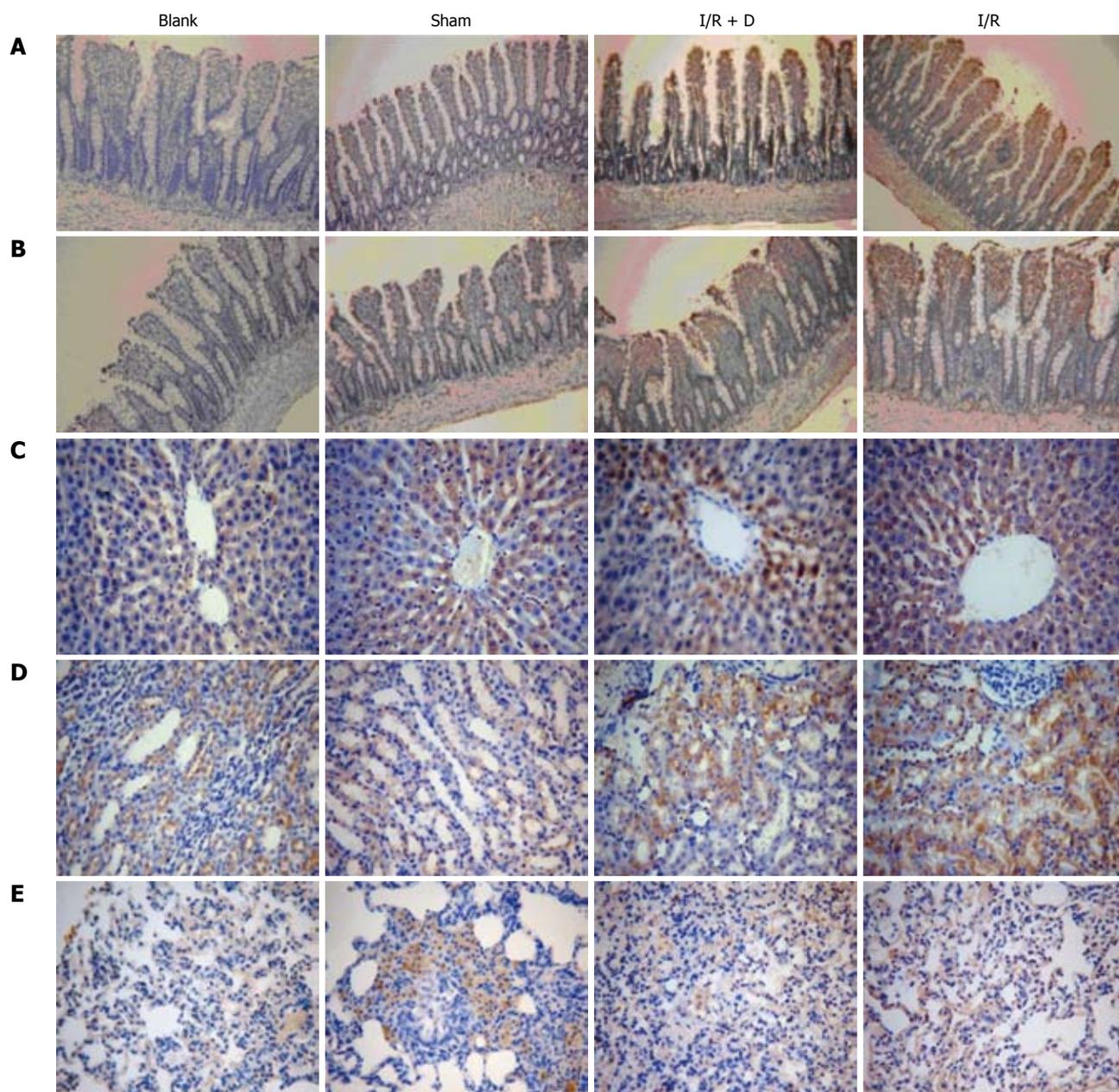


Figure 1 Immunohistochemistry staining of high mobility group protein box 1 in the jejunum (A), ileum (B), liver (C), kidney (D) and lung (E) (n = 8). I/R + D: Ischemia/reperfusion and drainage; I/R: Ischemia/reperfusion. Images shown at 100× magnification (A and B) and 200× magnification (C, D and E).

Table 2 Serum levels of cytokines in the ischemia/reperfusion and ischemia/reperfusion + drainage groups compared to respective controls (mean ± SD, n = 8)

Groups	TNF-α (pg/mL)	IL-1β (pg/mL)	IL-6 (pg/mL)	sICAM-1 (ng/mL)
Blank	13.799 ± 6.456	22.476 ± 8.498	8.687 ± 0.761	30.901 ± 6.962
Sham	18.863 ± 6.679	38.178 ± 7.157	11.566 ± 2.973	33.073 ± 5.957
I/R + D	25.381 ± 9.281 ^{a,c}	65.829 ± 10.888 ^{a,c,e}	19.204 ± 4.136 ^{a,c,e}	48.401 ± 6.547 ^{a,c,e}
I/R	45.863 ± 11.553 ^{a,c}	144.646 ± 29.378 ^{a,c}	41.773 ± 9.753 ^{a,c}	97.360 ± 12.714 ^{a,c}

^aP < 0.05 vs the blank group; ^cP < 0.05 vs the sham group; ^eP < 0.05 vs the I/R group. I/R: Ischemia/reperfusion; I/R + D: Ischemia/reperfusion + drainage; TNF: Tumor necrosis factor; IL: Interleukin; sICAM: Soluble cell adhesion molecule.

num and ileum mucosa in the I/R group showed swelling and atrophy, and appeared fragile and black in color in some segments of the intestine. In the I/R + D group, the intestinal mucosa showed slight swelling, no breakage, and less apparent damage than the I/R group.

Analysis of the jejunum and ileum in the blank group confirmed that there was no HMGB1 staining or apparent injury. A small amount of staining at the top of the microvillus was detected in the sham group, indicating slight injury. In the I/R group, both the top of villi and the basement membrane were stained for HMGB1 in most areas. Injury in the I/R + D group was less than that in the I/R group, although we did find some areas showing positive staining (Figure 1A and B).

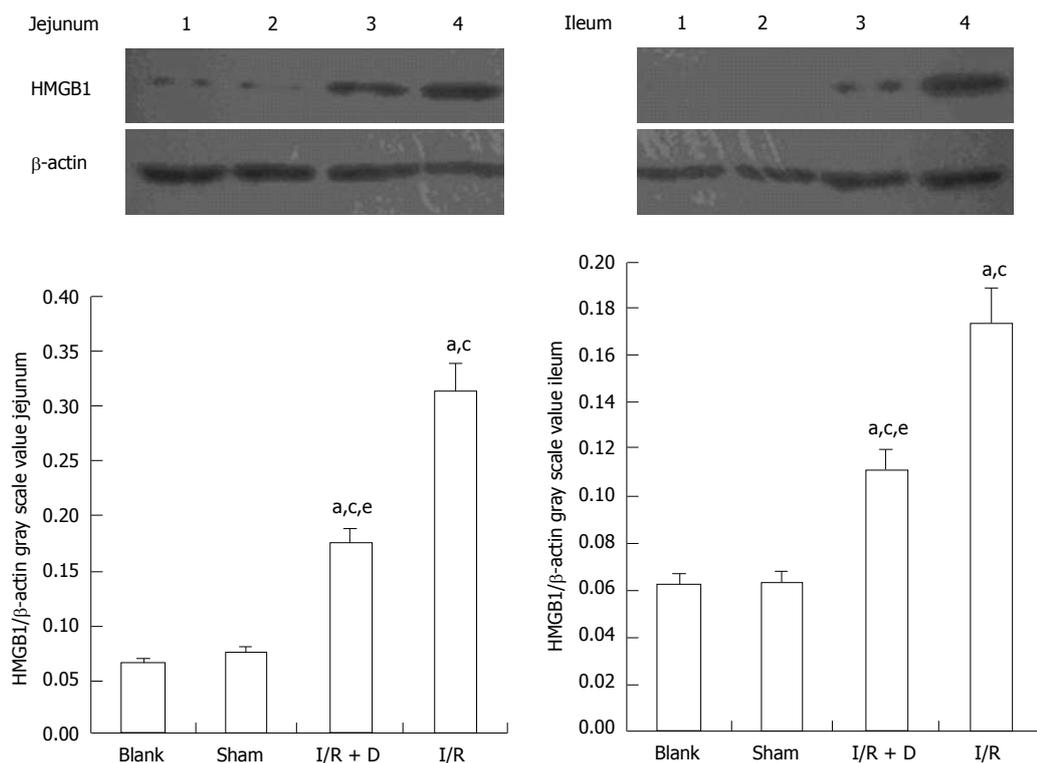


Figure 2 Expression levels of high mobility group protein box 1 protein in the jejunum and ileum ($n = 8$). Top: Western blotting analysis of high mobility group protein box 1 (HMGB1) in the jejunum (left) and ileum (right). Lane 1: Blank; Lane 2: Sham; Lane 3: I/R + D; Lane 4: I/R; Bottom: Quantification of HMGB1 protein levels in the jejunum (left) and ileum (right). HMGB1 levels were normalized to the levels of the β -actin internal control. ^a $P < 0.05$ vs the blank group; ^c $P < 0.05$ vs the sham group; ^e $P < 0.05$ vs the I/R group. I/R + D: Ischemia/reperfusion and drainage; I/R: Ischemia/reperfusion.

There was a significant level of HMGB1 staining in the liver of I/R group, while only a few liver cells were stained in the I/R + D group. HMGB1 staining in the medullary region and the outer medulla of the kidney were obviously increased in the I/R group compared to controls. Analysis of HMGB1 expression in the lung showed that a large number of cells, including endothelial cells and macrophages, were positively stained. Immunohistochemistry in the blank and sham groups showed almost no yellow staining in the liver, kidney, or lung (Figure 1C-E).

Intestinal, liver, kidney and lung HMGB1 expression by western blot

The expression of HMGB1 levels in the jejunum and ileum was significantly higher ($P < 0.05$) in the I/R and I/R + D groups than in the blank and sham groups (which corresponded to the immunohistochemistry data) and HMGB1 expression was significantly lower in the I/R + D group than in the I/R group ($P < 0.05$) (Figure 2). Furthermore, the expression of HMGB1 protein levels in the liver, kidney, and lung was significantly higher in the I/R and I/R + D groups than in the blank and sham groups ($P < 0.05$), and levels in all three organs in the I/R + D group were significantly lower than those in the I/R group ($P < 0.05$) (Figure 3). Together, these data showed that rats in the I/R + D group had significantly less injury than those of the I/R group, suggesting that blocking the “gut-lymph” pathway may attenuate the increase in HMGB1 levels incurred by I/R, and consequently decrease distant

tissue injury.

DISCUSSION

Intestinal I/R injury can lead to severe intestinal damage and increased intestinal permeability. This study showed that intestinal I/R causes intestinal morphological changes, such as intestinal mucosal injury, visible erosion, necrosis, interstitial congestion in the lamina propria of villi top, edema, inflammation, and mucosa and submucosa hemorrhage. In the jejunum and ileum, slight staining was observed at the top of microvilli in the sham group, and both the top villi and the basement membrane were stained in most areas in the I/R group. Moreover, we found that injury in the I/R + D group was less than that of the I/R group. Our previous study in rats showed that the intestinal permeability increased and bacteria translocated after I/R injury, and that the mucosal thickness and villus height were significantly lower than in the control group^[29].

Recent studies have shown that the key mechanism of intestinal I/R injury that leads to a systemic inflammatory response and injury of distant tissues and organs may be mediated by several gut-derived cytokines released in the circulatory system through the “gut-lymph” pathway, which causes downstream injurious effects. The “gut-lymph” pathway theory is based on several observations. Firstly, lymph drainage before hemorrhagic shock can prevent organ dysfunction caused by the shock^[8,30];

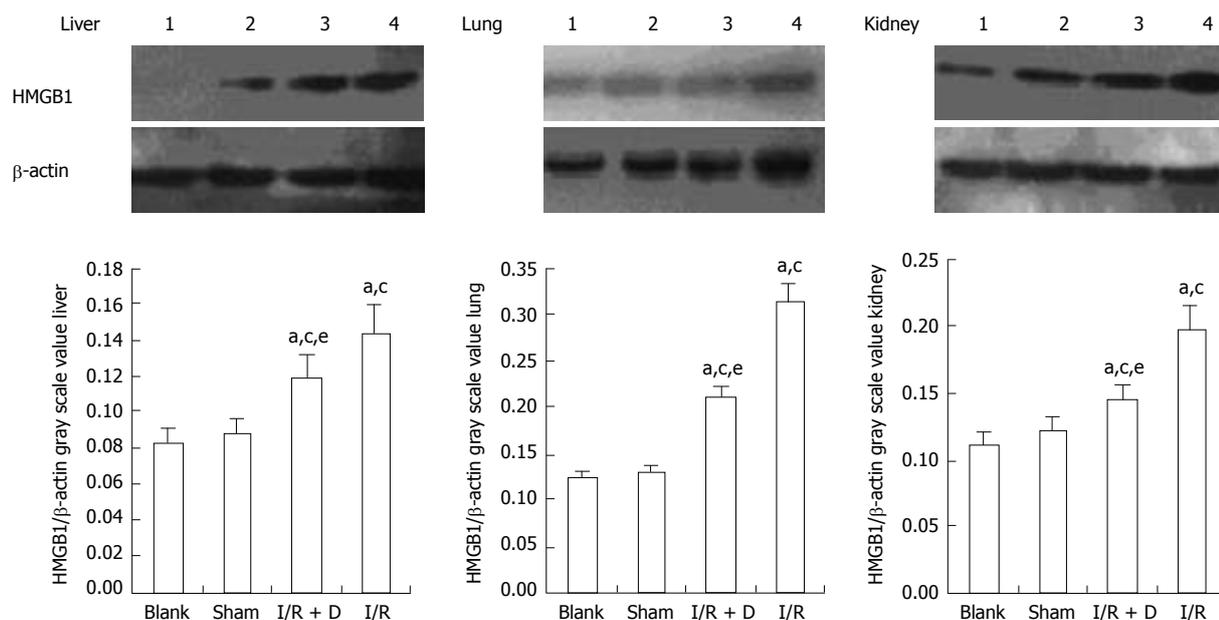


Figure 3 Expression levels of high mobility group protein box 1 in the liver, lung, and kidney ($n = 8$). Top: Western blotting analysis of high mobility group protein box 1 (HMGB1) in the liver (left), lung (middle), and kidney (right). Lane 1: Blank; Lane 2: Sham; Lane 3: I/R + D; Lane 4: I/R; Bottom: Quantification of HMGB1 protein levels in the liver (left), lung (middle), and kidney (right). HMGB1 levels were normalized to the levels of the β -actin internal control. ^a $P < 0.05$ vs the blank group; ^b $P < 0.05$ vs the sham group; ^c $P < 0.05$ vs the I/R group. I/R + D: Ischemia/reperfusion and drainage; I/R: Ischemia/reperfusion.

and secondly, injecting normal animals with the lymph from animals undergoing hemorrhagic shock can lead to a variety of pathological injuries^[12], such as increased permeability, alveolar cell apoptosis, and accumulated neutrophils in the lung.

In this study, our results demonstrated a change in intestinal morphology and an increase in endotoxin, HMGB1, and cytokine serum levels in rats with intestinal I/R injury. Importantly, we found that intestinal lymph drainage could significantly reduce the symptoms of I/R injury. The endotoxin, HMGB1, and cytokine serum levels in the I/R + D group were significantly reduced compared to the I/R group. Cavriani *et al*^[15] observed that lymphatic ligation changed the serum levels of IL-1 β and IL-10, and alleviated the inflammatory response and lung injury in rats. Our previous study also indicated that intestinal lymph duct ligation could effectively reduce intestinal permeability and inflammatory cytokine and endotoxin levels in the circulation in rats after I/R injury^[14,29]. Another study^[31] showed that D-lactate could be detected in the circulation during ischemia for 5 min in I/R rats, and as time progressed, the degree of intestinal mucosa damage increased. Therefore, the D-lactate level could be an early indicator of mucosa damage and permeability changes. The results reported in this study are consistent with previous findings. These studies suggest that both lymph ligation and drainage can block the “gut-lymph” pathway and exert a protective effect on intestinal barrier function.

HMGB1 is the endogenous ligand of TLR4, and the HMGB1-TLR4 combination is an important step in I/R injury^[32]. HMGB1, which was initially found to be a late lethal inflammatory factor in sepsis, was recently considered to be an inflammatory cytokine in ALI and hepatic

injury. In the early stages, HMGB1 levels increases immediately and continue to slowly increase as reperfusion time is extended. Moreover, an anti-HMGB1 antibody was able to alleviate the inflammation response, as well as significantly reduce myeloperoxidase, IL-1 β , and IL-6 expression levels, and especially decrease the accumulation of neutrophils and pulmonary edema^[23,33]. A recent study^[4] found no bacteria or endotoxin in the circulation in rats with some trauma, surgery-induced SIRS, distant organ injury, and the administration of a TLR4 blocker significantly reduced inflammation and tissue damage. Therefore, it may not be endotoxin, but rather the endogenous ligand that activates TLR4.

During I/R injury, the intestinal epithelial cells and macrophages synthesize and release TLR4 endogenous ligands, which bind to TLR4. TLR4 then activates signaling pathways, such as nuclear factor- κ B, and regulates the synthesis of proteins and enzymes that promote the synthesis and release of a variety of cytokines. This results in the subsequent injury of the distant organs^[19,23,27]. This study showed that serum levels of HMGB1 were significantly higher in the I/R and I/R + D groups than in the blank and sham groups, and that HMGB1 in the I/R + D group was significantly lower than in the I/R group. These results were consistent with HMGB1 protein expression in the intestine, liver, kidney, and lung as determined by western blot. The immunohistochemistry analysis of HMGB1 in the liver, kidney, and lung also showed that the distant organs were damaged during intestinal I/R injury. Yang *et al*^[34] and Liu *et al*^[35] found that treatment of transient ischemic rats with a neutralizing anti-HMGB1 antibody could reduce IL-6 mRNA and TNF- α mRNA on the surface of intestinal mucosa, attenuate injury, and improve the

survival rate. This indicates that HMGB1 released during the early stage may be the factor that promotes intestinal injury and a systemic inflammatory response. In this study, we observed an improvement in morphology, and the serum levels of endotoxin, inflammatory cytokines, and HMGB1 in the I/R + D group were significantly lower than in the I/R group. This could be due to the low blood flow caused by ischemia and hypoxia when organs were subjected to perfusion. It could also be due to the fact that factors such as inflammatory cytokines diffuse throughout the entire body through reperfused blood^[7,36]. The degree of the injury in the liver, kidney, and lung, as well as the expression of HMGB1 in the I/R group, were significantly greater than in the I/R + D group ($P < 0.05$). This demonstrates that HMGB1 participates in the occurrence and development of the injury during intestinal I/R, and that blocking the “gut-lymph” pathway can effectively reduce the injury of the intestinal barrier function and the levels of systemic inflammatory cytokines, as well as attenuate the stimulation of HMGB1 on intestine and distant organs.

In conclusion, intestinal injury after I/R can stimulate the release of HMGB1, endotoxin, and inflammatory cytokines, which may be related to intestinal barrier dysfunction and distant organ injury. Intestinal lymph drainage may improve the morphology and function of the intestine, reduce the levels of cytokines and endotoxin in circulation, and attenuate the injury of distant organs by blocking the “gut-lymph” pathway; providing a reference for clinical treatment.

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COMMENTS

Background

Intestinal ischemia/reperfusion (I/R) injury is the “motor” of systemic inflammatory response syndrome (SIRS), acute respiratory distress syndrome (ARDS), and multiple organ dysfunction syndrome (MODS). The ligating lymph duct in a variety of species after hemorrhagic shock can prevent distant organ injury. However, the composition of lymph that is responsible for distant organ injury remains unknown.

Research frontiers

Recent studies proposed that mesenteric lymph-induced distant organ injury in intestinal I/R was directly mediated by gut-derived endogenous ligands, one of which is the high mobility group protein box 1 (HMGB1). HMGB1 acts as a late mediator of lethal sepsis and an early mediator of inflammation and necrosis following I/R injury. The research hot spot is whether HMGB1 in the intestinal mesenteric lymph after I/R mediates distant organ injury.

Innovations and breakthroughs

This study is a continuation of previous study. The authors hypothesized that the HMGB1 and toll-like receptor 4 (TLR4) combination plays an important role in the distant organ injury caused by intestinal I/R injury. The purpose of this study was to determine the impact of intestinal I/R injury and lymph drainage on the intestine and distant organs in rats, and to clarify whether HMGB1 in the intestinal mesenteric lymph after I/R mediates distant organ injury.

Applications

The study results suggest that intestinal injury after I/R stimulate the release of HMGB1, endotoxin, and inflammatory cytokines, and that lymph drainage could block the “gut-lymph” pathway, improve intestinal barrier function, reduce cytokine and endotoxin, and attenuate the injury of distant organs incurred by intestinal I/R; providing a reference for future clinical practice.

Terminology

Intestinal I/R injury: Intestinal I/R injury is the “motor” of SIRS, ARDS and MODS, and can be associated with severe trauma, acute necrotizing pancreatitis, major surgery, extensive burns, and other stresses; HMGB1: HMGB1 is one of the endogenous ligands which can be recognized by and combine with TLR4, and is also the key factor of inflammation of aseptic injury.

Peer review

This is a good descriptive study in which the authors have analyzed the impact of intestinal I/R injury and lymph drainage on distant organs in rats. The authors conclude that lymph drainage could block the “gut-lymph” pathway, improve intestinal barrier function, reduce cytokine and endotoxin, and attenuate the injury of distant organs in intestinal I/R lesions. The experiments were well designed, and the results were clearly demonstrated and analyzed. The techniques are appropriate and the conclusions are supported by the data presented.

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Clinical features and risk factors of acute hepatitis E with severe jaundice

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Abstract

AIM: To compare the clinical features of patients infected with hepatitis E virus (HEV) with or without severe jaundice. In addition, the risk factors for HEV infection with severe jaundice were investigated.

METHODS: We enrolled 235 patients with HEV into a cross-sectional study using multi-stage sampling to select the study group. Patients with possible acute hepatitis E showing elevated liver enzyme levels were screened for HEV infection using serologic and molecular tools. HEV infection was documented by HEV antibodies and by the detection of HEV-RNA in serum. We used χ^2 analysis, Fisher's exact test, and Student's *t* test where appropriate in this study. Significant predictors in the univariate analysis were then included in a forward, stepwise multiple logistic regression model.

RESULTS: No significant differences in symptoms, alanine aminotransferase, aspartate aminotransferase, al-

kaline phosphatase, or hepatitis B virus surface antigen between the two groups were observed. HEV infected patients with severe jaundice had significantly lower peak serum levels of γ -glutamyl-transpeptidase (GGT) (median: 170.31 U/L vs 237.96 U/L, $P = 0.007$), significantly lower ALB levels (33.84 g/L vs 36.89 g/L, $P = 0.000$), significantly lower acetylcholine esterase (CHE) levels (4500.93 U/L vs 5815.28 U/L, $P = 0.000$) and significantly higher total bile acid (TBA) levels (275.56 μ mol/L vs 147.03 μ mol/L, $P = 0.000$) than those without severe jaundice. The median of the lowest point time tended to be lower in patients with severe jaundice (81.64% vs 96.12%, $P = 0.000$). HEV infected patients with severe jaundice had a significantly higher viral load (median: 134 vs 112, $P = 0.025$) than those without severe jaundice. HEV infected patients with severe jaundice showed a trend toward longer median hospital stay (38.17 d vs 18.36 d, $P = 0.073$). Multivariate logistic regression indicated that there were significant differences in age, sex, viral load, GGT, albumin, TBA, CHE, prothrombin index, alcohol overconsumption, and duration of admission between patients infected with acute hepatitis E with and without severe jaundice.

CONCLUSION: Acute hepatitis E patients may naturally present with severe jaundice.

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Key words: Hepatitis E virus; Acute hepatitis E; Clinical features; Severe jaundice; Risk factor

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INTRODUCTION

Although hepatitis E was recognized as a new disease in 1980, the virus was first visualized in 1983 and its genome was cloned and characterized in 1991, however, the disease is probably ancient but was not recognized until modern times^[1-6]. Hepatitis E is the most important or the second most important cause of acute clinical hepatitis in adults throughout Asia, the Middle East and Africa^[1]. Hepatitis E virus (HEV) is a small round RNA-containing virus that is the only member of the genus *Hepevirus* in the family *Hepeviridae*^[7]. The clinical course of hepatitis E is usually more severe than hepatitis A, and is frequently complicated by protracted coagulopathy and cholestasis. HEV accounts for more than 50% of acute viral hepatitis in young adults in developing countries^[8]. The disease course is benign, and severe jaundice is rarely reported. Severe icteric forms are unusual in patients without cirrhosis and have been documented in immunocompromised patients^[9]. These cases did not progress to fulminant hepatitis or severe acute hepatitis. However, the differences in the demography, mode of transmission, and clinical features of patients infected with HEV with and without severe jaundice have not been sufficiently studied. Thus, the present study was designed to determine the clinical features and risk factors of acute hepatitis E with severe jaundice in patients without cirrhosis. Jaundice was very marked in all patients. HEV infection was documented by HEV antibodies and by the detection of HEV-RNA in serum. Here, we studied a well-characterized population of HEV-infected individuals enrolled in a large Chinese cohort study. Acute HEV in our patients was characterized by severe jaundice with serum bilirubin levels above 171 $\mu\text{mol/L}$.

MATERIALS AND METHODS

Patients

Between December 2005 and December 2010, we diagnosed 235 cases of acute hepatitis E at Beijing YouAn Hospital affiliated to Capital Medical University in Beijing. Patients with possible acute hepatitis E showing elevated liver enzyme levels were screened for HEV infection using serologic and molecular tools. Biliary tract complications were ruled out by abdominal ultrasonography. Toxin- and drug-related causes of abnormal liver function tests were ruled out by patient history. 235 patients tested positive for serum HEV RNA. Serum samples which had been stored frozen (or below $-20\text{ }^{\circ}\text{C}$) were thawed prior to analysis. The samples were screened for immunoglobulin M (IgM) antibody to hepatitis A virus (AxSYM System, Abbott), hepatitis B virus surface antigen (HBsAg), IgM antibody to hepatitis B core antigen (AxSYM System, Abbott), polymerase chain reaction for HBV DNA and antibody to hepatitis C virus (AxSYM System, Abbott). Subsequently, the serum was also tested for IgM anti-Epstein-Barr virus antibody [anti-HEV IgM, enzyme-linked immunosorbent assay (ELISA), Biotest], and IgM anti-cytomegalovirus antibody (AxSYM System,

Abbott). Diagnosis of drug-induced hepatitis was based on patient history and clinical course; the lymphocyte stimulation index for drugs was used as an adjunct. Acute hepatitis of unknown etiology was a diagnosis of exclusion. Acute hepatitis E was defined by the presence of HEV RNA in acute-phase sera. Drugs and alcohol were excluded as likely etiological agents by patient history and a review of patient medications. Other causes of liver disease were excluded by testing for serum ceruloplasmin, 24-h urinary copper, antinuclear antibody, antismooth muscle antibody, serum iron and iron-binding capacity and alpha-1 antitrypsin levels.

We enrolled 235 patients with HEV. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review Board at each institution. Informed consent was obtained from each patient.

Epidemiological survey and laboratory examinations

All patients enrolled in the study provided detailed medical history, including general data such as age, sex, onset of illness, travel history before onset of illness, history of blood transfusion, alcohol intake, medicine use, and disease complications. Acute hepatitis E with severe jaundice was defined as total bilirubin $> 171\text{ }\mu\text{mol/L}$. In this study, an underlying disease was defined as an active illness where the patient had received medical care before the onset of hepatitis. Overconsumption of alcohol was defined as daily ethanol ingestion over 50 g for 5 years or longer. To minimize recall bias and/or interview bias, we performed the following: we visited each patient at home twice in 3 mo and using an open-ended questionnaire assisted them to recall their daily ingestion and/or alcohol intake during the 2 mo preceding the onset of illness, and compared that with their current eating and drinking habits. In the laboratory examinations, total bilirubin, bilirubin direct, alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ -glutamyltranspeptidase (GGT), alkaline phosphatase (ALP), albumin (ALB), acetylcholine esterase (CHE), total bile acid (TBA) and prothrombin index (PTA) were measured, using a sequential multi-autoanalyzer at each hospital.

Detection of anti-HEV IgM

Serum samples were tested for HEV IgG and IgM using a commercial ELISA kit (Wan Tai Pharmaceutical Co., Beijing, China) produced with two recombinant peptides corresponding to amino acid residues 396-603 of the major structural protein specified by open reading frame 2 of the HEV genome. The sensitivity and specificity of the ELISA kit was demonstrated by Zhang *et al.*^[10]. Serum samples were tested according to the manufacturer's instructions with three negative and positive control wells included on each plate. The samples with an optical density less than the cutoff value (cutoff value for IgG was equal to the mean optical density for the three negative controls on each plate plus 0.12, and the cutoff value for IgM was equal to the mean optical density for the three

Table 1 Comparison of background characteristics and clinical manifestations, laboratory data and outcome

	Acute hepatitis E with severe jaundice	Acute hepatitis E without severe jaundice	P value
Characteristic			
Age, yr, mean (range)	56 (20-85)	51 (18-85)	0.015
Sex, men, n (%)	64 (70)	125 (165)	0.003
Occupation, n (%)			
Handling with animals	0	0	
Handling with raw food	0 (70)	5 (165)	0.580
Underlying disease	4 (70)	32 (165)	0.008
History, n (%)			
Blood transfusion	0	0	
Intake wild animals	0	0	
Intake raw pig liver or intestine	0	0	
Intake raw fish or shellfish	0 (70)	5 (165)	0.580
Alcohol overconsumption	27 (70)	16 (165)	0.003
Clinical features			
Symptoms (%)			
Fever	1.47 ± 0.737	1.35 ± 0.725	0.243
Jaundice	1.00 ± 0.000	1.01 ± 0.029	0.892
Laboratory data			
Peak ALT (U/L)	1089.23 ± 1607.77	1035.53 ± 869.49	0.741
Peak AST (U/L)	660.83 ± 675.99	604.34 ± 715.72	0.574
Peak TBil (μmol/L)	276.31 ± 91.63	78.70 ± 48.06	0.000
Peak DBil (μmol/L)	185.06 ± 64.64	48.52 ± 35.16	0.000
Lowest ALB (g/L)	33.84 ± 3.72	36.89 ± 4.76	0.000
Peak GGT (U/L)	170.31 ± 117.55	237.96 ± 181.18	0.007
Peak ALP (U/L)	184.96 ± 67.22	187.76 ± 89.82	0.826
Peak CHE (U/L)	4500.93 ± 175.32	5815.28 ± 151.04	0.000
Peak TBA	275.56 ± 28.81	147.03 ± 10.53	0.000
HBsAg	8 (69)	27 (159)	0.203
Lowest PTA (%)	81.64 ± 22.69	96.12 ± 24.63	0.000
PT ≤ 60%, n (%)	7 (60)	11 (146)	0.424
Viral load	134 ± 68	112 ± 69	0.025
Duration for admission (d)	38.17 ± 17.13	18.36 ± 91.35	0.073

TBil: Total bilirubin; DBil: Bilirubin direct; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: γ -glutamyl-transpeptidase; ALP: Alkaline phosphatase; ALB: Albumin; CHE: Acetylcholine esterase; TBA: Total bile acid; PTA: Prothrombin index; PT: Point time; HBsAg: Hepatitis B virus surface antigen.

negative controls on each plate plus 0.26) were considered negative. Samples with an optical density greater than or equal to the cutoff value were tentatively considered reactive and were retested in duplicate to confirm the result.

Detection of HEV RNA

Using the QIAamp RNA Blood Mini Kit (QIAGEN, Germany), RNA was reverse transcribed into cDNA with 10 units of AMV Reverse Transcriptase (Promega, Madison, WI, United States) at 42 °C for 60 min. The primers used in this study were synthesized as previously described^[10]. The primer positions indicated below are relative to the HEV strain JYI-Chisai01C (GenBank accession number AB 197674): P1: 5’AAC(T)TATGC ACAGCCGGGTTG3’ (forward position 5725-5746) P2: 5’CCCTTATCCTGCTGAGCATTCTC3’ (reverse position 6433-6455); P3: 5’GTC(T)ATGC(T)TC(T)TGC ATACATGGCT-3’ (forward position 6010-6031) P4: 5’AGCCGACGAAATC(T)AATTCTGTC-3’ (reverse po-

sition 6336-6357). The first round of polymerase chain reaction (PCR) was carried out in a 50 μL reaction volume with 8 μL cDNA, 25 pmol of each primer (P1, P2), 1 μL of 10 mmol/L dNTPs, and 0.5 μL TaKaRa Ex Taq polymerase. PCR was conducted for 35 cycles at 94 °C for 1 min, 55 °C for 45s and 72 °C for 1 min with a final extension time of 72 °C for 7 min. The second round reaction were carried out in a 50 μL volume with 8 μL of the first-round product, 25 pmol of each primer (P3/P4), and 0.5 μL TaKaRa Ex Taq polymerase. The parameters were the same as the first round. The second-round PCR products were separated by 2% agarose gel.

Statistical analysis

We used χ^2 analysis, Fisher’s exact test, and Student’s *t* test where appropriate in this study. Significant predictors in the univariate analysis were then included in a forward, stepwise multiple logistic regression model. A *P* value of < 0.05 was considered statistically significant; all tests were two-tailed. Data were analyzed using the statistical software StatView for Macintosh (Version 5.0 StatView, SAS Institute Inc., NC, United States).

RESULTS

Patients infected with HEV: Characteristics, laboratory data and outcome

Of the 235 patients with acute hepatitis E, 70 were diagnosed with acute hepatitis E and severe jaundice and 165 were diagnosed with acute hepatitis E without severe jaundice. Of the patients with acute hepatitis E and severe jaundice, 64 were male and 6 were female (all non-pregnant) with a median age of 56 years (range: 20-85years). Twenty-seven (39%) of these patients demonstrated alcohol overconsumption. Of the patients with acute hepatitis E without severe jaundice, 125 were male and 40 were female (all non-pregnant) with a median age of 51 years. Sixteen (10%) of these patients demonstrated alcohol overconsumption.

Comparison between patients infected with HEV

Comparison of baseline characteristics between the two groups. A comparison of characteristics between acute hepatitis E patients with severe jaundice and acute hepatitis E patients without severe jaundice is shown in Table 1.

There were significant differences in age and the male-female ratio between the two groups. There were no significant differences in occupation, history of traveling to endemic areas, blood transfusion, or the presence of underlying disease(s) between the two groups. Overconsumption of alcohol was observed in a higher proportion of patients infected with severe jaundice compared with those without severe jaundice (27/70 vs 16/165, *P* = 0.003). None of the patients had a history of ingesting raw wild deer, boar, bear or rabbits, or a history of blood transfusion, ingestion of raw pig liver, and/or undercooked pig intestines before the onset of illness. There were no significant differences in the intake of raw fish or shellfish between the two groups.

Table 2 Relation between acute hepatitis E with severe jaundice and risk factors

Risk factors	SE	P value	OR	95%CI
Viral load	0.002	0.026	1.005	1.001-1.009
Age	0.011	0.016	1.026	1.005-1.048
Sex	0.464	0.008	0.291	0.117-0.772
Fever	0.203	0.243	1.268	0.851-1.888
ALT	0.000	0.741	1.000	1.000-1.000
AST	0.000	0.573	1.000	1.000-1.001
GGT	0.001	0.009	0.997	0.995-0.999
ALP	0.002	0.825	1.000	0.996-1.003
ALB	0.036	0.000	0.857	0.799-0.919
TBA	0.001	0.000	1.006	1.004-1.009
CHE	0.000	0.000	1.000	0.999-1.000
PTA	0.007	0.000	0.975	0.963-0.988
HBsAg	0.431	0.303	1.560	0.670-3.632
Alcohol overconsumption	0.309	0.003	2.476	1.350-4.542
Duration for admission	0.009	0.000	1.038	1.020-1.056

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: γ -glutamyl-transpeptidase; ALP: Alkaline phosphatase; ALB: Albumin; CHE: Acetylcholine esterase; TBA: Total bile acid; PTA: Prothrombin index; HBsAg: Hepatitis B virus surface antigen; OR: Odds ratio.

Comparison of clinical features between the two groups

The clinical features of the study population are shown in Table 1. There were no significant differences in symptoms, ALT, AST, ALP, or HBsAg between the two groups. HEV infected patients with severe jaundice had significantly lower peak serum levels of GGT (median: 170.31 U/L *vs* 237.96 U/L, $P = 0.007$), significantly lower ALB levels (33.84 g/L *vs* 36.89 g/L, $P = 0.000$), significantly lower CHE levels (4500.93 U/L *vs* 5815.28 U/L, $P = 0.000$) and significantly higher TBA levels (275.56 μ mol/L *vs* 147.03 μ mol/L, $P = 0.000$) than those without severe jaundice. The lowest median PT tended to be lower in the severe jaundice group (81.64% *vs* 96.12%, $P = 0.000$). The proportion of patients with PT $\leq 60\%$ was not significantly different in the severe jaundice group (7/60 *vs* 11/146, $P = 0.424$). HEV infected patients with severe jaundice had a significantly higher viral load (median: 134 *vs* 112, $P = 0.025$) than those without severe jaundice. HEV infected patients with severe jaundice showed a trend toward longer median hospital stay (38.17 d *vs* 18.36 d, $P = 0.073$).

Relation between acute hepatitis E with severe jaundice and risk factors

The multivariate logistic regression analysis indicated that there were significant differences in age, sex, viral load, GGT, ALB, TBA, CHE, PTA, alcohol overconsumption, and duration of admission between acute hepatitis E patients with and without severe jaundice. The relations between acute hepatitis E patients with severe jaundice and risk factors are shown in Table 2.

DISCUSSION

Clinically, HEV usually causes a self-limiting illness, with an average incubation period of 40 d, moderate jaundice,

and complete resolution of hyperbilirubinemia and normalization of aminotransferases within 1-6 wk. Laboratory findings in patients with HEV are also similar to other forms of viral hepatitis and include elevated serum bilirubin, alanine aminotransferase, aspartate transaminase, alkaline phosphatase, and gamma-glutamyl-transferase levels^[11]. The cases presented here are exceptional in that some patients had severe jaundice. We report 70 cases of acute hepatitis E with severe jaundice, no pruritus, normal serum creatinine levels, and normal PTA. Most cases of acute hepatitis E do not have severe jaundice. In this study, 70 of 235 cases with acute hepatitis E had severe jaundice. Our report is rather unique in that detailed personal interviews were performed to explore patients' exposures which may not have been recalled when the patient was originally investigated for hepatitis E. We maintained an attitude of strict neutrality during the interview to minimize recall bias and/or interview bias. The characteristics of the patients with hepatitis E and severe jaundice included in our study are summarized as follows: many patients were 4060 years of age, similar to endemic cases reported in Asia^[12,13], 64 patients (90%) were male; none of the patients had a history of ingesting wild animal meat or undercooked intestines. Raw or partially cooked shellfish are a common cause of HEV infection as shellfish filter large volumes of water. Five patients (7%) had a history of ingesting raw fish or shellfish. Four patients seemed to have underlying diseases. Recently, Mizuo *et al*^[14] reported that the presence of underlying disease(s) influence the severity of hepatitis E. The presence of underlying diseases was also significantly different between patients with severe jaundice and patients without severe jaundice. Sixteen patients (10%) demonstrated alcohol overconsumption. In our series, patients with severe jaundice consumed alcohol on a regular basis compared to those without severe jaundice, there were also significant differences in other background characteristics between the two groups. Alcohol overconsumption, however, was not associated with illness severity. Whether overconsumption of alcohol is associated with susceptibility to acute hepatitis E requires further research.

Physical examination revealed 235 icteric subjects, in otherwise good health, with no signs of hepatic failure. Specifically, there was no history of medication or drug use, and the patients denied taking any herbal supplements. Cholelithiasis is an unlikely explanation for jaundice, as there was no evidence of cholecystitis found on repeat imaging studies or during surgery, and there was no evidence of choledocholithiasis. There were no significant differences in symptoms, ALT, AST, ALP, or HBsAg between the two groups. Patients with severe jaundice showed more severe clinical manifestations (including laboratory data such as ALB, CHE and TBA levels, PTA and duration of hospital stay) than those without severe jaundice. Patients with severe jaundice had significantly lower peak serum GGT levels ($P = 0.007$) which requires further research. The proportion of patients with PT $\leq 60\%$, however, was not significantly different between the two groups. Bernardin *et al*^[15] reported that the severity of

hepatitis E was related to dose of the virus. We found that patients with severe jaundice had a significantly higher viral load than those without severe jaundice. The mechanisms underlying the development of severe jaundice during acute hepatitis E are likely to be multifactorial. It is well known that inflammatory liver cell necrosis is a factor in jaundice in the setting of acute viral hepatitis. Other cofactors of hyperbilirubinemia may include concomitant etiologies such as autoimmune cholangitis and accentuation of preoperative jaundice after laparotomy^[16,17]. Such cofactors can be ruled out in our patients as none of the patients underwent abdominal surgery, and autoimmune cholangitis is unlikely since serum autoantibodies were absent. It is therefore possible to hypothesize that HEV itself had a direct effect on the development of high-grade hyperbilirubinemia in these patients. Some data indicate that HEV has not only hepatocytic but also biliary tropism, and replicates within bile epithelial cells^[18-20]. However, the mechanisms underlying high-grade hyperbilirubinemia in HEV infection remain unclear.

According to our results, age, sex, GGT, ALB, CHE, TBA, viral load, and alcohol overconsumption seem to be risk factors affecting patients with acute HEV and severe jaundice (odds ratio = 1.026, 0.291, 0.997, 0.857, 1.000, 1.006, 1.005 and 2.476, respectively). In multivariate analysis, fever symptoms, ALT, AST, ALP, and HBsAg were not significant risk factors for acute HEV infection with severe jaundice. Taking into account that the majority of HEV infections in endemic areas are caused by genotype 1, a definitive picture of the differences in acute HEV infected patients with severe jaundice according to the genotype need to be obtained from more in-depth studies.

In conclusion, patients with acute hepatitis E may naturally present with severe jaundice. However, further clinical, epidemiological and virological studies are needed to elucidate HEV infection with severe jaundice and the association between HEV genotype and severe jaundice as well as its underlying mechanism.

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COMMENTS

Background

Clinically, hepatitis E virus (HEV) usually causes a self-limiting illness, with an average incubation period of 40 d, moderate jaundice, and complete resolution of hyperbilirubinemia and normalization of aminotransferases within 1-6 wk. Laboratory findings in patients with HEV are also similar to other forms of viral hepatitis and include elevated serum bilirubin, alanine aminotransferase, aspartate transaminase, alkaline phosphatase, and gamma-glutamyl-transferase levels. The clinical course of hepatitis E is usually more severe than hepatitis A, and is frequently complicated by protracted coagulopathy and cholestasis.

Research frontiers

HEV accounts for more than 50% of acute viral hepatitis in young adults in developing countries. Severe icteric forms are unusual in patients without cirrhosis and have been documented in immunocompromised patients. These cases did not progress to fulminant hepatitis or severe acute hepatitis. However, the differences in the demography, mode of transmission, and clinical features of

patients infected with HEV with and without severe jaundice have not been sufficiently studied.

Innovations and breakthroughs

In this study, 70 of 235 cases with acute hepatitis E had severe jaundice, no pruritus, normal serum creatinine levels, and normal prothrombin index. Most cases of acute hepatitis E do not have severe jaundice. The report is rather unique in that detailed personal interviews were performed to explore patients' exposures which may not have been recalled when the patient was originally investigated for hepatitis E. The authors maintained an attitude of strict neutrality during the interview to minimize recall bias and/or interview bias.

Applications

The study was designed to determine the clinical features and risk factors of acute hepatitis E with severe jaundice in patients without cirrhosis. Jaundice was very marked in all patients. Acute HEV in the patients was characterized by severe jaundice with serum bilirubin levels above 171 $\mu\text{mol/L}$. These patients with severe jaundice might represent true cases of acute hepatitis E.

Terminology

The HEV is a small, non-enveloped, icosahedral, positive-sense, single-strand RNA virus. HEV is responsible for the majority of cases of what was previously called enterically transmitted non-A, non-B hepatitis. Hepatitis E is endemic in many subtropical and tropical areas. In these areas, hepatitis E occurs both epidemically and sporadically. Hepatitis E is a self-limiting disease of varying severity, presenting as acute, icteric hepatitis, with clinical and morphological findings similar to those of hepatitis A. Recent studies have found immunoglobulin G to HEV in several wild and domestic animal species native to developing and industrialized countries. Molecular evidence for natural HEV infection in swine has been reported for HEV-endemic and -nonendemic countries worldwide.

Peer review

The paper describes the characteristics of patients with a more severe jaundice than the remainder of the group.

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Growth-inhibitory effects of MOB2 on human hepatic carcinoma cell line SMMC-7721

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Abstract

AIM: To investigate the growth-inhibiting and apoptosis-inducing effects of the gene MOB2 on human hepatic carcinoma cell line SMMC-7721.

METHODS: The full-length cDNA of the MOB2 gene was amplified from human umbilical vein endothelial cells. The correct full-length MOB2 cDNA was subcloned into the eukaryotic expression vector pEGFP-C1. After lipofection of the MOB2 gene into cancer cells, the levels of MOB2 protein in the cancer cells were detected by immunoblotting. To transfect the recombinant plasmid vector pEGFP-CI-MOB2 into SMMC-7721 cells, the cells were cultured in Dulbecco's Modified Eagle's

Medium with 10% fetal calf serum and glutamine, and then mixed with liposomes, Lipofectamine 2000 and the plasmid vector pEGFP-CI-MOB2.

RESULTS: We observed the growth and proliferation of SMMC-7721 cells containing pEGFP-CI-MOB2 and analyzed their apoptosis and growth cycle phases by flow cytometry. We successfully transfected the recombinant plasmid vector pEGFP-CI-MOB2 into SMMC-7721 cells and screened for a single clone cell containing MOB2. After transfection, MOB2 enhanced growth suppression, induced apoptosis, increased the ratio of G0/G1, significantly inhibited the advance of cell cycle phase, and arrested cells in G0/G1 phase.

CONCLUSION: MOB2 overexpression induces apoptosis and inhibits the growth of human hepatic cancer cells, which may be useful in gene therapy for hepatic carcinoma.

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Key words: Gene expression; SMMC-7721; Growth inhibition; Apoptosis

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INTRODUCTION

Mps-one-binder (Mob) family proteins belong to a conserved gene family found in yeast^[1-3], *Drosophila*^[4,5] and mammals^[6]. Mob1 protein was originally identified as a regulator of mitotic exit and cytokinesis in yeast^[1]. In *Drosophila*, Mob1 is a key component of the Hippo

signaling pathway^[5,7] and thus plays a role in tumor suppression. Mob2 binds to Cbk1, a conserved protein kinase that is similar to human myotonic dystrophy kinase. Mob2 promotes polarized cell growth and induces asymmetric cell fate in fission yeast^[3,8-10]. In *Drosophila*, the Mob2 protein binds directly to the Tricornered protein, an ortholog of nuclear Dbf2-related (Ndr) kinase^[11]. Previous work has also shown that Dmob2 is involved in photoreceptor morphogenesis by regulating the subcellular localization of Crumbs, a cell polarity gene, and phosphorylated moesin^[12]. Knockdown of Dmob2 expression causes abnormal actin rearrangement and results in abnormal rhabdomere formation, which suggests that Dmob2 may regulate actin dynamics during rhabdomere formation^[12].

Ndr kinase belongs to a subfamily of serine/threonine protein kinases that control cell division and morphogenesis in various cell types^[13]. Ndr2 is induced in the mouse amygdala during fear memory consolidation. Other studies have demonstrated that this kinase plays an important role in neurite formation^[14]. Tricornered, the Ndr kinase ortholog in *Drosophila*, interacts with actin filaments and regulates dendritic tiling and branching^[11]. SAX-1, the ortholog of Ndr kinase in nematodes, activates the RhoA-GTPase signaling pathway during neurite formation^[14]. These results strongly indicate the importance of Ndr kinases in neurite formation. Mob proteins have been shown to bind Ndr kinases, making them important in kinase activation^[6]. In this study, we investigated what role Mob proteins play in the human hepatic carcinoma cell line SMMC-7721.

MATERIALS AND METHODS

Materials

The eukaryotic expression vector pEGFP-C1 and competent cells of *Escherichia coli* DH5 α were routinely preserved by the central laboratory of our hospital. We used the following materials in our studies: RNeasy Protect mini kit (Qiagen, Germany); SMARTTM PCR cDNA synthesis kit (Clontech, United States); DNA gel extraction kit (Dalian TaKaRa, China); plasmid mini preparation kit (Shanghai Huasun Biotechnology, China); KOD-Plus DNA polymerase (TOYOBO, United States); T4 DNA ligase and restriction enzymes (*Hind*III and *Kpn*I) (New England Biolabs, United States); Lipofectamine 2000 (Invitrogen, United States); rabbit anti-MOB2 monoclonal antibody (Santa Cruz Biotechnology, United States); and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Zhongshan, China). Nucleic acid sequencing was performed by Shanghai Yingjun Bioengineering (China). MOB2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were synthesized by Shanghai Yingjun Bioengineering.

Construction of recombinant MOB2 vector

Total RNA was isolated from human umbilical vein endothelial cells. Full-length MOB2 cDNA was amplified

by reverse-transcriptase polymerase chain reaction. The primer pairs for MOB2 fragment amplification were designed based on the MOB2 mRNA sequence provided by GenBank using the Premier Primer 5.0 software package. The primer sequences used were as follows:

5'-CCCAAGCTTCCATGGACTGGCTCATGGGGAAGTC-3' (forward primer) and 5'-CGGGGTACCTCATCTCCTTCACGTGGTTCTG-3' (reverse primer). The reaction conditions were as follows: 94 °C for 1 min, 94 °C for 30 s, 60 °C for 30 s, and 68 °C for 75 s. After 35 amplification cycles, the product was extended at 68 °C for 2 min. After removal of the pEGFP cDNA with *Hind*III and *Kpn*I, the amplified fragment was inserted into pEGFP-C1 to make the expression vector pEGFP-C1-MOB2.

Cell culture and gene transfection

SMMC-7721 cells were maintained in Dulbecco's Modified Eagle's Medium containing 10% calf serum, 100 U/mL penicillin and 100 μ g/mL streptomycin in a humidified chamber at 37 °C with 5% CO₂. Transient transfections were performed. Gene transfer was conducted using the liposome-mediated method (Lipofectamine 2000; Invitrogen, United States) according to the manufacturer's instructions. Blank control, empty vector (pEGFP-C1) and pEGFP-C1-MOB2 transfection groups were constructed in this experiment.

Western blotting

To harvest cells under non-denaturing conditions, we removed the medium and rinsed the cells with ice-cold PBS. Ice-cold lysis buffer (500 μ L) was added to each well. The cells were scraped from the surface and transferred to an Eppendorf tube on ice. Each sample was then sonicated four times for 5 s while being kept cold. The mixture was centrifuged for 10 min at 4 °C, and the supernatant was saved as lysate for later use. The proteins (150 μ g) in the lysate were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (10% polyacrylamide gel containing 0.1% SDS) and then transferred to a polyvinylidene difluoride membrane by electrophoretic blotting. The membranes were probed with specific antibodies and then treated with anti-rabbit IgG-HRP (1:2000). Protein bands were visualized by enhanced chemiluminescence using the procedure recommended by the manufacturer (Thermo Scientific, United States). The expression of GAPDH was measured as a control.

Flow cytometry analysis

Cell cycle parameters were determined by propidium iodide labeling of nuclear DNA. Cells (10⁶) were trypsinized and resuspended in 70% ethanol before being stored for later use. The cells were treated with RNase and Triton X-100 as well as with propidium iodide to label the nuclear DNA. The labeled cells were analyzed by flow cytometry at 630 nm for propidium iodide. The cell cycle parameters were determined using the DNA cell cycle analysis in the software provided by the manufacturer (Invitrogen).

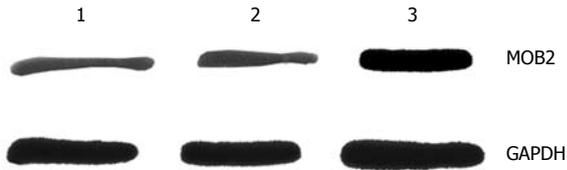


Figure 1 MOB2 protein level in SMMC-7721 cells. 1: Control group; 2: Empty vector transfection group; 3: pEGFP-C1-MOB2 transfection group.

Determination of growth of cancer cells using trypan blue staining

SMMC-7721 cells were trypsinized and seeded into 24-well plates at a density of 10^5 /mL. After transfection, cells were digested and counted using trypan blue staining under an inverted light microscope for five consecutive days.

Plate colony formation assay

Approximately 100 cells were added to each well of a six-well culture plate. After incubation at 37 °C for 15 d, cells were washed twice with PBS and stained with Giemsa solution. The number of colonies containing ≥ 50 cells was counted under a microscope [plate clone formation efficiency = (number of colonies/number of cells inoculated) \times 100%]. Each experiment was performed in triplicate.

Statistical analysis

Student's *t* test and analysis of variance were performed for statistical analysis with SPSS version 10.0.

RESULTS

Verification of MOB2 expression vector

After the recombinant plasmid pEGFP-C1-MOB2 was digested by both *Hind*III and *Kpn*I, two bands appeared in the gels, with the larger band at about 3945 bp and the smaller one at 734 bp, showing that the eukaryotic expression vector had been successfully constructed. The results of nucleic acid sequencing indicated that the full-length Chinese MOB2 cDNA had been cloned successfully. BLAST analysis demonstrated that the cloned sequence was 100% homologous to the previously reported MOB2 sequence.

Detection of MOB2 expression

Western blotting indicated that uninfected SMMC-7721 cells and empty-vector-infected SMMC-7721 cells showed only faint traces of MOB2 gene product. In contrast, a bright MOB2 band was obtained from pEGFP-C1-MOB2-infected SMMC-7721 cells. Western blotting indicated that 72 h after transfection with pEGFP-C1-MOB2, MOB2 protein expression increased by 3.8 times compared with that of the control group ($P < 0.01$). These results suggested that the transduction of the MOB2 gene was successful and that expression of the protein was significantly higher after transfection (Figure 1).

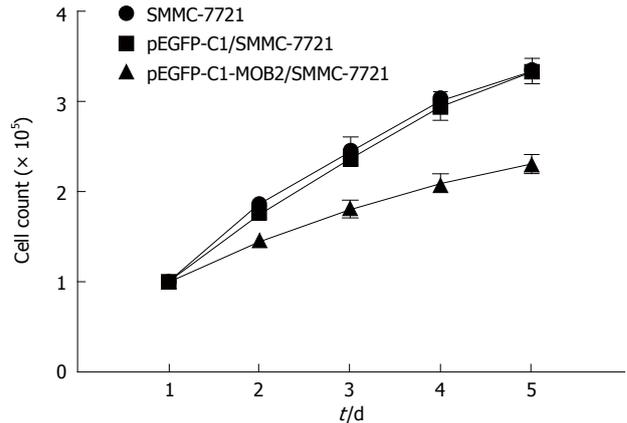


Figure 2 Effects of pEGFP-C1-MOB2 transfection on proliferation of SMMC-7721 cells.

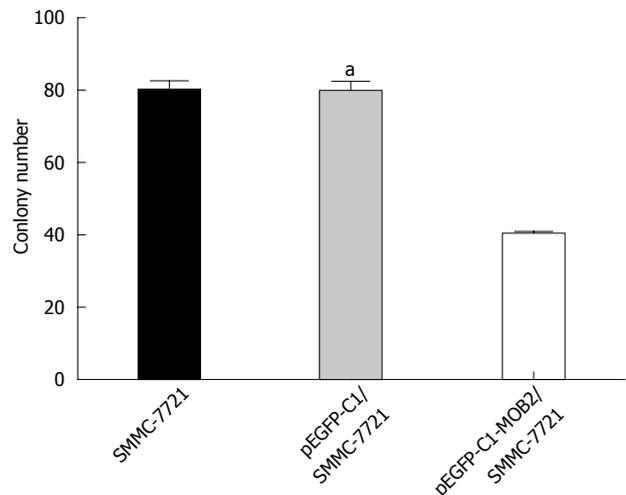


Figure 3 The growth of SMMC-7721 cells was examined by plate colony formation assay. ^a $P < 0.05$ when compared with pEGFP-C1-MOB2 cells and control vector cells.

MOB2 inhibits cell proliferation in vitro

To analyze the function of MOB2, we studied the rate of cell proliferation of MOB2-expressing SMMC-7721 cells. The growth curves, as determined by a trypan blue staining assay, showed that MOB2 significantly inhibited proliferation of SMMC-7721 cell lines when compared with parental SMMC-7721 cells and control clone cells (Figure 2). A colony formation assay showed that MOB2-overexpressing SMMC-7721 cells formed significantly fewer colonies than control clone cells ($P < 0.001$) (Figure 3), suggesting an inhibitory effect of MOB2 on human hepatic carcinoma SMMC-7721 cells.

Inhibition of cell cycle by MOB2

To determine the effect of MOB2 on the cell cycle, we measured cell cycle distribution in MOB2-expressing SMMC-7721 cells. In these cell lines, the S-phase population was markedly decreased while the G1 population was significantly increased compared with the control-vector cells and the SMMC-7721 cells ($P < 0.01$). Neither

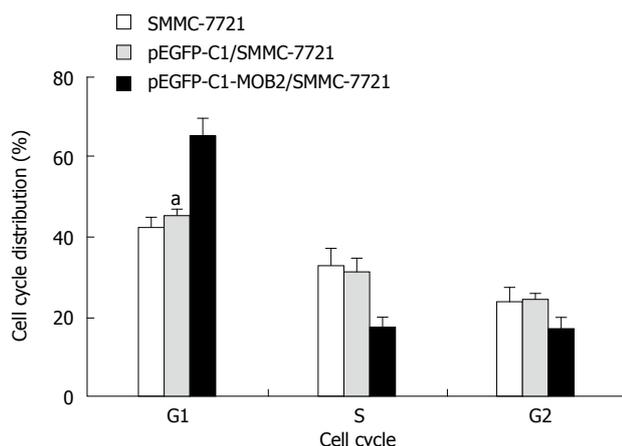


Figure 4 The cell cycle in parental SMMC-7721 cells, control vector cells and pEGFP-C1-MOB2 cells was determined by FACS Caliber cytometry. ^aP < 0.05 when compared with pEGFP-C1-MOB2 cells and control vector cells.

cell line had significant changes in the G2 population (Figure 4, Table 1).

DISCUSSION

Ndr kinase belongs to a subfamily of serine/threonine protein kinases that control cell division and morphogenesis in various cell types^[13]. Ndr2 is induced in the mouse amygdala during fear memory consolidation. Further studies have demonstrated that this kinase plays an important role in neurite formation^[14]. Tricornered, the Ndr kinase ortholog in *Drosophila*, interacts with actin filaments and regulates dendritic tiling and branching^[11]. SAX-1, the ortholog of Ndr kinase in nematodes, activates the RhoA-GTPase signaling pathway during neurite formation^[14]. These results strongly indicate the importance of Ndr kinases in neurite formation. Mob proteins have been shown to bind Ndr kinases, making them important in kinase activation^[6]. These results suggest that MOB2 might play a suppressive role in tumor pathogenesis.

To examine whether MOB2 plays a suppressive role in cancer cells, we applied a gain-of-function approach by introducing MOB2 into cells to investigate its biological function. SMMC-7721 cells were transfected with a MOB2-pEGFP-expressing eukaryotic vector and selected using G418. We successfully established cell lines that stably expressed MOB2 protein at dramatically elevated levels compared with control cells. Subsequent functional studies demonstrated that overexpression of MOB2 led to G1/S transition blockage and significantly reduced *in vitro* cell growth.

A tumor suppressor gene is a gene that slows down cell growth and thus reduces the probability that a cell in a multicellular organism will turn into a tumor cell. A mutation or deletion of such a gene increases the probability of the formation of a tumor and fails to keep cancer from growing. The results of the present study indicate that the MOB2 gene plays a role in carcinogenesis and may be a candidate tumor suppressor gene. Furthermore,

Table 1 Overexpression of MOB2 retarded cell cycle progression from G1 to S phase

Group	Cell cycle		
	G1	S	G2
SMMC-7721	42.41 ± 2.32	32.52 ± 4.53	23.54 ± 3.42
pEGFP-C1/SMMC-7721	45.45 ± 1.32	31.3 ± 3.11	24.24 ± 1.32
pEGFP-C1-MOB2/SMMC-7721	65.33 ± 4.34	17.33 ± 2.65	16.82 ± 3.14

our results clearly suggest that overexpression of MOB2 can inhibit proliferation and result in apoptosis of ovarian cancer cells. Therefore, reactivation of the MOB2 signaling pathway might provide an effective gene therapy strategy for human hepatic carcinoma.

COMMENTS

Background

Some reports suggest that knockdown of MOB2 expression causes abnormal actin rearrangement and results in abnormal rhabdomere formation in *Drosophila*. However, few reports exist regarding the effects in MOB2 on cancer cells. In this study, the authors investigated what role the MOB2 proteins play in the human hepatic carcinoma cell line, SMMC-7721.

Innovations and breakthroughs

In this study, by cloning the MOB2 gene and transferring it into hepatocellular carcinoma cell line SMMC-7721, the authors investigated the growth-inhibiting and apoptosis-inducing effects of MOB2 gene on carcinoma cells and concluded that the over-expression of MOB2 gene could induce apoptosis and inhibit the growth of hepatic carcinoma cell line SMMC-7721.

Applications

These results suggest that MOB2 functions as an inhibitor of hepatic carcinoma and might serve as a new novel biological target to cure hepatic carcinoma.

Terminology

Mps-one-binder (Mob) family proteins belong to a conserved gene family found in yeast, *Drosophila*, and mammals. In *Drosophila*, MOB2 acts as a tumor suppressor through its function as a key component of the Hippo signaling pathway.

Peer review

In this study, the authors applied a gain-of-function approach to examine the biological processes regulated by MOB2 in SMMC-7721 cells. They demonstrated the functional importance of MOB2 in the suppression of SMMC-7721 cell growth. This is an important result, and it can bring out a new field in clinical tumor treatment.

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Ex-situ liver surgery without veno-venous bypass

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Abstract

AIM: To evaluate the results of hepatic resection with *ex-situ* hypothermic perfusion and without veno-venous bypass.

METHODS: In 3 patients with liver tumor, the degree of the inferior vena cava and/or main hepatic vein involvement was verified when the liver was dissociated in the operation. It was impossible to resect the tumors by the routine hepatectomy, so the patients underwent *ex-situ* liver surgery, vein cava replacement and hepatic autotransplantation without veno-venous bypass. All surgical procedures were carried out or supervised by a senior surgeon. A retrospective analysis was performed for the prospectively collected data from patients with liver tumor undergoing *ex-situ* liver surgery, vein cava replacement and hepatic autotransplantation without veno-venous bypass. We also compared our data with the 9 cases of Pichlmayr's group.

RESULTS: Three patients with liver tumor were analysed. The first case was a 60-year-old female with a huge haemangioma located in S1, S4, S5, S6, S7 and S8 of liver; the second was a 64-year-old man with cholangiocarcinoma in S1, S2, S3 and S4 and the third one was a 55-year-old man with a huge cholangiocarcinoma in S1, S5, S7 and S8. The operation time for the three patients were 6.6, 6.4 and 7.3 h, respectively. The anhepatic phases were 3.8, 2.8 and 4.0 h. The volume of blood loss during operation were 1200, 3100, 2000 mL in the three patients, respectively. The survival periods without recurrence were 22 and 17 mo in the first two cases. As for the third case complicated with postoperative hepatic vein outflow obstruction, emergency hepatic vein outflow extending operation and assistant living donor liver transplantation were performed the next day, and finally died of liver and renal failure on the third day. Operation time (6.7 ± 0.47 h vs 13.7 ± 2.6 h) and anhepatic phase (3.5 ± 0.64 h vs 5.7 ± 1.7 h) were compared between Pichlmayr's group and our series ($P = 0.78$).

CONCLUSION: *Ex-situ* liver resection and liver autotransplantation has shown a potential for treatment of complicated hepatic neoplasms that are unresectable by traditional procedures.

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Key words: Liver autotransplantation; *Ex-situ* resection; Total vascular exclusion; Liver tumor

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INTRODUCTION

In 1988, *ex vivo* liver resection and liver autotransplantation were pioneered by Pichlmayr *et al*^[1], who tried to open a new platform for unresectable hepatobiliary malignancies and explore new modalities for liver surgery. In 1990, the whole liver was explanted and then perfused with cold preservation solution. The hepatic resection was performed on the back table and the remnant liver was reimplanted orthotopically. The aim of the extracorporeal hypothermic parenchymal resection was to extend oncological radicality by achieving an R0 margin with a better tumor-free cutting edge. But *ex-situ* liver surgery with veno-venous bypass aggravated ischemia-reperfusion injury of liver and resulted in the liver failure, which is the most common reason for the operation abortion. Subsequently, Hannoun *et al*^[2] and Sauvanet *et al*^[3] endeavored to improve the *ex-situ* technique. However, it is still difficult to avoid the ischemia-reperfusion injury and liver failure. To resolve this problem, we performed *ex-situ* liver surgery without veno-venous bypass in three patients with tumors involving inferior vena cava (IVC) based on the animal experiments. We introduce our experience in this operation as follows.

MATERIALS AND METHODS

Patients

From December 1999 to December 2008, a total of 1700 patients with hepatic tumors underwent routine hepatectomy at our hepatobiliary department. The extent of IVC and/or main hepatic vein (MHV) involved by the tumors was assessed by preoperative radiographic inspection. However, the degree of the tumor-involved IVC and/or main hepatic vein could not be confirmed by the preoperative inspection in three patients. Finally, it was verified when the liver was dissociated during the operation. The tumors were unresectable by the routine hepatectomy, so we performed *ex-situ* liver surgery, vein cava replacement and hepatic autotransplantation without veno-venous bypass. All surgical procedures were carried out or supervised by a senior surgeon (Dong JH). Patients' conditions are summarized in Table 1. There were two males and one female, with a mean age of 60 years (range, 55-65 years). Two patients suffered from cholangiocarcinoma and the other one from hemangioma.

Preoperative assessment

All patients were examined with computed tomography (CT) as indicated to exclude extrahepatic malignant disease of the abdomen, pelvis or chest. The location of the tumor and extent of involvement of intrahepatic vessel and IVC (Table 1) were assessed before surgery with CT (Figure 1A) and magnetic resonance imaging (Figure 1B). Cardiovascular, renal and pulmonary fitness for surgery was evaluated with an exercise electrocardiogram, chest radiography, and pulmonary function testing in all patients.

Table 1 General conditions of the three patients with liver tumor

No.	Sex	Age (yr)	Disease	Segments involved	Tumor diameter (cm)	Vessels involved or infiltrated
1	Female	60	Hemangioma	1, 4, 5, 6, 7, 8	20	IVC (circum 190°, length 5 cm)
2	Male	64	Cholangiocarcinoma	1, 2, 3, 4	6	IVC (circum 80°, length 3 cm)
3	Male	55	Cholangiocarcinoma	1, 5, 7, 8	5	IVC (circum 60°, length 2 cm) RHV, MHV, RPV

IVC: Inferior vena cava; RHV: Right hepatic vein; MHV: Main hepatic vein; RPV: Right portal vein; Circum: Circumference.

Anesthetic management

This type of surgery should be performed always with the presence of anesthesiologists who have experience with liver resection and are able to manage prolonged IVC occlusion and the changes that occur with liver reperfusion. During surgery, all patients were monitored by standard non-invasive techniques. In addition, a Swann-Ganz catheter and an arterial line should be placed before surgery in the patients who planned to undergo total vascular exclusion (TVE) with hypothermic perfusion. Body warmers were routinely employed to prevent hypothermia intraoperatively.

Method and techniques

We routinely used a bilateral subcostal incision which provided adequate exposure for almost all types of liver resection. After mobilization of liver, a double examination of the liver by palpation and ultrasonography was performed to confirm the number and size of the lesions, to define their relationship to intrahepatic vascular structures, exclude undiagnosed liver metastases and lastly to determine the resection line.

The approach of vascular control was chosen depending on the tumor location and extent of tumor involvement. The retrohepatic cava was partially occluded by a side clamp^[4] if the extent of involved IVC was small ($\leq 60^\circ$ circumferentially and ≤ 2 cm longitudinally). However, the TVE was needed to guarantee clear surgical field^[4-8] if the IVC was involved in a larger extent. The technique of TVE we used involves mobilization of the liver, exposure and control of the suprahepatic and infrahepatic vena cava as well as the portal structures (portal vein and hepatic artery)^[9,10]. *En bloc* liver and a segment of the vena cava were removed finally (Figure 2A).

IVC was quickly replaced with a 20-mm ringed polytetrafluoroethylene (PTFE) graft if it was infiltrated by the tumor. After that, IVC was unclamped (Figure 2B).

Hypothermic hepatic perfusion and bench hepatectomy: After parenchymal transection was completed, the liver was perfused and harvested as described for whole organ procurement. The hilar structures were divided on the bench. After cannulation of the portal and arterial

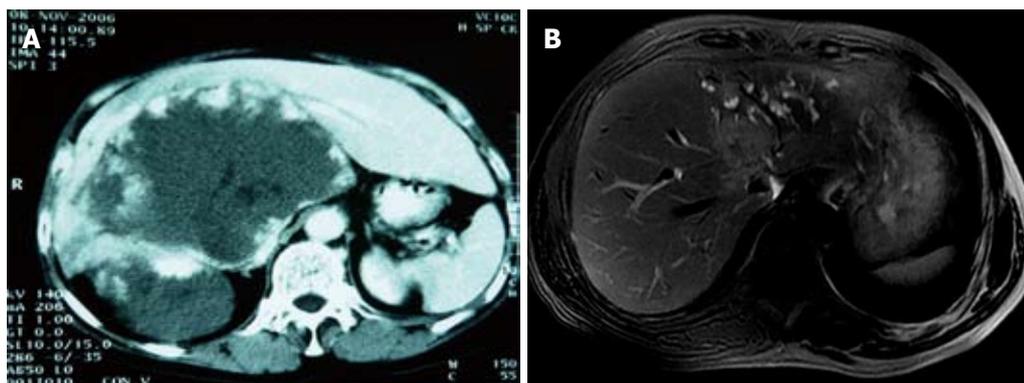


Figure 1 Computed tomography and magnetic resonance imaging of tumor. A: Preoperative computed tomography scan (case 1) demonstrated a large hemangioma located in S1, S4, S5, S6, S7 and S8 of liver, and inferior vena cava (IVC) was involved and compressed severely (IVC circumference 190°, longitude 5 cm); B: Magnetic resonance imaging (case 3) showed the tumor located in S1, S5, S7 and S8, with a diameter of 5 cm; IVC (circumference 60°, longitude 2 cm), right hepatic vein thrombus, main hepatic vein as well as right portal vein were infiltrated by the tumor.

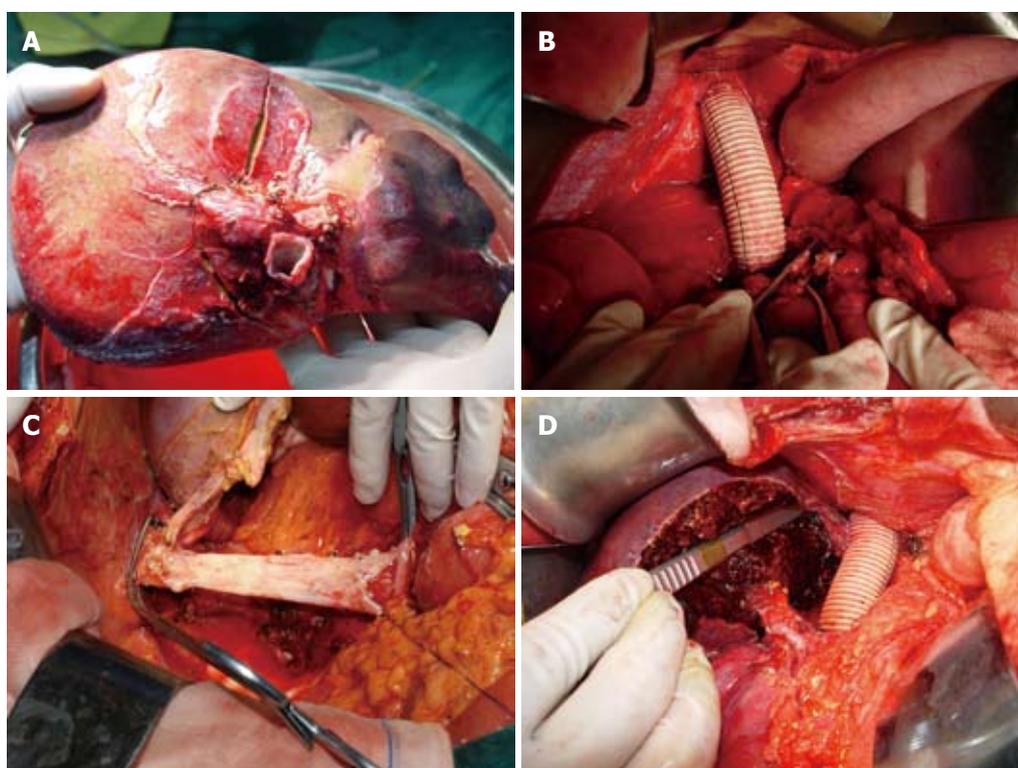


Figure 2 Main technical aspects of *ex-situ* liver surgery without veno-venous bypass. A: Bench hepatectomy-cutting line of removed liver (case 3); B: Infiltrated inferior vena cava was replaced with a 20-mm ringed polytetrafluoroethylene graft; C: Reconstruction of inferior vena cava when polytetrafluoroethylene graft was removed (case 1); D: Anastomosis was performed in excised portal vein, hepatic artery and hepatic duct of residual right hepatic lobe (case 2).

systems, the liver was flushed with preservation solution [histidine-tryptophan-ketoglutarate (HTK)-Bretschneider] at 4 °C and packed with ice immediately. The volume of HTK used was 1 L. Proper hepatic artery and common bile duct were flushed respectively as well. The tumor was excised with cavipulse ultrasonic surgical aspirator and dipolar electro-surgical unit under intraoperative ultrasonographic guide, and the involved blood vessels were partially excised, repaired, formed and reconstructed. Figure 2C shows that the involved IVC was peeled off, trimmed and replaced anastomosed PTFE graft in right

trisegmentectomy in the case of hemangioma.

The hepatic resection was performed on the back table and the remnant liver was reimplanted orthotopically (Figure 2D). After meticulous hemostasis, a fibrin glue sealant was sprayed on the cut surface. After irrigation, a closed suction drain was inserted, and the wound was closed in layers.

Statistical analysis

Fisher's exact tests were used to compare variables between Pichlmayr *et al*^[1] and the present series. All statisti-

Table 2 Details of *ex-situ* hepatectomy without venous bypass in the three patients

No.	Hepatectomy	Segments resected	IVC reconstruction	Operation time (h)	Anhepatic phase (h)	Blood loss (mL)	Complication	Hospital stay (d)	Outcome
1	<i>Ex vivo</i> hepatectomy with reimplantation of segment 2 and 3	1, 4, 5, 6, 7, 8	Synthetic graft and autologous IVC	6.6	3.8	1200	Pleural effusion	28	Alive without disease 22 mo after operation
2	<i>Ex vivo</i> hepatectomy with reimplantation of segment 4b, 5, 6, 7, 8	1, 2, 3, 4	Synthetic graft	6.4	2.8	3100	No	38	Alive without disease 17 mo after operation
3	<i>Ex vivo</i> hepatectomy with reimplantation of segment 2, 3, 4	1, 5, 6, 7, 8	Synthetic graft	7.3	4.0	2000	Liver and renal failure	11	Died

IVC: Inferior vena cava.

Table 3 Difference of operation time and anhepatic phase between our study and Pichlmayr's venous bypass group

	n	Operation time (h)	Anhepatic phase (h)	Postoperative death
Pichlmayr's	9	13.7 ± 2.6 (11-18)	5.7 ± 1.7 (4-9)	3
Present series	3	6.7 ± 0.47 (6.4-7.3)	3.5 ± 0.64 (2.8-4.0)	1

cal analyses were performed using Stat View 5.0J (Abacus Concepts, Berkeley, CA). $P < 0.05$ was defined as significant difference.

RESULTS

Neither major perioperative surgical nor anesthesiological complications were observed and no respiratory complications were noticed during surgery or the immediate postoperative period. Patient general features and tumor characteristics are summarized in Table 1. The intraoperative clinical outcomes and postoperative clinical course are summarized in Table 2. The first case was a 60-year-old female with a huge hemangioma located in S1, S4, S5, S6, S7 and S8 of the liver; the second was a 64-year-old man with cholangiocarcinoma in S1, S2, S3 and S4 and the third one was a 55-year-old man with a huge cholangiocarcinoma in S1, S5, S7 and S8 (Table 1). The operation time for the three patients were 6.6, 6.4 and 7.3 h, respectively. The anhepatic phases were 3.8, 2.8 and 4.0 h. The volume of blood loss during operation were 1200, 3100 and 2000 mL, respectively. The survival period without recurrence was 22 and 17 mo in the first two cases, respectively. As for the third case, which was complicated with hepatic vein outflow obstruction after operation, emergency hepatic vein outflow extending operation and assistant living donor liver transplantation (LDLT) were performed the next day, and finally he died of liver and renal failure on the third day. Operation time (6.7 ± 0.47 h *vs* 13.7 ± 2.6 h) and anhepatic phase (3.5 ± 0.64 h *vs* 5.7 ± 1.7 h) were compared between Pichlmayr *et al*^[11] and our series (Table 3) ($P = 0.78$).

DISCUSSION

Ex-vivo liver resection and liver autotransplantation represents a new approach for liver surgery. We simplified the operation by omitting the veno-venous bypass, which shortened the operative time and anhepatic period.

Most liver tumors can be removed with conventional resection techniques using partial or total vascular occlusion when needed. But it is difficult to resect the central liver or posterior segments where hepatic veins are involved. The procedure would result in massive hemorrhage or air embolism if it was performed in such patients.

The accessory left hepatic artery should be occluded carefully, or it will lead to hepatic congestion or excessive bleeding. Huguet *et al*^[9] and Choi *et al*^[10] reported that TVE resulted in blood congestion of IVC and portal vein definitely and caused a series of physiopathologic problems. The congested blood re-enters the circulation and aggravates the injury of ischemia-reperfusion when the influx was recovered, especially in patients with liver cirrhosis. The mortality can reach up to 75% after the hepatectomy using the TVE method.

With the development of surgical techniques, the veno-venous bypass has been applied to complicated hilum hepatectomy^[11,11]. The blood in portal vein and IVC was by-passed by the bio-pump to ensure the stability of general circulation and lessen the ischemia-reperfusion injury when the TVE was performed. The total veno-venous bypass technique enhances the tolerance of liver to warm ischemia. In this condition, the safe time limit of liver tolerance to total vascular occlusion seems to be 30-120 min^[7,12,13], which is short for extensive and complex tumors in a direct contact with major vascular structures.

To overcome this limitation and to reduce liver damage, the concept of hypothermic perfusion has come into consideration. This technique enables liver resection to proceed *in situ*, *ante situm* or *ex vivo*. The liver was filled by Belzer's University of Wisconsin solution. The tolerance to the ischemia phase is extended to 4 h with hypothermic liver perfusion. Azoulay *et al*^[14] completed complicated liver resections using combined veno-venous bypass and *in situ* hypothermic perfusion, and they testified that the duration of liver tolerance to ischemia could last 90

min at least in 85% cases. The IVC must be replaced and reconstructed if it is infiltrated by tumors extensively.

Ante situm liver resection (*in vivo* hypothermic perfusion and veno-venous bypass) has been applied widely to resect the tumors located in hepatic hilum, confluence of hepatic vein and IVC, but veno-venous bypass was used during the operation which led to severe water, electrolyte and acid-base imbalance during the first post-operative week. Azoulay *et al*^[14] reported that the mortality from *ex-situ* liver resection with hypothermic perfusion (28%) was higher than that from *in vivo* hepatectomy (8%). We reviewed the pertinent literature published from 1992 to 2007 and found that 41 cases underwent *ex-situ* liver surgery with hypothermic perfusion. But only one patient underwent *ex-situ* liver surgery with hypothermic perfusion without veno-venous bypass, and the patient is still alive^[15]. However, 10 patients died after the resection with veno-venous bypass with a mortality of 24%^[15-22]. Cabezuelo *et al*^[23] suggested that, in the resection with veno-venous bypass, the hemodynamic stability of the body and regional organs (kidney, gastrointestinal tract) was improved during the anhepatic phase, but the rate of kidney failure was not decreased. The procedure without veno-venous bypass has been verified to be feasible and safe during the past few years. The cost was reduced because this technique shortens the total operation time, reduces hemorrhage during operation and decreases the rates of reperfusion injury.

Taking into account these controversial facts and the deficiency of liver graft, we performed *ex-situ* liver surgery, vein cava replacement and hepatic autotransplantation without veno-venous bypass in three cases. The general conditions of the three cases are described in Table 1.

The first case is a patient with a huge hemangioma located in S1, S4, S5, S6, S7 and S8 (Figure 1A) where IVC was involved and compressed severely. It is likely to lead to grave hemorrhage if IVC is dissected in the operation because of involvement of IVC (circumference 190°, longitude 5 cm). The other two cases were patients with cholangiocarcinoma located at S1, S2, S3 and S4 and S1, S5, S7 and S8, respectively. The extension of infiltrated IVC was large (circumference $\geq 60^\circ$, longitude ≥ 2 cm)^[6,24].

Although the tumor diameter was about 5 cm, the IVC, right hepatic vein, MHV and right portal vein were involved in the third patient. And the aim of cholangiocarcinoma resection was to achieve an R0 margin with a better tumor-free cutting edge. So it is difficult to complete the resection in a short time if the *in situ* or *ante situm* procedure was performed. But the *ex-situ* liver resection with hypothermic perfusion could decrease the chance of blood disseminated, and provide more chances to achieve an R0 margin^[25].

We have accumulated much experience in performing complicated hepatectomy and liver transplantation. Before we performed the *ex-situ* liver surgery under hypothermic perfusion and without veno-venous bypass, we had done the operation in the animal experiments with the protocol approved by the ethics committee of

the hospital. In order to shorten the operation time, we repaired the IVC, and performed the *ex-vivo* hepatectomy when the IVC was resected at back table in the first case. The segment S2 and S3 was autotransplanted when the PTFE graft was removed. The operation time and the anhepatic phase were 6.6 and 3.8 h, respectively.

For the other two patients with cholangiocarcinoma, we performed *ex-vivo* hepatectomy with liver reimplantation. The segment 4b, 5, 6, 7 and 8 and segment 2, 3 and 4 were autotransplanted, respectively. Their operation time was 6.4 and 7.3 h and anhepatic phase was 2.8 and 4.0 h, respectively.

We compared our data with the Pichlmayr *et al*^[11] of 9 cases undergoing *ex-vivo* hepatectomy with veno-veno bypass. Our procedure shortened the time of surgery and anhepatic phase because we avoided the veno-venous bypass, although there was no significant difference between the different techniques ($P = 0.78$), possibly due to the small number of cases. Furthermore, no hepatic dysfunction resulting from the delayed liver hemoperfusion occurred in our group after operation. But in the 9 cases of Pichlmayr *et al*^[11], four cases suffered from hepatic dysfunction because of the delayed liver hemoperfusion. Although 3 cases received liver transplantation, they were all died of liver failure finally. In our study, the patients 1 and 2 are still alive without recurrence. The third case was complicated with acute liver failure because of outflow obstruction of hepatic vein after operation. Although emergency hepatic vein outflow extending operation and assistant LDLT were performed the next day, he died of liver and kidney failure at last. Therefore, it is important to reconstruct the outflow tract of hepatic vein for *ex-vivo* hepatectomy.

In conclusion, the *ex-situ* liver surgery under hypothermic perfusion and without veno-venous bypass extends the group of surgical patients, and offers a chance for the patients with unresectable liver tumor by traditional procedures. But further clinical studies are needed to verify the feasibility of this technique in clinical practice.

COMMENTS

Background

Primary liver malignancies together with metastatic liver tumors are the most common tumor types in human. Experimental studies are being conducted in hepatic resection with *ex-situ* hypothermic perfusion if huge lesions are located in hepatic hilum or hepatic vein confluence, which involved and/or infiltrated main hepatic vein and inferior vena cava (IVC).

Research frontiers

In some patients, the degree of the IVC and/or main hepatic vein involved by tumors is so extensive that it is impossible to resect the tumors by the routine hepatectomy. The *ex-situ* liver surgery, vein cava replacement and hepatic autotransplantation without veno-venous bypass have been performed.

Innovations and breakthroughs

The extracorporeal hypothermic parenchymal resection was to extend the oncological radicality by achieving an R0 margin with a better tumor-free cutting edge. But *ex-situ* liver surgery with veno-venous bypass aggravates ischemia-reperfusion injury of liver and results in liver failure. To resolve this problem, the authors performed *ex-situ* liver surgery without veno-venous bypass in three cases with tumors involving IVC based on animal experiments.

Applications

Ex-vivo liver resection and liver autotransplantation has shown a potential for treatment of complicated hepatic neoplasms that are unresectable by traditional procedures.

Peer review

The authors described their experience in hepatic resection with total vascular exclusion, *ex-situ* liver surgery, without the need of veno-venous bypass because of immediate replacement of inferior vena cava. The topic is of interest in the specific surgical field.

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Comparison between air and carbon dioxide insufflation in the endoscopic submucosal excavation of gastrointestinal stromal tumors

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Abstract

AIM: To evaluate the safety and efficacy of CO₂ insufflation compared with air insufflation in the endoscopic submucosal excavation (ESE) of gastrointestinal stromal tumors.

METHODS: Sixty patients were randomized to undergo endoscopic submucosal excavation, with the CO₂ group ($n = 30$) and the air group ($n = 30$) undergoing

CO₂ insufflation and air insufflation in the ESE, respectively. The end-tidal CO₂ level (pETCO₂) was observed at 4 time points: at the beginning of ESE, at total removal of the tumors, at completed wound management, and 10 min after ESE. Additionally, the patients' experience of pain at 1, 3, 6 and 24 h after the examination was registered using a visual analog scale (VAS).

RESULTS: Both the CO₂ group and air group were similar in mean age, sex, body mass index (all $P > 0.05$). There were no significant differences in PetCO₂ values before and after the procedure ($P > 0.05$). However, the pain scores after the ESE at different time points in the CO₂ group decreased significantly compared with the air group (1 h: 21.2 ± 3.4 vs 61.5 ± 1.7 ; 3 h: 8.5 ± 0.7 vs 42.9 ± 1.3 ; 6 h: 4.4 ± 1.6 vs 27.6 ± 1.2 ; 24 h: 2.3 ± 0.4 vs 21.4 ± 0.7 , $P < 0.05$). Meanwhile, the percentage of VAS scores of 0 in the CO₂ group after 1, 3, 6 and 24 h was significantly higher than that in the air group (60.7 ± 1.4 vs 18.9 ± 1.5 , 81.5 ± 2.3 vs 20.6 ± 1.2 , 89.2 ± 0.7 vs 36.8 ± 0.9 , 91.3 ± 0.8 vs 63.8 ± 1.3 , respectively, $P < 0.05$). Moreover, the condition of the CO₂ group was better than that of the air group with respect to anal exsufflation.

CONCLUSION: Insufflation of CO₂ in the ESE of gastrointestinal stromal tumors will not cause CO₂ retention and it may significantly reduce the level of pain, thus it is safe and effective.

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Key words: Carbon dioxide insufflation; Endoscopic submucosal excavation; Gastrointestinal tract; Stromal tumor; Treatment

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common tumors of mesenchymal tissue in the digestive system, with a property of non-directional differentiation. They are formed as a result of the overproliferation of immature spindle cells or epithelioid cells^[1], and characterized by overexpression of CD117 and CD34 based on the pathology. Thus, they can be distinguished from other mesenchymal tissue tumors such as leiomyoma, leiomyosarcomas, Schwann tumors and neurofibroma, *etc.*

GISTs appear unexpectedly, and the significance of EUS in the diagnosis and differentiation of GISTs has now been defined^[2]. However, GISTs should be considered as tumors inclined to recur and metastasize because their potential malignancy is difficult to predict. GISTs which are derived from the mucous layer and submucosa in the digestive tract are usually referred to endoscopic treatment, but lesions originating from the muscularis propria are difficult to completely resect; furthermore, multiple complications such as acute perforation can occur.

In recent years, with rapid advances in endoscopic submucosal excavation (ESE) to treat GISTs, ESE has been the preferred choice instead of surgical excision and follow-up observation as in the past. However, abdominal pain and abdominal discomfort after operation are apparent in patients undergoing ESE with the insufflation of air. Using CO₂ as a replacement for air could mean a remarkable improvement, because CO₂ can be absorbed by the gastrointestinal mucosa rapidly and be discharged from the body by respiration.

Foreign experts and scholars have begun investigating the application of CO₂ in endoscopic submucosal dissection^[3], endoscopic retrograde cholangiopancreatography^[4], as well as in double-balloon enteroscopy^[5], *etc.* So far, however, there has no report about the effect of endoscopic submucosal excavation with the insufflation of CO₂. In this study, we evaluate the safety and efficacy of CO₂ insufflation in ESE compared with the insufflation of air as control. We conducted this work as a prospective, comparative pilot study.

MATERIALS AND METHODS

Patients

From March 2011 to February 2012, 61 patients diagnosed as having submucosal lesions of the digestive tract derived

from muscularis propria distinguishable by endoscopic ultrasound (EUS) and computed tomography (CT) (26 were transferred from other hospitals) were enrolled in this study. One patient diagnosed with rectal malignant mesenchymoma with liver metastasis was not in conformity with the inclusion criteria. Sixty patients signed the medical informed consent form before the ESE. All 60 patients gave their consent to be randomized to undergo endoscopic submucosal excavation with insufflation of air ($n = 30$) or CO₂ ($n = 30$) by means of random numbers generated from the computer. The 60 patients were clinically manifested by abdominal discomfort, abdominal mass and hematochezia without intestinal obstruction; 13 of the 60 patients did not experience any noticeable symptoms.

The exclusion criteria were as follows: malignant GISTs with metastasis; intestinal obstruction; vascular invasion; large lesion (> 10 cm) that failed complete resection; young age (< 14 years) and incapable of finishing the relevant questionnaire; chronic obstructive pulmonary disease patients with the retention of CO₂; acute digestive tract hemorrhage or variation from normal; shock with various causes; severe cardiopulmonary cerebral diseases; inability to tolerate the preoperative preparation; allergic to propofol; pregnancy, breast-feeding, *etc.*

Experimental equipment

The main instruments used included a gastroscope (GIF-Q260J, Olympus, Japan), enteroscope (CF-Q260AI, Olympus, Japan), ultrasound gastroscope (UE-260, Olympus, Japan), CO₂ air-insufflation equipment (UCR, Olympus, Japan), needle (INJ1-A1-07.160, Medwork, Germany), hot biopsy forceps (FD-1U-1, Olympus, Japan), hemoclips (HX-610-135 and HX-610-090, Olympus, Japan), needle knife (3281, Boston, America), TT knife (KD-640L, Olympus, Japan), snare (99052012225MW, MTW/Edoscopic, Germany), high-frequency electric knife (ICC-200, ERBE, Germany) and argon plasma coagulation (APC300, ERBE, Germany). A transparent cap (D-201-11802/D-201-13404/D-201-10704, Olympus, Japan) was added to the tip of the endoscope during the endoscopic submucosal excavation.

Experimental methods

Intravenous anesthesia was executed by an anesthetist, who used propofol (AstraZeneca) to maintain general anesthesia. Routine oxygen treatment was performed (2-3 mL/min); meanwhile, heart rate, respiration, blood pressure, blood oxygen saturation and PetCO₂ were continuously monitored under anesthesia. Propofol was started as normal with 1.5-2.5 mg/kg, observing the change of life signs. Routine endoscopy was performed as soon as the absence of consciousness was seen, while respiration, heart rate, blood oxygen saturation, *etc.*, were essentially normal. Additional propofol at 0.3-0.5 mg/kg was given to sustain proper sedation in the event that the patient had a reaction.

No eating or drinking for 8 h prior to surgery was allowed. EUS was performed to ensure the stage and identification of the main lesions. CT examination was also used to observe the composition of the tumor and the

Table 1 Patient characteristics

Group	CO ₂ insufflation (n = 30)	Air insufflation (n = 30)	P value ¹
Age, yr, mean ± SD	52.1 ± 5.1	50.9 ± 6.6	0.83
Sex (male/female)	17/13	16/14	0.80
Mean BMI (kg/m ²)	21.63	21.79	0.54
Previous surgeries, n (%)			
Any prior abdominal surgery	17 (57)	16 (53)	0.80
Cholecystectomy	9 (30)	11 (37)	0.58
Hysterectomy	3 (10)	2 (7)	
Liver transplantation	2 (7)	1 (3)	
Other ²	5 (17)	2 (7)	0.30

¹By χ^2 for categorical variables and *t* test for continuous variables; ²Other surgeries include ovarian cystectomy^[2], appendectomy^[2], laparoscopic gastrectomy^[1]. BMI: Body mass index.

relationship with the surrounding organs and vessels, in order for it to be distinguished from other lesions. Like GISTs, advanced gastric cancer or gastric lymphoma can also grow outward, while GIST always showed the uneven thickening of stomach wall, obvious local invasion as well as the swelling of perigastric, hilar and abdominal lymph nodes, with an evenly enhanced mass. Patients with GISTs originating from the muscularis propria without metastasis to other regions were treated by endoscopic submucosal excavation under anesthesia.

The standard operating procedures were as follows: (1) Marking: it is recommended to carry out electrical coagulation at the edge of the distinguished lesion marked by use of an argon knife; (2) Submucosal insufflation: multiple parts of the submucosa outside the marker points were irrigated with normal saline (including methylene blue and epinephrine); (3) Incising mucosa at the edge of lesion: a needle knife was employed to spot-incise up till the submucosa, and a TT knife was used to incise the mucosa along the lateral margin of the marking point; (4) Excision: a snare was used to enclose the mucosa and submucosa of the lesions and expose the muscularis propria, then the lesion was excised along the edge with a TT knife. If lesions which were clinging to the serosa layer could not be excised completely, it was recommended to perform full-thickness isolation and carry out a perforation initiative. The wound was then observed carefully to see if there were tumors remaining. No remaining tumors existed under endoscopy. Subsequently all the removed tissues were sent for pathological examination to rule out positive margins, which proved that the tumor was excavated completely; and (5) Wound management: for the small blood vessels which were visible in the wound, electrocoagulation hemostasis was recommended by use of argon knife, and if need be, the wound was closed by suture with a metallic hemostatic clamp, as well as by spraying tissue glue on the wound to prevent hemorrhaging.

In order to ensure the double-blind nature of the trial, both the endoscope and the valve of the CO₂ insufflation equipment were covered by black cloth. Someone was put in charge of the valve of the insufflation equipment and

the switch of the gas pump; both the operator and the patient did not know what type of gas had been used.

Measuring of PetCO₂

Studies have shown that the partial pressure of end-tidal carbon dioxide (PetCO₂) and the arterial partial pressure of carbon dioxide [p(CO₂)] of a normal adult are very similar and close to each other, so that [p(CO₂)] is usually replaced by PetCO₂ because of its noninvasive characteristic^[6]. In this trial, we use the portable CO₂ analyzer (ULT-1, Datex-Ohmeda, Finland) to measure PetCO₂. The PetCO₂ was measured by nurses randomly at the following four time-points: at beginning of ESE, at total removal of the tumors, after completed wound management, and 10 min after ESE.

Grading of abdominal pain

The 100 mm visual analog scale (VAS) was applied to grade pain according to the varying degrees of severity^[7]. The spectrum of VAS is 0-100; the minimal point is 0 which means no pain, the maximal one is 100 which means unbearable agony. Patients' abdominal pain was assessed at 1, 3, 6 and 24 h after ESE. Consequently, questionnaires were collected at the endoscopy center in our hospital.

Statistical analysis

Statistical analysis was performed by using the software SPSS13.0 (SPSS Inc, Chicago, IL, United States). The results were expressed as mean ± SD, variables in the two groups were analyzed with a Student's *t* test. The comparison of mean VAS at all time points was analyzed with the nonparametric, rank-sum test for two independent samples (Wilcoxon, 1945). Percentage of pain scores of 0 at each time point between the two groups were preceded with chi-square test. The value of PetCO₂ at each time point was analyzed through the repeated measures of analysis of variance. *P* value < 0.05 was considered statistically significant.

RESULTS

Comparison of baseline characteristics between the two groups

All 60 patients completed the study protocol. Thirty patients were enrolled in the CO₂ group (17 males, 13 females, mean age 52.1 years) and 30 patients in the air group (16 males, 14 females, mean age 50.9 years). The body type was indicated by body mass index; in the CO₂ group the value was 21.63 kg/m², while in the air group this was 21.79 kg/m², there was no statistical difference between the two groups (*P* > 0.05). The actual clinical data are shown in Table 1.

Endoscopic submucosal excavation treatment in the two groups

The patients' diseased regions in the CO₂ group consisted of esophagus (4/30), stomach (22/30), rectum (2/30) and sigmoid colon (2/30), while in the air group, the diseased regions included esophagus (6/30), stomach (21/30),

Table 2 Endoscopic submucosal excavation treatment characteristics

Group	Location (n)	Diameter (cm)	Operating time (min)	Success rate (%)	Full-thickness isolation (n)
CO ₂	Esophagus (4)	1.7	41	100	3
	Stomach (22)	1.3	23		
	Rectum (2)	1.5	40		
	Sigmoid colon (2)	1.9	47		
Air	Esophagus (6)	1.1	43	100	2
	Stomach (21)	0.7	31		
	Rectum (2)	0.9	38		
	Sigmoid colon (1)	1.7	51		

Table 3 Comparison of PetCO₂ between the two groups

Time point	CO ₂ group (mmHg)	Air group (mmHg)	P value
Beginning of ESE	34.01 ± 2.03	33.32 ± 2.21	0.78
Removal of the tumors	31.21 ± 2.35	30.59 ± 2.73	0.73
Wound management	32.75 ± 2.69	32.01 ± 2.22	0.92
10 min after ESE	33.23 ± 2.56	32.61 ± 2.78	0.79

ESE: Endoscopic submucosal excavation.

rectum (2/30) and sigmoid colon (1/30). The mean diameters of tumors of the CO₂ group and the air group were 1.6 ± 0.3 cm (range 0.5-5.0 cm) and 1.2 ± 0.5 cm (range 0.5-4.0 cm), respectively. The mean operating time was 35 ± 12 min in the CO₂ group, and this was 41 ± 10 min in the air group. The success rate for complete resection of tumor was 100%. Both the CO₂ group and the air group had light intraoperative bleeding; the mean bleeding volume was approximately 10 mL, without postoperative bleeding. Five cases were diagnosed with extraluminal type under endoscopic ultrasonography; it was recommended to perform full-thickness isolation and a perforation initiative for 3 cases of 5 belonging to the CO₂ group, and for 2 cases of 5 belonging to the air group. There was no statistical difference in the ESE between the two groups (*P* > 0.05). The actual clinical data are shown in Table 2.

Comparison of PetCO₂ between the two groups

The value of PetCO₂ was compared at the following four time points: beginning of ESE, at removal of the tumors, at completed wound management, and 10 min after ESE. The value was expressed as mean ± SD; the clinical data are shown in Table 3. From the mean value at each time point above, we could conclude that the value of PetCO₂ at each time point between the two groups had no statistical difference (*P* > 0.05, Figure 1A).

Comparison of abdominal pain between the two groups after revival from anesthesia

The VAS was applied to evaluate the level of abdominal pain of the patients at the following four time points^[8]: 1, 3, 6 and 24 h after anesthesia revival, respectively. The results showed that there was a significant difference of the value of VAS at 1, 3 and 6 h after revival from anesthesia between the two groups (*P* < 0.05, Figure 1B).

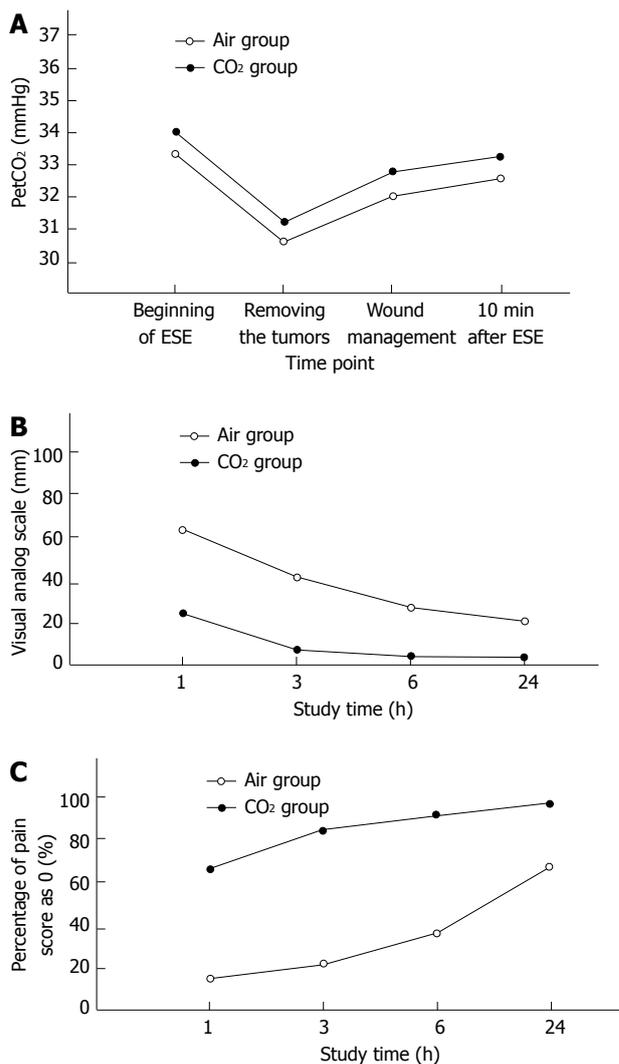


Figure 1 The comparison of PetCO₂ (A), visual analog scale (B) and percentage of pain score of 0 (C) at each time point between the CO₂ group and the air group. ESE: Endoscopic submucosal excavation.

Moreover, a comparison of the value of VAS at 24 h after revival from anesthesia between the two groups still had statistical difference. From Figure 1B, we can see that there was a slow decline in the air group, while the curved line declined more obviously in the CO₂ group (1 h: 21.2 ± 3.4 vs 61.5 ± 1.7; 3 h: 8.5 ± 0.7 vs 42.9 ± 1.3; 6 h: 4.4 ± 1.6 vs 27.6 ± 1.2; 24 h: 2.3 ± 0.4 vs 21.4 ± 0.7, *P* < 0.05). Furthermore, the curved line returned to the baseline at 6 h after anesthesia revival in the CO₂ group; however, in the air group the curved line never returned to baseline. The percentage of VAS scores of 0 at each time point was subjected to chi-square test, and the result demonstrated that the percentage of VAS scores of 0 in the CO₂ group was significantly higher than that in the air group (1 h: 60.7 ± 1.4 vs 18.9 ± 1.5; 3 h: 81.5 ± 2.3 vs 20.6 ± 1.2; 6 h: 89.2 ± 0.7 vs 36.8 ± 0.9; 24 h: 91.3 ± 0.8 vs 63.8 ± 1.3, respectively, *P* < 0.05, Figure 1C). Consequently, in the ESE of gastrointestinal stromal tumors, the condition of abdominal pain at each time point after revival from anesthesia could be clearly aggravated by the application of CO₂.

Comparison of anal exsufflation between the two groups

In checking the anal exsufflation of patients in the two groups at 1, 2 and 4 h after treatment, only 21% of the patients in the CO₂ group had anal exsufflation at 1 h after anesthesia revival, while 7% lasted for 2 h or more. However, 73.8% of the patients in the air group had anal exsufflation, and nearly 14.3% had a moderate or great amount of flatus, 28.6% lasted for 4 h or more. A comparison of the two groups at 1, 2 and 4 h after revival from anesthesia was carried out with a chi-square test, and a *P* value < 0.01 was found which was considered statistically significantly different. The amount of anal exsufflation had a negative correlation with the degree of abdominal pain and distension; furthermore, it also had a negative correlation with recovery time of abdominal distension in the air group.

DISCUSSION

GISTs should be considered as potentially malignant tumors owing to their unpredictable recurrence and metastasis; however, there are no definite clinical criteria for the diagnosis and treatment of GISTs^[9]. EUS, especially an EUS-fine needle aspiration, plays an important part in the diagnosis of GISTs, can determine the nature of submucosal lesions of the digestive tract and is instructive in the choice of treatment methods. GISTs with a diameter of 3-5 cm shown in the endoscopic examination and by pathology are more likely to be malignant; therefore, such GISTs are supposed to be thoroughly surgically excised^[10-14]. Although large GISTs are more inclined to be malignant, the small ones also have the possibility, so it is irrational to regard tumor size as the only standard for the malignancy of GISTs^[11,15]. In this study, we defined the risk classification of GISTs according to the National Institutes of Health^[16]. Consequently, the GISTs with definite diagnosis should be treated as much as possible.

Nowadays, a variety of surgical methods (as well as chemotherapy) for the treatment of GISTs are recognized in foreign and domestic studies. Surgical operation is still the traditional treatment; many patients with GISTs have been reported as being excised by undergoing laparoscopy^[17,18], and it is significantly important to excise larger lesions by surgical treatment. Imatinib, a tyrosine kinase inhibitor, is currently being used to treat GISTs which have unique kinase mutations that serve as targets for medical therapy, but some disadvantages exist such as high cost of therapy, long-term treatment and indeterminate side-effects; meanwhile few studies are reported about the treatment for GISTs with unclear symptoms^[19,20]. However, endoscopic therapy for these is much rarer. Choosing the treatment for GISTs that has lesser invasive injury and lower cost under endoscopy is rather clinically valuable.

Endoscopic mucosal resection (EMR) can be applied to the treatment of patients with distinguishable lesions of the digestive tract, such as early carcinoma and submucosal tumor. Moreover, EMR has not only the same therapeutic effect as surgical operation, but a short oper-

ating time, short hospitalization time, rapid recovery and low medical costs. However, it is hard to accomplish *en bloc* resection by the use of EMR for those lesions whose size is 2 cm or more. As a result, the remains are likely to recur and lead to many complications such as bleeding and perforation. Compared with EMR, ESE is able to excise a large majority of GISTs and provide intact data for pathological diagnosis. For preoperative evaluation of benign stromal tumors whose size is 5 cm or less, ESE is able to accomplish *en bloc* resection. ESE fully demonstrates the superiority of minimally invasive surgery as it has the advantage of rapid recovery, short hospitalization time and low medical costs. In our study, ESE was preferable for the GISTs originating from the muscularis propria, but not from the muscularis mucosae.

ESE is appropriate for GISTs originating from the muscularis propria; however, too much air insufflation because of a long operating time leads to pain for the patients in various degrees after revival from anesthesia. Pain caused by abdominal distension is the most common type, resulting from gastrointestinal gaseous tension. Therefore, it is recommended to select inhaling CO₂ instead of air, as the CO₂ is easily soluble in blood and other body liquids. It is not only rapidly absorbed by the gastrointestinal tract, but easily eliminated from the body by respiration. Patients never appear to have a metabolic disorder such as CO₂ retention. Yamano *et al*^[21] has reported that the usage of CO₂ in enteroscopy could effectively alleviate the subjective pain of patients. In summary, our study investigated the comparison between the application of CO₂ and air insufflation for the ESE operation; the postoperative subjective pain of patients was measured by VAS and results suggested that the absolute VAS was lower in the CO₂ group than in the air group, and the number of patients with severe postoperative pain was also fewer in the CO₂ group.

We compared the value of PetCO₂ at the following four time points: beginning of ESE, at total removal of the tumors, at completed wound management, and 10 min after ESE. From the above data, we could draw conclusions that there were no significant differences of PetCO₂ at each time point between the two groups, suggesting that CO₂ is not able to cause postoperative retention as well not influencing the safety during the operation.

Comparing postoperative anal exsufflation between the two groups, the results revealed that the time of anal exsufflation in the CO₂ group is shorter than that in the air group, and that the flatus of patients in the CO₂ group is also less, which demonstrates that CO₂ is much easier to be absorbed. Both the difficulty of operation and the ratio of various related complications will increase in the case of the existence of a large amount of remaining gas.

The GISTs partly derived from muscularis propria are diagnosed as extraluminal type or clinging to the serosa by EUS. Those tumors clinging to the serosa layer cannot be excised completely by ESE; it is suggested to perform full-thickness excision and bring out a perforation initiative. In our study, there were five patients with full-thickness excision of GISTs who had little gas entry

into the abdominal cavity so that there was less obvious abdominal pain, and no postoperative abnormal conditions happened compared with other patients by ESE. However, the patients with full-thickness excision among the air group had severe abdominal pain as well as long-term gastrointestinal decompression.

In summary, CO₂ insufflation could effectively alleviate the pain of patients when the GISTs were excised by ESE, without the risk of CO₂ retention. The safety of CO₂ insufflation is comparable to that of air insufflation, and less pain exists after operation. Therefore, it is hopeful that CO₂ insufflation will become the standard method for ESE with full-thickness excision and it is apparent that this method will be widely applied in the future.

COMMENTS

Background

Gastrointestinal stromal tumors are the most common tumors of mesenchymal tissue in the digestive system. In recent years, endoscopic submucosal excavation has been used to treat gastrointestinal stromal tumors (GISTs) instead of surgical excision. The application of CO₂ in endoscopic submucosal excavation (ESE) could reduce the complications of the procedure effectively.

Research frontiers

Foreign experts and scholars have begun investigating the application of CO₂ in endoscopic submucosal dissection, endoscopic retrograde cholangiopancreatography, as well as in double-balloon enteroscopy, *etc.* So far, there has not been a report about the effect of endoscopic submucosal excavation with the insufflation of CO₂. In this study, the authors evaluate the safety and efficacy of CO₂ insufflation in ESE compared with the insufflation of air as control.

Innovations and breakthroughs

In this study, the authors have detailed the superiority of CO₂ insufflation in ESE. Compared with air insufflation, the pain scores after ESE at different time points in the CO₂ group decreased significantly. Meanwhile, the percentage of visual analog scale (VAS) scores of 0 in the CO₂ group after 1, 3, 6 and 24 h was significantly higher than that in the air group. Moreover, the condition of the CO₂ group was better than that of the air group in respect of anal exsufflation.

Applications

CO₂ insufflation could effectively alleviate the pain of patients when GISTs are excised by ESE without the risk of CO₂ retention. Therefore, it is hopeful that CO₂ insufflation will become the standard method for ESE with full-thickness excision and it will certainly be widely applied in the future.

Peer review

The authors examined the application of CO₂ insufflation in endoscopic submucosal excavation. The results suggested that the postoperative pain of patients measured by VAS seems to be lower in the CO₂ group than that in the air group, and the time of anal exsufflation in the CO₂ group is also shorter than that in the air group. So, CO₂ insufflation may be the standard method for the ESE with full-thickness excision in the future.

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Predictors and clinical outcomes for spontaneous rupture of hepatocellular carcinoma

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Abstract

AIM: To determine the risk factors for hepatocellular carcinoma (HCC) rupture, and report the management and long-term survival results of patients with spontaneous rupture of HCC.

METHODS: Among 4209 patients with HCC who were diagnosed at Eastern Hepatobiliary Surgery Hospital from April 2002 to November 2006, 200 (4.8%) patients with ruptured HCC (case group) were studied retrospectively in term of their clinical characteristics and prognostic factors. The one-stage therapeutic approach to manage ruptured HCC consisted of initial management by conservative treatment, transarterial embolization (TACE) or hepatic resection. Results of various treatments in the case group were evaluated and compared with the control group (202 patients) without ruptured HCC during the same study period. Continuous data were expressed as mean \pm SD or me-

dian (range) where appropriate and compared using the unpaired *t* test. Categorical variables were compared using the Chi-square test with Yates correction or the Fisher exact test where appropriate. The overall survival rate in each group was determined using the Kaplan-Meier method and a log-rank test.

RESULTS: Compared with the control group, more patients in the case group had underlying diseases of hypertension (7.5% vs 3.0%, $P=0.041$) and liver cirrhosis (87.5% vs 56.4%, $P<0.001$), tumor size >5 cm (83.0% vs 57.4%, $P<0.001$), tumor protrusion from the liver surface (66.0% vs 44.6%, $P<0.001$), vascular thrombus (30.5% vs 8.9%, $P<0.001$) and extrahepatic invasion (36.5% vs 12.4%, $P<0.001$). On multivariate logistic regression analysis, underlying diseases of hypertension ($P=0.002$) and liver cirrhosis ($P<0.001$), tumor size >5 cm ($P<0.001$), vascular thrombus ($P=0.002$) and extrahepatic invasion ($P<0.001$) were predictive for spontaneous rupture of HCC. Among the 200 patients with spontaneous rupture of HCC, 105 patients underwent hepatic resection, 33 received TACE, and 62 were managed with conservative treatment. The median survival time (MST) of all patients with spontaneous rupture of HCC was 6 mo (range, 1-72 mo), and the overall survival at 1, 3 and 5 years were 32.5%, 10% and 4%, respectively. The MST was 12 mo (range, 1-72 mo) in the surgical group, 4 mo (range, 1-30 mo) in the TACE group and 1 mo (range, 1-19 mo) in the conservative group. Ninety-eight patients in the control group underwent hepatic resection, and the MST and median disease-free survival time were 46 mo (range, 6-93 mo) and 23 mo (range, 3-39 mo) respectively, which were much longer than that of patients with spontaneous rupture of HCC undergoing hepatic resection ($P<0.001$). The 1-, 3-, and 5-year overall survival rates and the 1-, 3- and 5-year disease-free survival rates in patients with ruptured HCC undergoing hepatectomy were 57.1%, 19.0% and 7.6%, 27.6%, 14.3% and 3.8%, respectively, compared with those of 77.1%, 59.8% and 41.2%, 57.1%, 40.6% and 32.9% in 98 patients with-

out ruptured HCC undergoing hepatectomy ($P < 0.001$).

CONCLUSION: Prolonged survival can be achieved in selected patients undergoing one-stage hepatectomy, although the survival results were inferior to those of the patients without ruptured HCC.

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Key words: Hepatocellular carcinoma; Spontaneous rupture; Predictors; Hepatectomy; Overall survival; Disease-free survival

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common neoplasms, and its incidence is increasing worldwide because of the increasing prevalence of hepatitis B and C virus infection^[1,2]. One of the life-threatening complications of HCC is the spontaneous rupture of the tumor, with intra-peritoneal hemorrhage. Spontaneous rupture of HCC occurs in 3%-26% of all patients with HCC, and the mortality rates are high in a range of 32%-66.7%^[3-7].

There are few reports of predictors for spontaneous rupture of HCC^[8-10]. In addition, clinicians often feel helpless when facing these complicated situations, and little information is available in the literature to guide clinicians as to the most appropriate management of this complication. The prognosis of ruptured HCC was poor, a median survival of 1.2-4 mo if left untreated^[11], but some studies have reported better survival with staged hepatectomy^[3]. There is still a debate concerning the best approach in cases of HCC rupture^[12]. The following treatments have been employed for the ruptured HCC: hepatic resection, conservative treatment and transcatheter arterial embolization (TACE)^[8,11-14]. It is also unclear whether the clinical outcome of definitive treatment including hepatic resection was affected by the complication of tumor rupture.

We, therefore, conducted this retrospective study to determine the risk factors for HCC rupture, and to report the management of patients with spontaneous rupture of HCC and long-term survival results during a 5-year period at a single center in China.

MATERIALS AND METHODS

Patients

From April 2002 to November 2006, 4209 patients with

HCC visited Eastern Hepatobiliary Surgery Hospital. Among them, 200 patients (4.8%) had hemoperitoneum due to spontaneous rupture of the tumor. The clinical records of these 200 patients (case group) were retrospectively reviewed and compared with 202 patients without ruptured HCC (control group) who were randomly chosen by matching age, sex and time of admission from the patients who visited our hospital in the same period.

All patients had a chest X-ray, ultrasonography (USG) of abdomen, and contrast computed tomography (CT) or magnetic resonance imaging (MRI) of abdomen. Laboratory blood tests were performed, including antigen to hepatitis B surface, antibodies to hepatitis C, alpha-fetoprotein (AFP), carcinoembryonic antigen, carbohydrate antigen 19-9, serum albumin, total bilirubin, aspartate aminotransferase, alanine aminotransferase, prothrombin time and so on. Progression profile of HCC (number of tumors, maximum tumor size, macroscopic tumor thrombus and extrahepatic invasion), treatments employed, and survival time were recorded. The liver function status was evaluated using the Child-Pugh classification system. HCC was staged according to the tumor node metastasis (TNM) staging system proposed by the American Joint Committee on Cancer (AJCC)^[15].

HCC was diagnosed by at least two radiologic imaging examinations showing characteristic features of HCC, or one radiologic imaging examination showing characteristic features of HCC associated with AFP ≥ 400 ng/mL. Ruptured HCC was diagnosed by the identification of a space-occupying lesion(s) in the liver using USG, CT or MRI. The following CT findings are useful for diagnosing a ruptured HCC: hemoperitoneum, HCC with surrounding perihepatic hematoma, active extravasation of contrast materials, tumor protrusion from the hepatic surface, focal discontinuity of the hepatic surface and an enucleation sign^[5].

Therapeutic options were determined for each patient according to the tumor feature and liver function. Selected patients with resectable tumor(s) and well preserved liver function were considered for surgery, and these patients with Child-Pugh class A liver function underwent major hepatic resection, while those patients with Child-Pugh class B liver function received minor hepatic resection. Patients with unresectable tumor(s) and well preserved liver function were considered for TACE, while those with poor liver function (Child-Pugh class C) were suggested to receive conservative treatment.

Statistical analysis

Information of patient details, intraoperative parameters, postoperative course, and disease progress was collected and analyzed retrospectively. Continuous data were presented as mean \pm SD or median (range) where appropriate and compared using the unpaired *t* test. Categorical variables were compared using the χ^2 test with Yates correction or the Fisher exact test where appropriate. Factors associated with a *P* value < 0.05 in the univariate analysis were sequentially entered into the multivariate

Table 1 Univariate analysis of risk factors related to spontaneous rupture of hepatocellular carcinoma

Variables	Case group (n = 200)	Control group (n = 202)	P value
Age (yrs)	47.9 ± 12.4	50.5 ± 11.4	0.209
Sex			0.322
Male	184	180	
Female	16	22	
Diabetes			0.388
Presence	8	5	
Absence	192	197	
Hypertension			0.041
Presence	15	6	
Absence	185	196	
Liver cirrhosis			< 0.001
Yes	175	114	
No	25	88	
Liver function status			0.096
Child-Pugh class A	133	157	
Child-Pugh class B	49	32	
Child-Pugh class C	18	13	
PT (s)	13.8 ± 2.3	13.3 ± 1.5	0.328
PLT (×10 ⁹ /L)	161.4 ± 94.8	159.1 ± 64.6	0.187
TB (μmol/L)	34.4 ± 40.6	30.0 ± 6.7	0.097
Albumin (g/L)	37.2 ± 5.8	36.8 ± 4.2	0.101
ALT(IU/L)	86.0 ± 91.6	59.4 ± 48.1	0.261
AST(IU/L)	136.2 ± 176.4	59.8 ± 47.5	0.643
Creatinine (μmol/L)	72.6 ± 20.2	74.3 ± 18.7	0.209
AFP (μg/L)	586.6 (1.3-95600.5)	499.2 (1.8-10521.6)	0.125
Positive HBsAg status	181	166	0.514
Maximum tumor size (cm)			< 0.001
≤ 5	34	86	
> 5	166	116	
Tumor location			0.927
Right lobe	142	146	
Left lobe	38	38	
Both lobes	20	18	
Protrusion from the liver surface			< 0.001
Yes	132	90	
No	68	112	
Vascular thrombus			< 0.001
Presence	61	18	
Absence	139	184	
Ascites			0.301
Presence	24	16	
Absence	176	186	
Extrahepatic invasion			< 0.001
Yes	73	25	
No	127	177	

PT: Prothrombin time; PLT: Platelet count; TB: Total bilirubin; AFP: α-feto-protein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

logistic regression analysis to indicate the relatively independent risk factors.

Group comparisons were performed using the Chi-square test for independence or the Fisher exact test for comparisons of the two groups. The overall survival rate in each group was determined using the Kaplan-Meier method and a log-rank test. The survival period defined as the length of time from the onset of the spontaneous rupture of the HCC to the death of the patient or the closing date of the study. The closing date for the study was November 31, 2011.

Table 2 Multivariate analysis for factors related with spontaneous rupture of hepatocellular carcinoma

Variables	HR	95%CI for HR	P value
Hypertension (presence <i>vs</i> absence)	7.75	2.10-28.57	0.002
Liver cirrhosis (presence <i>vs</i> absence)	7.16	3.96-12.97	< 0.001
Tumor size (≤ 5 cm <i>vs</i> > 5 cm)	3.97	2.28-6.93	< 0.001
Protrusion from the liver surface (yes <i>vs</i> no)	6.51	2.31-10.84	0.008
Vascular thrombus (presence <i>vs</i> absence)	2.90	1.48-5.70	0.002
Extrahepatic invasion (yes <i>vs</i> no)	3.78	2.04-7.00	< 0.001

HCC: Hepatocellular carcinoma; HR: Hazard ratio; CI: Confidence interval.

RESULTS

Clinical data between the case group and the control group are presented in Table 1. The initial symptoms of spontaneous rupture of HCC were the sudden onset of abdominal pain (134 patients, 67%), shock at admission (102 patients, 51%), and abdominal distension (66 patients, 33%).

Risk factors related to spontaneous rupture of HCC

Compared with the control group, more patients in the case group had underlying diseases of hypertension (7.5% *vs* 3.0%, *P* = 0.041) and liver cirrhosis (87.5% *vs* 56.4%, *P* < 0.001), tumor size > 5 cm (83.0% *vs* 57.4%, *P* < 0.001), tumor protrusion from the liver surface (66.0% *vs* 44.6%, *P* < 0.001), vascular thrombus (30.5% *vs* 8.9%, *P* < 0.001) and extrahepatic invasion (36.5% *vs* 12.4%, *P* < 0.001) (Table 1). On multivariate logistic regression analysis, underlying diseases of hypertension and liver cirrhosis, tumor size > 5 cm, tumor protrusion from the liver surface, vascular thrombus and extrahepatic invasion remained predictive for spontaneous rupture of HCC (Table 2).

Management of patients with spontaneous rupture of HCC

Among the 200 patients with spontaneous ruptured HCC, 126 (63%) had stage I or II HCCs according to the TNM staging system proposed by the AJCC^[15]. After recovery from the initial insult with blood replacement, correction of coagulopathy and cardiovascular monitoring and complete clinical evaluation, 105 patients were considered suitable for curative hepatic resection which was performed on a median of 15 d (range, 7-35 d) after rupture, 33 received TACE, and 62 were given conservative treatment. Of the 105 patients considered for hepatic resection, 72 (58.1%) underwent major hepatic resection while the remaining ones received minor hepatic resection. Operative details of these 105 patients undergoing hepatectomy are shown in Table 3.

Acute hemorrhage was successfully controlled in all patients (100%) undergoing hepatic resection during the first hospital admission, compared with 31.0% patients receiving TACE and 16.5% patients receiving conservative treatment. One patient (0.9%) died of liver failure

Table 3 Operative detail of 105 patients undergoing hepatectomy

Detail	<i>n</i>
Type of hepatectomy	105
Minor hepatectomy	43
Major hepatectomy	62
Operating time (min)	166.8 ± 70.2
Duration of blood inflow occlusion (min)	16.9 ± 9.0
Blood loss (mL)	811.4 (50-5000)
Surgical margins	
R0 resection	92
R1 resection	12
R2 resection	1
Hospital mortality	1
Major complications	
Post-operative bleeding	0
Liver failure	1
Bile leak	1
Pleural effusion	51
Postoperative hospital stay (d)	17.3 ± 6.7

on day 20 after hepatic resection, 10 (30.3%) patients died of liver failure after TACE and 36 (58.1%) patients died of bleeding after conservative treatment within 30 days of hospitalization, respectively.

Survival analysis of patients with spontaneous rupture of HCC

At the closing date of the study, 187 patients with spontaneous rupture of HCC died, including 92 (87.6%) in the surgical group, 33 (100%) in the TACE group and 62 (100%) in the conservative group. The median survival time (MST) of all patients with spontaneous rupture of HCC was 6 mo (range, 1-72 mo), and the overall survival rates at 1, 3 and 5 years were 32.5%, 10% and 4%, respectively. The MST was 12 mo (range, 1-72 mo) in the surgical group, 4 mo (range, 1-30 mo) in the TACE group and 1 mo (range, 1-19 mo) in the conservative group. The 1-, 3-, and 5-year overall survival rates in the surgical group were 57.1%, 19.0% and 7.6%, respectively, while they were 12.1%, 0% and 0% in the TACE group and 1.6%, 0% and 0% in the conservative group. In addition, the 1-, 3-, and 5-year disease-free survival rates of surgical group were 27.6%, 14.3% and 3.81%, respectively, while they were 9.1%, 0% and 0% in the TACE group and none in the conservative group.

In the 98 patients in the control group who underwent hepatic resection, the MST and median disease-free survival time were 46 mo (range, 6-93 mo) and 23 mo (range, 3-39 mo), respectively, which were much longer than in the patients with spontaneous rupture of HCC undergoing hepatic resection ($P < 0.001$). Accordingly, the 1-, 3- and 5-year overall survival rates of these 98 patients were 77.1%, 59.8% and 41.2%, and the 1-, 3- and 5-year disease-free survival rates were 57.1%, 40.6% and 32.9%, both of which were significantly longer than that of patients with spontaneous rupture of HCC undergoing hepatic resection ($P < 0.001$) (Figure 1).

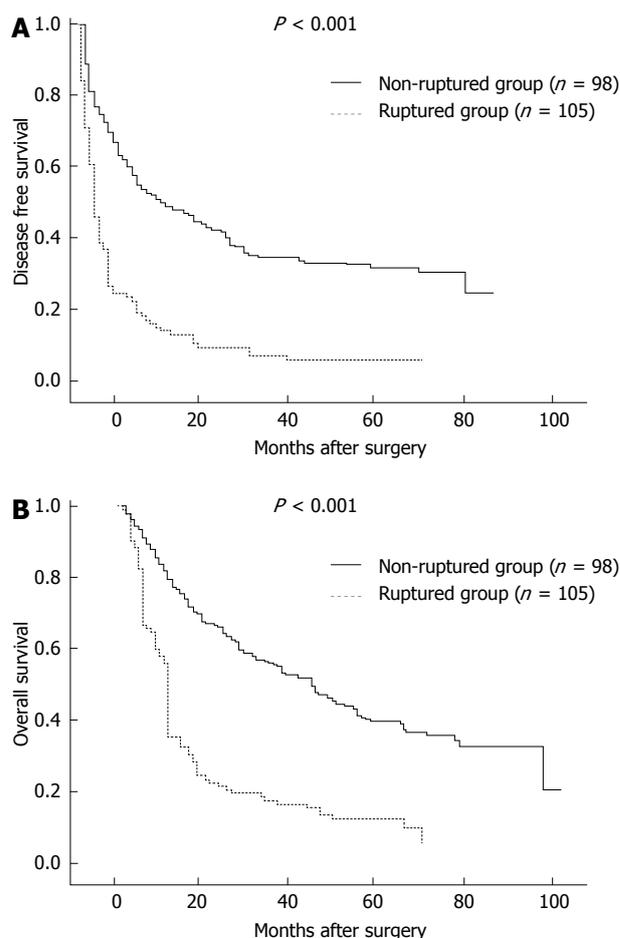


Figure 1 Overall survival and disease-free survival in patients with and without ruptured hepatocellular carcinoma undergoing hepatic resection.

DISCUSSION

As a life-threatening complication of HCC, spontaneous rupture of HCC is one of the most common emergencies in liver surgery. Seeking for the risk factors failed to conclude a widely accepted result^[5,16].

A widely accepted hypothesis for the mechanism of rupture of HCC suggests that rupture is usually preceded by rapid expansion of the tumor secondary to bleeding from within its substance, and a high intratumoral pressure due to occlusion of the hepatic venous outflow by tumor invasion results in rupture^[9]. In our study, we found that tumor protrusion from the liver surface, vascular thrombus and extrahepatic invasion were predictive for spontaneous rupture of HCC, which were consistent with previous studies^[5,9,16]. The protruded tumor without surrounding normal parenchyma may easily rupture due to compression or friction to the adjacent diaphragmatic muscle, abdominal wall and gastrointestinal tracts. Therefore, the presence of extrahepatic invasion more likely leads to rupture. Moreover, the maximum tumor size > 5 cm was one of the risk factors predicting rupture of HCC. However, in our study, rupture of tumors as small as 2 cm was found, which was consistent with a report by Tanaka *et al*^[17]. It is difficult to explain how a small HCC

located in the periphery of the liver would rupture by above mechanism. More recent studies suggested that underlying vascular dysfunction may play a role^[8]. The vessels in the ruptured HCC tend to be more friable due to increased collagenase expression and increased collagen IV degradation^[10]. This proposed mechanism may help to explain why some of the small tumors also rupture.

Interestingly, in our study, the presence of hypertension and liver cirrhosis remained predictive for spontaneous rupture of HCC. The reasons may be as follows. HCC is a vascular tumor and bleeding secondary to rupture can cause tearing of vessels with uncontrollable blood loss, hypertension can directly result in an increase of pressure within the tumor. In addition, patients with cirrhosis have underlying coagulopathy. Both of the two factors may promote the process of rupture described above.

Previous studies have shown a very poor prognosis, with a 30-d mortality rate in the range of 30%-70%^[13,19,21], and recent reports have indicated a significant decrease in the mortality rate. In our study, we observed an overall 30-d mortality rate of 23.5%, while the mortality rate was only 0.95% (one patient) among the patients in whom hepatectomy was successfully conducted.

In another series, the 1-year and 3-year survival rates for patients who underwent emergency resection were only 60% and 42%, respectively^[22]. Miyamoto *et al.*^[23] reported that half of the hospital mortality of emergency hepatectomy for ruptured HCC was due to liver failure. One group reported that only 12.5% of the patients with ruptured HCC could be managed with hepatic resection, while another group reported a percentage of 59.3%^[4,24,25]. In our study, which yielded similar results, 52.5% (105/200) of patients with ruptured HCC could be managed with hepatic resection. In two case series of delayed resection for ruptured HCCs from Japan, no in-hospital mortality was observed, and 1- and 3-year survival rates of 71%-77% and 48%-54%, respectively, were achieved^[26,27]. In our series, the 1-year, 3-year and 5-year survival rates were 57.1%, 19.0% and 7.6%, respectively, for the patients with one-stage resection, and the MST in the surgical group was 12 mo.

For patients with unresectable HCC, TACE was the best option for the treatment of patients with ruptured tumor, and the MST in the TACE group was significantly longer than that in the conservative group. Most of the patients in the conservative group died within 1 mo as a result of re-rupture, re-bleeding or hepatic failure.

Historically, the prognosis for ruptured HCC is thought to be worse than for non-ruptured HCC, due to multiple factors. The patients with ruptured HCC harbor advanced disease at presentation, the incidence of coexisting cirrhosis is high, and peritoneal seeding may occur at the time of rupture. However, recent studies have challenged this concept, stating that the long-term survival for ruptured HCC may be equivalent to non-ruptured HCC^[20], especially when the comparison was adjusted for tumor stage^[28]. However, in our study, patients with ruptured HCC had a much worse long-term prognosis

than those without.

In conclusion, spontaneous ruptured HCC is a life threatening condition and a commonly encountered surgical emergency. For HCC patients who had underlying diseases of hypertension and liver cirrhosis, extrahepatic invasion and tumor size > 5 cm, high propensity to rupture should be considered. As long as preoperative clinical evaluation meets surgery requirements, elective one-stage hepatectomy in patients with ruptured HCC should be suggested. Prolonged survival can be achieved in selected patients undergoing one-stage hepatectomy, although the survival results were inferior to those of the patients who did not have the complication of rupture.

COMMENTS

Background

Spontaneous ruptured hepatocellular carcinoma (HCC) is a life threatening condition and a commonly encountered surgical emergency. There are few reports of predictors for spontaneous rupture of HCC. It was also not clear whether the clinical outcome of definitive treatment including hepatic resection is affected by the complication of tumor rupture.

Research frontiers

Seeking for the risk factors failed to conclude a widely accepted result. Several recent studies reported better survival with staged hepatectomy, and challenged traditional concept, stating that the long-term survival for ruptured HCC may be equivalent to non-ruptured HCC. There is still a debate concerning the best approach in cases of HCC rupture.

Innovations and breakthroughs

In this study, the presence of hypertension and liver cirrhosis remained predictive for spontaneous rupture of HCC. Patients with ruptured HCC had a much worse long-term prognosis than those without.

Applications

The study results suggest that for HCC patients who had underlying diseases of hypertension and liver cirrhosis, extrahepatic invasion and tumor size > 5 cm, high propensity to rupture should be considered. Prolonged survival can be achieved in selected patients undergoing one-stage hepatectomy, although the survival results were inferior to those of the patients who did not have the complication of rupture.

Terminology

HCC is the most common primary malignant tumor of the liver which originates from liver parenchymal cells. Spontaneous rupture of HCC refers to the HCC ruptured without any sign.

Peer review

The authors analyzed the clinical data of patients with spontaneous ruptured HCC and non-ruptured HCC for detecting the risk factors for HCC rupture, and reporting the management and long-term survival results of patients with spontaneous ruptured of HCC. They determined the risk factors and evaluated the prognosis for spontaneous rupture of HCC. This conclusion well guided clinicians as to the most appropriate management of this complication. The study was carefully designed and the evaluation of the results was also appropriate. The manuscript was well organized and well written.

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Short-term outcomes of laparoscopic total mesorectal excision compared to open surgery

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Abstract

AIM: To investigate the short-term outcome of laparoscopic total mesorectal excision (TME) in patients with mid and low rectal cancers.

METHODS: A consecutive series of 138 patients with middle and low rectal cancer were randomly assigned to either the laparoscopic TME (LTME) group or the open TME (OTME) group between September 2008 and July 2011 at the Department of Colorectal Cancer of Shanghai Cancer Center, Fudan University and pathological data, as well as surgical technique were reviewed retrospectively. Short-term clinical and oncological outcome were compared in these two groups. Patients were followed in the outpatient clinic 2 wk after the surgery and then every 3 mo in the first year if no adjuvant chemoradiation was indicated. Statistical analysis was performed using SPSS 13.0 software.

RESULTS: Sixty-seven patients were treated with LTME and 71 patients were treated with OTME (sex ratio 1.3:1

vs 1.29:1, age 58.4 ± 13.6 years vs 59.6 ± 9.4 years, respectively). The resection was considered curative in all cases. The sphincter-preserving rate was 65.7% (44/67) vs 60.6% (43/71), $P = 0.046$; mean blood loss was 86.9 ± 37.6 mL vs 119.1 ± 32.7 mL, $P = 0.018$; postoperative analgesia was 2.1 ± 0.6 d vs 3.9 ± 1.8 d, $P = 0.008$; duration of urinary drainage was 4.7 ± 1.8 d vs 6.9 ± 3.4 d, $P = 0.016$, respectively. The conversion rate was 2.99%. The complication rate, circumferential margin involvement, distal margins and lymph node yield were similar for both procedures. No port site recurrence, anastomotic recurrence or mortality was observed during a median follow-up period of 21 mo (range: 9-56 mo).

CONCLUSION: Laparoscopic TME is safe and feasible, with an oncological adequacy comparable to the open approach. Further studies with more patients and longer follow-up are needed to confirm the present results.

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Key words: Rectum; Neoplasms; Colorectal surgery; Laparoscopy; Treatment outcome

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INTRODUCTION

Rectal cancer accounts for approximately half of all colorectal cancer cases in China. Significant advances have been achieved in the treatment of rectal cancer in the past

few decades. With the introduction of total mesorectal excision (TME), laparoscopic technique, as well as pre- and postoperative chemo-radiotherapy, the local control rate and survival of rectal cancer patients have dramatically improved. The TME principles, which were first described by Heald *et al.*^[1], are currently considered the standard practice for mid and low rectal cancer as local recurrence is reduced to less than 5%^[2]. A complete TME consists not only of the routine excision of intact mesorectum, but also preservation of the autonomic nervous system and the sphincters.

Laparoscopic surgery has been used in the treatment of colorectal cancer since the end of the 1990s with the purpose of ameliorating postoperative recovery without compromising oncological adequacy. Although laparoscopy in colon cancer has gained acceptance due to its proven benefits^[3,4], which include fewer perioperative complications, faster postoperative recovery and comparable survival rates, laparoscopy in rectal cancer is still not recommended as the treatment of choice by National Comprehensive Cancer Network guidelines. Laparoscopic rectal surgery is more complex and technically demanding, especially for mid and low rectal cancer. As surgical techniques and equipment have developed, the feasibility and safety of laparoscopic TME (LTME) have been reported by many institutes^[5,6]. Moreover, long-term survival following LTME seems to be comparable to open TME (OTME)^[7,8]. However, there are few well-designed studies which have addressed this particular issue. Most of the randomized clinical trials on rectal cancer were performed in the early 2000s when the laparoscopic rectal surgical technique was still being developed, and the majority of these studies included both colon and sigmoid cancer^[9,10]. Thus it is inclusive whether laparoscopic surgery for rectal cancer is comparable to open surgery.

The aim of this study was to compare the short-term outcomes of LTME and OTME in mid and low rectal cancers in a series of unselected patients. The primary endpoints were operative details (operating time, blood loss, sphincter preservative rates and conversions), perioperative complications (anastomotic leak, obstruction, and wound infections) and postoperative recovery (urinary function, use of analgesics, and return of bowel movement). The secondary endpoints were pathological evaluations (positive margin involvement, number of lymph nodes harvested and length of inferior margin).

MATERIALS AND METHODS

Patients

Between September 2008 and July 2011, a consecutive series of 138 patients diagnosed with middle and low rectal cancer underwent surgical treatment at the Department of Colorectal surgery of Shanghai Cancer Center, Fudan University. The diagnosis in these patients was confirmed by a full colonoscopy plus a biopsy. The inclusion criteria were: patients diagnosed with rectal cancer with the tumor located ≤ 10 cm from the anal verge.

All patients received a systematic preoperative assessment including physical examination, biochemical analysis and a five-marker panel (carcinoembryonic antigen, carbohydrate antigen 19-9, cancer antigen 724, cancer antigen 242 and cancer antigen 125 for female patients) assay. Chest, abdominal and pelvic computed tomography scans were performed to rule out any pulmonary or liver metastasis. In addition, magnetic resonance imaging or endorectal ultrasound was performed to evaluate the preoperative staging. Patients staged at cT3/4 cTxN+ were assigned to neoadjuvant treatment in the absence of contraindications, and therefore were excluded from this series. The exclusion criteria also comprised patients with a former history of radiotherapy or chemotherapy, patients with distant metastases and patients with contraindication to laparoscopic surgery. An American Society of Anesthesiologists (ASA) grade was assigned to each patient by an anesthetist before surgery. Low molecular weight heparin was administered subcutaneously as prophylactic anticoagulant treatment and D-dimer was monitored regularly at day 1, 4 and 7 postoperatively.

Follow-up

Patients were routinely followed in the outpatient clinic 2 wk after surgery and every 3 mo for the first year, then every 6 mo for the second year, and every year thereafter. If there was an indication for adjuvant chemoradiation, follow-up data was complemented by phone contact with the patients as well as contact with the patients' current treating physicians. Data were collected and reviewed retrospectively, including patients' demographics, preoperative staging, surgical technique, pathological evaluations and postoperative recovery.

Surgical technique

All patients underwent low anterior resection (LAR) or abdominoperineal resection (APR) according to accepted TME principles. All surgeries were performed by the same team of surgeons with proven expertise in colorectal cancer surgeries who perform more than 100 laparoscopic and open colorectal surgeries annually. All patients were operated on under general anesthesia.

Four trocars were introduced after CO₂ pneumoperitoneum was established at 12 to 15 mmHg. A 10-mm port was positioned 0.5 cm above the umbilicus for observation. Another 10-mm port was introduced one-third of the distance from the right anterior superior iliac spine to the navel as the major operative site. Two additional 5-mm ports were placed for assistance. One port was set one-third of the distance from the left anterior superior iliac spine to the navel. The other was positioned at a digit inferior to the umbilicus crossing the left parasternal line reserved for possible colostomy.

Firstly, the peritoneal cavity was inspected carefully for any metastasis or tumor implantation. Adopting a median-to-lateral approach, the sigmoid was held by the assistant to the left and the right mesorectum was dissected starting from the sacral promontory. Sharp dis-

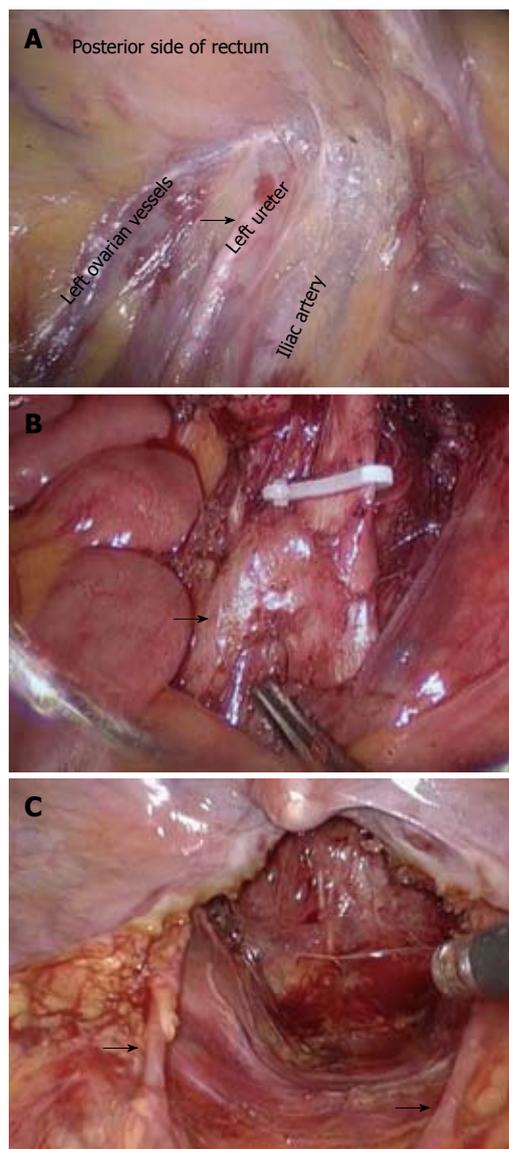


Figure 1 Surgical technique. A: Care must be taken not to injure the left ureter (arrow) while dissecting the inferior mesenteric artery; B: Careful preservation of the left colic artery (arrow); C: Preservation of bilateral sympathetic hypogastric nerves (arrows).

section continued along the “yellow-white boundary”, that is, between the adipose tissue in the sigmoid colon mesorectum and left peritoneum to the junction of the mesorectum. Dissection then proceeded as far as the origin of the inferior mesenteric artery (IMA). Care was taken not to injure the left ureter while dissecting the IMA (Figure 1A). The IMA and its concomitant vein were skeletonized and ligated using endoscopic clips close to the origin of the IMA. The left colic artery and vein were preserved (Figure 1B). It should be noted that the superior hypogastric plexus and sympathetic hypogastric nerves were at risk at this level (Figure 1C).

The next phase of surgery was pelvic dissection. The peritoneum was incised from the level of the sacral promontory posterior to the rectum down to the apex of the coccyx. It was important to recognize the loose

avascular plane between the parietal and visceral pelvic fascia before initiating sharp dissection of the presacral area. Anterior dissection occurred in the retrovesical septum in males and in the retrovaginal space in females. The rectosacral ligament and anococcygeal ligament were divided and incised at the level of the fourth sacral vertebra. The mesorectum was circumferentially mobilized while kept intact. A linear endoscopic cutting stapler was introduced to transect the rectum 2-5 cm below the tumor. Depending on the size of the lesion, a transverse incision of 3-4 cm was made to extract the specimen through a wound protector. The distal colon was transected 10 cm above the lesion. Before re-establishing the pneumoperitoneum, an anvil of the circular stapler was placed in the proximal colon and was fixed by a purse-string suture. The bowel was then returned to the peritoneal cavity, and the incision was sutured. The pneumoperitoneum was re-established. The last step of the procedure was to perform a low or ultra low rectal anastomosis with a circular stapler inserted transanally. The two tissue donuts created by the circular stapler were verified for integrity. The distal donut was sent for pathological examination as the circumferential margin.

For patients who have a deep, narrow pelvis, laparoscopic rectal transection is difficult. To guarantee a microscopically clear distal margin, the rectum was transected under direct vision with an assistant pulling the rectum outwards *via* the anus. Preservation of the sphincters was pursued based on the ability to achieve radical tumor removal. In patients with a very low tumor, the sphincters were unable to be preserved. The specimen was removed from the perineal incision and a permanent colostomy was performed at the anterolateral port.

Conversion was defined as performing any procedure using an open technique, other than extracting the specimen or transection of rectal cancer *via* the anus. The decision to convert was made when major complications occurred or when radical removal was impossible.

Patients in the OTME group underwent routine operation according to the TME principles. All patients were stratified based on tumor-node-metastasis classification. The duration of postoperative analgesia was as needed and was monitored. According to the National Comprehensive Cancer Network guidelines, patients who could possibly benefit received adjuvant chemotherapy or radiotherapy.

This study was approved by the Ethical Committee of Shanghai Cancer Center, Fudan University and all patients gave informed consent.

Statistical analysis

The statistical analysis was carried out using the SPSS software package version 13.0 (Chicago, IL, United States) and Windows 7. Parametric variables were expressed as mean \pm SD. The Student's *t* test was used to assess differences between the LTME and OTME groups. The χ^2 test (or Fisher's exact test where appropriate) and exact tests were performed to compare vari-

Table 1 Patients' clinical-pathological characteristics and comparisons of operative and perioperative data between laparoscopic and open total mesorectal excision

	LTME (n = 67)	OTME (n = 71)	P value
Characteristics			
Age (yr)	58.4 ± 13.6	59.6 ± 9.4	0.910
Sex ratio (male:female)	1.3:1	1.29:1	0.918
BMI	23.6 ± 2.6	23.4 ± 1.8	0.886
ASA			0.846
I	57	63	
II	9	7	
III	1	1	
pTNM classification			0.892
I	7	9	
II	46	49	
III	14	13	
Differentiation grades			0.964
Well-moderately differentiated	63	65	
Poorly differentiated	4	6	
Distal margin (cm)	3.6 ± 1.9	3.3 ± 1.7	0.648
Comparisons			
Sphincter-preserving surgery (%)	44 (65.7)	43 (60.6)	0.046
Operating time (min)	216.4 ± 68.3	162.7 ± 42.5	0.032
Blood loss (mL)	86.9 ± 37.6	119.1 ± 32.7	0.018
Postoperative analgesia (d)	2.1 ± 0.6	3.9 ± 1.8	0.008
Duration of urinary drainage (d)	4.7 ± 1.8	6.9 ± 3.4	0.016
Time to pass flatus (h)	46.9 ± 14.8	95.6 ± 54.8	0.004
Postoperative hospital stay (d)	10.4 ± 4.3	13.8 ± 5.9	0.036
Length of specimen (cm)	18.4 ± 4.2	19.7 ± 6.1	0.786
Number of lymph nodes harvested	20.3 ± 8.3	21.1 ± 6.7	0.924
Positive circumferential margin (%)	1.5	2.8	0.068

LTME: Laparoscopic total mesorectal excision; OTME: Open total mesorectal excision; BMI: Body mass index; pTNM: Pathologic tumor-node-metastasis; ASA: American Society of Anesthesiologists.

ables between the two groups. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Patients' characteristics

Between September 2008 and July 2011, a total of 138 patients were randomly assigned to either the LTME group or the OTME group. Sixty-seven patients underwent LTME and 71 patients underwent OTME. The sex ratio (male: female) was 1.3:1 and 1.29:1 for LTME and OTME, respectively. The mean age was 58.4 ± 13.6 years in the LTME group, and 59.6 ± 9.4 years in the OTME group. The two groups were well matched for age, sex ratio, body mass index, ASA, pathological tumor staging and differentiation grades as shown in Table 1.

Surgery and postoperative recovery

As shown in Table 1, there was no statistical difference in the types of surgical procedures chosen, including LAR and APR. The resection was considered curative in all cases. The sphincter-preserving rate was 65.7% (44/67) for LTME and 60.6% (43/71) for OTME and the difference was statistically significant. No protective diverting stoma was fashioned in either groups. Conver-

Table 2 Comparison of complications

Complications	LTME	OTME
Ureter damage	1	0
Anastomotic leak	1	1
Obstruction	1	3
Wound infection	1	2

LTME: Laparoscopic total mesorectal excision; OTME: Open total mesorectal excision.

sion to an open procedure was required in 2 cases (2.99%) due to ureter damage and severe adhesion, respectively.

Despite the fact that the operating time was significantly longer for LTME than OTME, there was significantly less hemorrhage in the LTME group than in the OTME group. Patients in the LTME group also enjoyed a significantly faster postoperative recovery, including lower requirement for analgesia and urinary drainage, faster return of bowel movement and earlier hospital discharge.

Complications

One patient (1.5%) suffered an anastomotic leakage in both the LTME group and in the OTME group (1.4%). A total of 4 patients suffered an obstruction, 1 (1.5%) in the LTME group and 3 (4.2%) in the OTME group. In addition, 1 patient and 2 patients in the LTME group and OTME group, respectively, suffered a wound infection. These differences were not statistically significant. There was no perioperative mortality (Table 2).

Oncological outcome and follow-up

With regard to oncological adequacy, the length of the specimen, distal margin of the tumor, number of harvested lymph nodes and positive circumferential margin were all comparable between the two groups. Short-term follow-up was available for all patients, with a median follow-up period of 21 mo (range: 9-56 mo). No port site recurrence, anastomotic recurrence or mortality was observed during the follow-up period.

DISCUSSION

Although laparoscopic surgery for colon cancer has been widely accepted due to its proven benefits, this technique for rectal cancer is controversial. In this report, we compared laparoscopic surgery to open surgery following TME principles in a consecutive series of patients who were operated on by a team of surgeons extensively experienced in laparoscopic colorectal surgery.

There is a growing body of literature reporting similar outcomes following LTME and OTME. Our results did not differ from the conclusion drawn from other publications that LTME was safe and feasible. Patients who underwent LTME benefited from a faster recovery, including less bleeding, less pain, faster return of urinary function and bowel movement, similar to that found in previous studies^[11].

However, there is still debate on whether LTME can

deliver an equally satisfying oncological radicality, allowing tumor-free margin and sufficient lymph node yield, while being minimally invasive^[12]. There is a lack of multicenter randomized trials addressing this particular issue. While we are still waiting for the results of the COLOR II trials, CLASICC remains the only randomized controlled trial available^[13,14]. The early results of CLASICC reported higher, but non-significant, rates of positive circumferential resection margin involvement, which did not translate into a difference in 3-year local recurrence rates. The 5-year follow-up results of the CLASICC trial were unable to show a difference in local recurrence, suggesting that this was probably the result of a learning curve effect. Not surprisingly, most investigations have reported different results. According to a meta-analysis conducted by Anderson *et al*^[15], the positive radical margin involvement rate of rectal cancer was not significantly different between laparoscopic surgery and open surgery. Similar results were obtained for distal margin involvement.

With regard to lymph node yield, our study reported a mean number of 20.3 lymph nodes for LTME and 21.1 for OTME with no statistical difference. This was slightly less than that found by Dulucq *et al*^[16]. They retrieved an average of 24.5 lymph nodes. However, our results were superior to most other studies where the mean number of lymph nodes harvested varied from 8 to 14^[17].

Due to the complicated nature of the TME procedure for middle and low rectal cancer, there are only a few publications which compare sphincter-preserving rates between laparoscopic and open approaches. It is worth noting that in our series, the sphincter-preserving rate was significantly higher in the LTME group. This may have been due to the fact that LTME provides the surgeon with a much more flexible operating space and allows them to dissect more easily down to the pelvic floor especially in patients with a deep, narrow pelvis. This clear, magnified and direct view is not available during OTME^[18,19]. Nonetheless, this study showed that having a deep, narrow pelvis with a large tumor complicated the LTME procedure and prolonged the operating time, but did not affect postoperative outcomes^[20]. The introduction of a linear endoscopic cutting stapler also guarantees a more satisfactory distal transection. Even when sphincter resection was inevitable, these advantages still facilitated pelvic surgery. Similar results were reported by Gezen *et al*^[21] who had a higher rate of neoadjuvant chemoradiation-treated patients in the LTME group. However, these advantages were not found in the largest series of rectal laparoscopic surgery, involving 612 patients, led by Zheng *et al*^[22]. It is noteworthy that an increased rate of APR was observed in the LTME group in a relatively small series of Greek patients with mid and rectal cancer. This might be explained by the significantly lower location of the tumors in the LTME patients^[23].

Another advantage of laparoscopic surgery is greater magnification and better illumination of the surgical field, thus allowing better exposure of the autonomic nerves and their protection. Our study showed that pa-

tients in the LTME group required a significantly shorter period of urinary drainage. Considering the mean age in this series, patients were predisposed to possible sexual dysfunction before surgery. However, the incidence of postoperative sexual dysfunction was not taken into account in this study.

Conversion has always been a major concern of laparoscopic rectal surgery. Studies in the early 2000s reported a conversion rate as high as 20%. The CLASICC study also associated conversion with a clear survival disadvantage. However, researchers in the CLASICC trial were unable to attribute this disadvantage to advanced tumor stage or to surgeon-related factors. Conversion was found to be associated with poor prognosis^[24]. The latest studies report a conversion rate of 3%-22%, suggesting the involvement of patient selection and surgeons. In the present study, our conversion rate was 2.99%, and was closer to that found by Leroy *et al*^[25] and Milsom *et al*^[26]. This may be explained by the exclusion of patients staged at cT3/4 cTxN+ or with a former history of pelvic radiotherapy. With regard to postoperative complications, the distribution and incidence was similar between the 2 groups. Furthermore, a recent meta-analysis led by Lin *et al*^[27] concluded that robotic surgery was superior to laparoscopic surgery in terms of conversion, and could be an alternative in patients who are more likely to undergo conversion.

Admittedly, a clear limitation of our study was that selection bias cannot be completely, even though the two groups were well balanced in terms of demographics and tumor statistics. However, by prospectively enrolling a consecutive series of patients operated on by the same team of surgeons, we hoped to avoid the learning curve effect. The follow-up period was too short to draw any conclusions as to the long-term outcome of LTME versus OTME, however, by continuing to enroll and follow-up patients, we hope to deliver more valuable information in the future.

Our study demonstrated that laparoscopic TME is safe and feasible, with an oncological adequacy comparable to the open approach. From our perspective, laparoscopic TME is performed through laparoscopic apparatus which are thin and long. In this way, tumors can be completely removed almost without being touched. Further studies with more patients and longer follow-up are needed to confirm the present results.

COMMENTS

Background

Rectal cancer accounts for approximately half of all colorectal cancer cases in China. With the introduction of total mesorectal excision (TME), laparoscopic technique, as well as pre- and postoperative chemo-radiotherapy, the local control rate and survival in rectal cancer patients have been dramatically improved.

Research frontiers

Although laparoscopy in colon cancer has gained acceptance due to its proven benefits, laparoscopy in rectal cancer is still not recommended as the treatment of choice by National Comprehensive Cancer Network guidelines. In this study, the authors demonstrated that laparoscopic TME (LTME) was advantageous in terms of clinical outcomes and comparable in oncological outcomes to open TME (OTME) in patients with mid and low rectal cancers.

Innovations and breakthroughs

Recent reports have highlighted similar outcomes following LTME and OTME in rectal cancer patients. This is the first study to prospectively compare LTME and OTME in a consecutive series of patients with middle and low rectal cancer regardless of the preservation of sphincters.

Applications

The present analysis confirmed the short-term benefits and comparable oncological adequacy of laparoscopic TME compared with the open procedure. This will be comforting to those surgeons performing the technique and should help to promote the laparoscopic TME approach so that a large multicenter randomized trial of LTME can be conducted to demonstrate its long-term benefits.

Peer review

The manuscript is generally well written, has an academic highlight that of the first study to prospectively compare LTME and OTME in a consecutive series of patients with middle and low rectal cancer. It is important that the shape and location of the intersigmoid recess for providing surgery of anus and rectum in laparoscopic total mesorectal excision and open mesorectal excision.

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Enteroenteroanastomosis near adjacent ileocecal valve in infants

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Abstract

AIM: To investigate the feasibility and the effectiveness of ileoileostomy in the region adjacent to the ileocecal valve, which can retain the ileocecal valve in infants.

METHODS: This is a retrospective review of 48 patients who underwent ileoileostomy in the region adjacent to the ileocecal valve (group 1) and 34 patients who underwent ileocecal resections and ileotransversanastomosis (group 2). Patients were monitored for the time to flatus, resumption of eating, length of hospital stay after surgery, serum total bile acid, vitamin B12 and postoperative complications.

RESULTS: The time to flatus, time until resumption of eating and post-operative length of hospital stay showed no statistically significant differences between the two groups. Serum total bile acid and vitamin B12

were not significantly different between the two groups at post-operative day 1 and day 3, but were significantly decreased at 1 wk after operation in group 2. None of the patients died or suffered from stomal leak in these two groups. However, the incidence of diarrhea, intestinal infection, disturbance of acid-base balance and water-electrolytes in group 1 was lower than in group 2.

CONCLUSION: Ileoileostomy in the region adjacent to the ileocecal valve is safe and results in fewer complications than ileotransversanastomosis in infants.

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Key words: Ileocecal valve; Ileoileostomy; Infants

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INTRODUCTION

Ileocecal valve plays a very important role in the regulation of intestinal transit. It acts as a barrier to delay passage for small bowel contents and hence increases absorption. It also prevents reflux from the caecum into the ileum^[1,2].

Anatomically, the terminal ileum has poor vascularization. The arterial supply of the terminal ileum in the region adjacent to the ileocecal valve comes from a single arch from the ileocolic artery. Therefore, conventional wisdom is, for a lesion involving the region of the ileum that is about 10-15 cm away from the ileoce-

cal valve, a right hemicolectomy should be selected^[3]. However, it was established in adults that the danger of incompetence of the anastomosis sutures near the blind gut was not greater than that in ileotransversanastomosis^[4]. Moreover, long-term results in adult patients after resection of the small intestine with the enteroenteroanastomosis near the blind gut were better than in patients with resection of the ileocecal valve, regarding passage of the intestinal contents^[4].

A previous study reported that an intact ileocecal valve was essential for better nutritive conditions in the newborn undergoing extensive small intestinal resection^[5]. In non-tumoral lesions, preservation of the ileocaecal valve to retain its important function should be considered. The terminal ileum has a high incidence of intussusception, transmesenteric hernia, intestinal atresia and necrotizing enterocolitis. Our aim was to determine the feasibility of ileoileostomy in the region adjacent to the ileocecal valve and compare the rate of complications of ileoileostomy in the region adjacent to the ileocecal valve and ileocolostomy after ileocecal resections in infants.

MATERIALS AND METHODS

Subjects

This is a retrospective review of 48 patients who underwent ileoileostomy in the region adjacent to the ileocecal valve (group 1) and 34 patients who underwent ileocecal resections and ileocolostomy (group 2) in the Department of Pediatric Surgery, Nanjing Children's Hospital, affiliated to Nanjing Medical University, between January 1, 2003 and May 3, 2011. In this study, all patients were without extensive ileal resection. We and our patients have established a long-term friendly relationship. The study was approved by the Ethics Committee of Nanjing Children's Hospital, affiliated to Nanjing Medical University. Informed consent was obtained from all the guardians of the subjects.

The patients were categorized into two groups. Group 1 included 48 patients (26 males and 22 females aged 1 d to 10.6 mo; median age 4.8 mo) who underwent ileoileostomy in the region adjacent to the ileocecal valve. Causes of surgery included intussusception (7), transmesenteric hernia (11), intestinal atresia (5), necrotizing enterocolitis (17), mesenteric cyst (5) and duplication of intestine (3). Group 2 included 34 patients (20 males and 14 females aged 1 d to 9.1 mo; median age 4.2 mo) who underwent ileocecal resections and ileocolostomy. Causes of surgery included intussusception (7), transmesenteric hernia (10), intestinal atresia (4), necrotizing enterocolitis (9), mesenteric cyst (2) and duplication of intestine (2) (Table 1).

No patient died during this study. The patients with underlying pathology associated with chronic diarrhea such as short bowel syndrome were excluded. Patients were monitored in the time to flatus, resumption of eating and length of hospital stay after surgery, serum total bile acid, vitamin B12 and post-operative complications.

Table 1 Subjects and their primary diseases

	Group 1	Group 2
Patients	48 (26 males and 22 females)	34 (20 males and 14 females)
Age	1 d to 10.6 mo (median age 4.8 mo)	1 d to 9.1 mo (median age 4.2 mo)
Primary diseases	Intussusception (7) Transmesenteric hernia (11) Intestinal atresia (5) Necrotizing enterocolitis (17) Mesenteric cyst (5) Duplication of intestine (3)	Intussusception (7) Transmesenteric hernia (10) Intestinal atresia (4) Necrotizing enterocolitis (9) Mesenteric cyst (2) Duplication of intestine (2)

Table 2 Time to flatus, resumption of eating, and length of hospital stay after surgery

	Time to flatus after surgery (d)	Time of resumption of eating after surgery (d)	Length of hospital stay after surgery (d)
Group 1	2 ± 0.9	6 ± 0.4	8 ± 3.1
Group 2	2 ± 0.7	5 ± 0.8	8 ± 2.5

Surgical process at ileocecal junction

In brief, for group 1, the mesentery close to the bowel wall was dissociated and the ileocolic artery trunk was reserved. After resection of the lesions of the terminal ileum, pulsatile bleeding was visible in the two ends of the bowel. An ileoileostomy was then performed with 5-0 PDS (2-5 cm away from the ileocecal valve at a median distance of 3.5 cm) with no embed. The arteria ileocolica was reserved. No intestinal decompression was added (Figure 1).

In group 2, after resection of the terminal ileum, ileocecal junction and the ascending colon and part of the colon transversum, an ileocolostomy was performed with 5-0 PDS.

The lesion resulting in the bowel resection was purple or gray, the mesenteric vascular pulse and bowel peristalsis disappeared, and the intestinal wall was inelastic. The resected bowel was approximately 10-30 cm.

Statistical analysis

Statistical analysis was carried out using SPSS software, version 14.0 (SPSS Inc., Chicago, IL, United States). A Pearson Chi-square test was used to compare the complications found in the two groups. The time of flatus, resumption of eating and length of hospital stay after operation were expressed as mean ± SD. Parameters were analyzed by Student's *t* test. For the above parameters, *P* < 0.05 was considered statistically significant.

RESULTS

Time to flatus, resumption of eating, and length of hospital stay after surgery

The time to flatus, resumption of eating, and length of hospital stay after surgery showed no statistically significant differences between the two groups (Table 2).

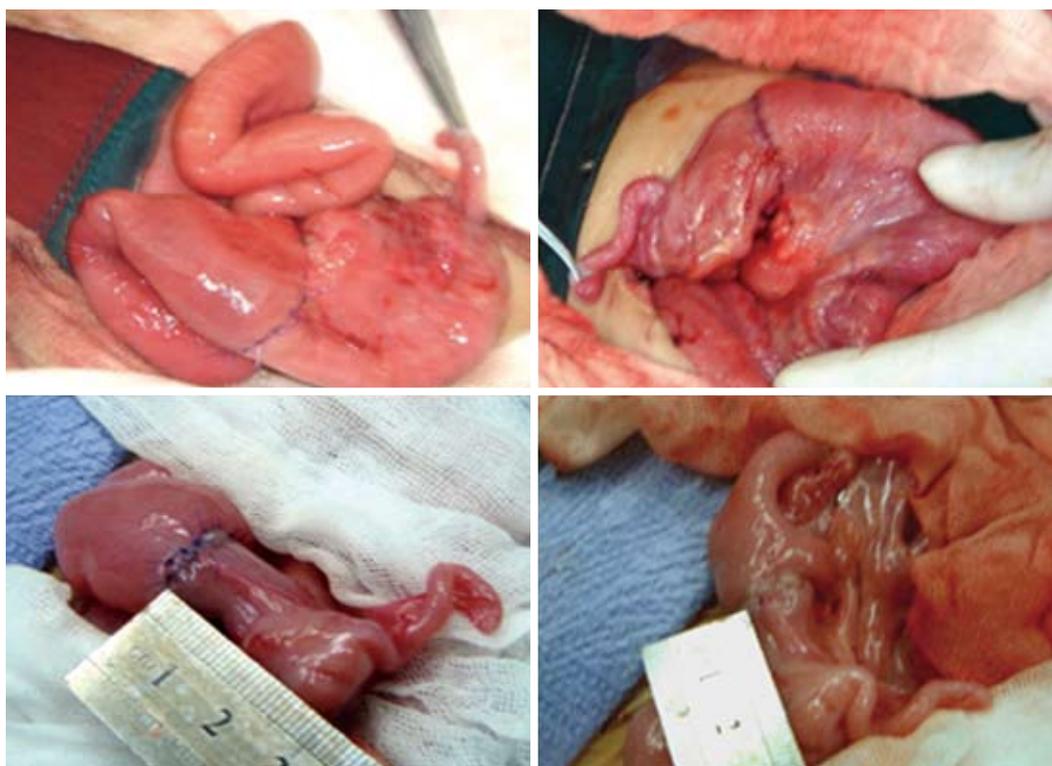


Figure 1 Enteroenteroanastomosis near the adjacent ileocecal valve.

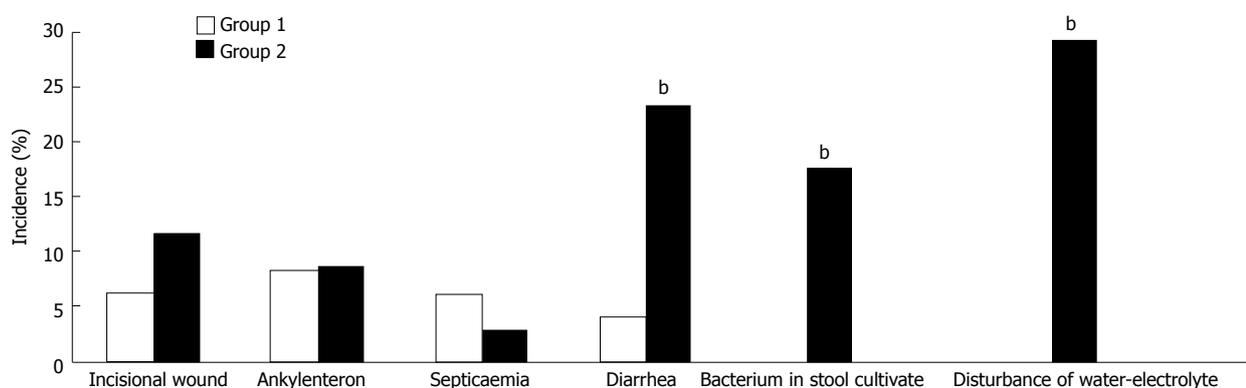


Figure 2 Incidence of diarrhea, intestinal infection, and disturbance of acid-base balance and water-electrolytes in group 1 was lower than in group 2, but the incidence of incisional wound, ankyloenteron, and septicaemia showed no differences. ^b*P* < 0.01 vs group 1.

Table 3 Serum total bile acid ($\mu\text{mol/L}$) and serum vitamin B12 (pg/mL)

	1 d after surgery	3 d after surgery	1 wk after surgery
Bile acid ¹			
Group 1	12.2 \pm 2.8	11.7 \pm 1.8	11.8 \pm 2.2 ²
Group 2	10.8 \pm 2.4	10.5 \pm 3.2	7.4 \pm 2.1
Vitamin B12 ²			
Group 1	712.5 \pm 143.4	696.2 \pm 108.5	683.2 \pm 104.2 ²
Group 2	685.3 \pm 131.5	623.8 \pm 132.1	560.8 \pm 99.2

¹Normal levels: 0-10 $\mu\text{mol/L}$; ²Normal levels (200-900 pg/mL). ^a*P* < 0.05 vs group 2.

After surgery, we advised nurses and parents to pay attention to the first flatus (anal exhaust) or defecation

of the children, and recorded the time as well.

Resumption of eating was guided and determined by little liquid in gastrointestinal decompression, no yellow or green liquid in gastrointestinal decompression, no flatulence, and successful defecation.

Serum total bile acid and vitamin B12

Serum total bile acid and vitamin B12 (Table 3) showed no significant differences between the two groups at post-operative day 1 and day 3, but were significantly decreased at 1 wk after operation in group 2.

Post-operative complications

None of the patients died or suffered from stomal leak in these two groups. However, the incidence of diarrhea,

bacterium in stool cultivante, and disturbance of water-electrolytes in group 1 was lower than in group 2 (Figure 2).

DISCUSSION

The present study showed that ileoileostomy in the region adjacent to the ileocecal valve was safe and was associated with fewer complications.

Previous studies have demonstrated that resection of the ileocaecal valve has several adverse effects. The ileocecal segment has been considered to possess a weak sphincteric action and functions in the flow of chyme and gut motility^[1]. The ileocaecal valve prevents the reflux of chyme, and thus prevents bacterial overgrowth from colonic bacterial flora in the small intestine. In the absence of an ileocecal valve, the colonic bacteria can reflux into the small intestine, resulting in infections of the small intestine and inflammation of the ileal mucosa^[6-11]. Moreover, in the absence of the ileocecal valve, transit time was decreased and there was reduced exposure to the absorptive surface. Without adequate detention time in the small intestine, accelerated chyme transport may lead to diarrhea, steatorrhea, malnutrition, dehydration, hypoalbuminemia and loss of minerals^[12]. Dorney *et al*^[5] found that the small bowel length required for survival was more than twofold when the ileocaecal valve was resected.

In animals and humans, bile salts and vitamin B12 have been shown to be major reclamation products from the ileum^[13-16]. Further studies showed that the ileal absorption of bile salts is mediated by an apical sodium-dependent bile acid transporter located in the terminal ileum^[17]. Previous studies have shown that patients with an ileal resection or disease of the distal ileum excreted bile salts in the feces significantly faster than healthy control subjects^[18]. Moreover, patients who underwent massive ileal resections, especially ileocecal resections, showed malabsorption of bile acids^[19,20]. Moreover, the anemia that occurred after ileocecal resection may have been due to deficiency in vitamin B12. As a result, reserving the ileal valve and terminal ileum might produce improved results.

Fernando *et al*^[21] found that in most of the specimens from human cadavers analyzed in their study, the arterial supply of the ileocecal junction was from the ileocolic artery, superior mesenteric artery, and terminal ileal branches of the superior mesenteric artery. In their study, anastomosis in the ileocecal junction with small windows was observed in 38 (70%) cases, more than the cases for large windows. Moreover, Serova^[4] demonstrated that the danger of incompetence of the anastomosis sutures in the region adjacent to the ileocecal valve was not higher than that in ileotransversanastomosis, and the immediate and long-term results in the enteroenteroanastomosis group were much better than in the group that had exclusion of the ileocecal segment. In our study, we demonstrated that after dissociating the mesentery close to the bowel wall and removing the part of the vascular network adjacent to the ileocecal junction, en-

teroenteroanastomosis near the blind gut was safe and no anastomotic leakage occurred.

Though there is a possibility of increasing the enteric cavity pressure of the ileocecal junction, we found that ileoileostomy only 2-5 cm away from the ileocecal valve is safe and effective in infants. This allowed a fluid diet to go through the ileal valve more easily than a solid diet. We also found that ileoileostomy in the region adjacent to the ileocecal valve was accompanied by fewer complications. As a result, the surgical method can be used clinically. Moreover, regarding the importance of the ileocecal valve, ileoileostomy in the region adjacent to the ileocecal valve might be helpful when a massive small bowel resection is necessary.

COMMENTS

Background

Ileocecal valve plays a very important role in the regulation of intestinal transit. It acts as a barrier to delay passage for small bowel contents and hence increases absorption. It also prevents reflux from the caecum into the ileum. In non-tumoral lesions, preservation of the ileocaecal valve to retain its important function should be considered.

Research frontiers

The terminal ileum has poor vascularization. The arterial supply of the terminal ileum in the region adjacent to the ileocecal valve comes from a single arch from the ileocolic artery. This study focuses on the feasibility of ileoileostomy in the region adjacent to the ileocecal valve and the complications of ileoileostomy in the region adjacent to the ileocecal valve.

Innovations and breakthroughs

Though there is a possibility of increasing the enteric cavity pressure of the ileocecal junction, the authors found that ileoileostomy only 2-5 cm away from the ileocecal valve is safe and effective in infants. This allowed a fluid diet to go through the ileal valve more easily than a solid diet. The authors also found that ileoileostomy in the region adjacent to the ileocecal valve was accompanied by fewer complications.

Applications

Enteroenteroanastomosis near the adjacent ileocecal valve can be used clinically. Moreover, regarding the importance of the ileocecal valve, ileoileostomy in the region adjacent to the ileocecal valve might be helpful when a massive small bowel resection is necessary.

Peer review

The presented paper is of some scientific interest and well-written.

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High mobility group-box 3 overexpression is associated with poor prognosis of resected gastric adenocarcinoma

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Abstract

AIM: To elucidate high mobility group-box 3 (HMGB3) protein expression in gastric adenocarcinoma, its potential prognostic relevance, and possible mechanism of action.

METHODS: Ninety-two patients with gastric adenocarcinomas surgically removed entered the study. HMGB3 expression was determined by immunohistochemistry through a tissue microarray procedure. The clinicopathologic characteristics of all patients were recorded, and regular follow-up was made for all patients. The inter-relationship of HMGB3 expression with histological and clinical factors was analyzed using non-parametric tests. Survival analysis was carried out by Kaplan-Meier (log-rank) and multivariate Cox (Forward LR) analyses between the group with overexpression of HMGB3 and the group with low or no HMGB3 ex-

pression to determine the prognosis value of HMGB3 expression on overall survival. Further, HMGB3 expression was knocked down by small hairpin RNAs (shRNAs) in the human gastric cancer cell line BGC823 to observe its influence on cell biological characteristics. The MTT method was utilized to detect gastric cancer cell proliferation changes, and cell cycle distribution was analyzed by flow cytometry.

RESULTS: Among 92 patients with gastric adenocarcinomas surgically removed in this study, high HMGB3 protein expression was detected in the gastric adenocarcinoma tissues *vs* peritumoral tissues ($P < 0.001$). Further correlation analysis with patients' clinical and histology variables revealed that HMGB3 overexpression was obviously associated with extensive wall penetration ($P = 0.005$), a positive nodal status ($P = 0.004$), and advanced tumor-node-metastasis (TNM) stage ($P = 0.001$). But there was no correlation between HMGB3 overexpression and the age and gender of the patient, tumor localization or histologic grade. Statistical Kaplan-Meier survival analysis disclosed significant differences in overall survival between the HMGB3 overexpression group and the HMGB3 no or low expression group ($P = 0.006$). The expected overall survival time was 31.00 ± 3.773 mo (95%CI = 23.605-38.395) for patients with HMGB3 overexpression and 49.074 ± 3.648 mo (95%CI = 41.925-57.311) for patients with HMGB3 no and low-level expression. Additionally, older age ($P = 0.040$), extensive wall penetration ($P = 0.008$), positive lymph node metastasis ($P = 0.005$), and advanced TNM tumor stage ($P = 0.007$) showed negative correlation with overall survival. Multivariate Cox regression analysis indicated that HMGB3 overexpression was an independent variable with respect to age, gender, histologic grade, extent of wall penetration, lymph nodal metastasis, and TNM stage for patients with resectable gastric adenocarcinomas with poor prognosis (hazard ratio = 2.791, 95%CI = 1.233-6.319, $P = 0.019$). In the gene function study,

after HMGB3 was knocked down in the gastric cell line BGC823 by shRNA, the cell proliferation rate was reduced at 24 h, 48 h and 72 h. Compared to BGC823 shRNA-negative control (NC) cells, the cell proliferation rate in cells that had HMGB3 shRNA transfected was significantly decreased ($P < 0.01$). Finally, cell cycle analysis by FACS showed that BGC823 cells that had HMGB3 knocked down were blocked in G1/G0 phase. The percentage of cells in G1/G0 phase in BGC823 cells with shRNA-NC and with shRNA-HMGB3 was $46.84\% \pm 1.7\%$, and $73.03\% \pm 3.51\%$ respectively ($P = 0.001$), whereas G2/M cells percentage decreased from $26.51\% \pm 0.83\%$ to $17.8\% \pm 2.26\%$.

CONCLUSION: HMGB3 is likely to be a useful prognostic marker involved in gastric cancer disease onset and progression by regulating the cell cycle.

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Key words: High mobility group-box 3; Gastric adenocarcinoma; Prognosis; Cell proliferation; Cell cycle

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INTRODUCTION

Gastric cancer is reported to be one of the common devastating types of cancer. It has a high occurrence rate, short survival period and high mortality rate. The mortality rate can be up to 25.2/100 000 in China, which accounts for 25% of deaths in malignant cancer. The occurrence ratio and mortality are ranked as No. 1 and No. 2 in malignant cancer^[1]. Usually gastric cancer is already at a late stage when it is diagnosed; the 5-year survival rate in clinical stage III and IV patients is only about 28.4% and 7.6%, respectively. There are many factors including lymph node metastasis, extent of wall penetration, pathological tumor-node-metastasis (pTNM) stage, and surgical mode *etc.*, associated with prognosis of the disease. Those factors are more clinipathologically related, however, investigating molecular biomarkers can not only provide indications for clinic prognosis, but also can identify potential targets for clinical therapy.

High mobility group-box 3 (HMGB3) belongs to the high mobility group box (HMG-box) subfamily. It has 99% homology with HMGB1 and HMGB2^[2]. This subfamily has a HMG-box, so it can regulate gene transcription by participating with formation of enhancesomes.

The HMG-box subfamily also plays an important role in DNA replication, transcription, recombination and repair, *etc.*^[3,4]. HMGB3 was reported to have high expression in embryos and weak expression in adult tissues. HMGB3 is important in keeping the balance between self-renewal and differentiation status in mouse hematopoietic stem cells^[5]. Also it was known that HMGB3 is indispensable in maintaining mouse leukemia stem cell self-renewal capability. Simultaneously overexpression of HMGB3, c-MyB and CBX5 can make hematopoietic stem/progenitor cells immortalized^[6]. Recent studies showed that HMGB3 and NPU98 fusion protein forms a new oncogenic gene in leukemia^[7]. HMGB3 also participates in recurrence of acute lymphoid leukemia and it shows high expression in the progression phase of breast cancer^[8]. HMGB3 was identified as one of the bio-markers detected in peripheral blood in lung cancer^[9]. Though HMGB3 is found in some types of cancers, its role in gastric cancer is still unclear.

In this study, we observed the expression of HMGB3 in gastric adenocarcinoma tissues by immunohistochemistry, and analyzed its correlation between expression level and clinicopathologic variables and prognosis. We found that HMGB3 showed a high expression level in gastric cancer *vs* peritumoral tissues. And HMGB3 overexpression was obviously associated with extensive wall penetration, a positive nodal status, advanced TNM stage and poor prognosis. Moreover, we silenced HMGB3 expression in BGC823 gastric cancer cell line by small interfering RNA (siRNA), and observed the changes in cell proliferation and cell cycle. BGC823 cells with HMGB3 knocked down showed a decreased proliferation rate and the ratio of G0/G1 phase cells in the cell cycle significantly increased. Results above indicate that the overexpression of HMGB3 is a marker for poor prognosis of gastric adenocarcinomas and that it may function by affecting gastric cell proliferation and cell cycle.

MATERIALS AND METHODS

Patients and specimens

Tissue microarrays (TMAs) from a total of 92 consecutive cases of gastric adenocarcinomas operated on in our hospital from December 2006 to October 2007 were prepared for immunohistochemical testing. All the patients were given radical resection and D1+ or D2 lymphadenectomy followed by adjuvant chemotherapy with the regimen epirubicin, cisplatin and fluorouracil. No preoperative therapy was given to any patient. The pathologic staging was made according to American Joint Committee on Cancer TNM staging system. The follow up end point was defined as the death of patients. The use of the tissue samples in TMA analysis and clinical data was approved by Medical Ethics Committee of Jiangsu University and the patients. Patients' clinical and histopathologic data were summarized in Table 1.

Tissue microarrays

For each case, we selected the tumor foci for the TMA

Table 1 Clinical and histopathologic data of the patients and expression of high mobility group-box 3 in correlation with clinicopathologic variables: High mobility group-box 3 overexpression predicts the clinical course ($n = 92$) (%)

Variables	Samples	HMGB3 overexpression	P value
Age at surgery (yr)			
≤ 60	34 (36.96)	20 (60.00)	0.247
> 60	58 (63.04)	30 (51.16)	
Gender			
Male	61 (66.30)	30 (50.00)	0.649
Female	31 (33.70)	20 (62.5)	
Extent of wall penetration			
pT1-2	22 (23.91)	6 (26.67)	0.005
pT3-4	70 (76.01)	44 (63.46)	
Lymph node metastasis			
N0	27 (29.3)	5 (14.29)	0.004
N1-3	65 (70.65)	45 (70.59)	
Tumor stage			
Stage I + II	37 (40.22)	9 (23.21)	0.001
Stage III + IV	55 (59.78)	41 (56.76)	
Tumor localisation			
Fundus gastricus and cardia	11 (11.96)	6 (54.55)	0.955
Gastric body	33 (35.87)	21 (63.64)	
Gastric antrum	48 (52.17)	23 (47.91)	
Histologic grade			
G1 + 2	22 (23.91)	9 (40.91)	0.073
G3 + 4	70 (76.09)	41 (58.57)	

HMGB3: High mobility group-box 3.

construction during routine diagnosis by marking them on the more representative hematoxylin-eosin-stained slide with a waterproof pencil. At the same time we chose corresponding peritumoral tissue as a control. The advanced tissue arrayer (ATA-100, Chemicon International, Temecula, CA, United States) was used to create holes in a recipient paraffin block to acquire cylindrical core tissue biopsies with a diameter of 1 mm from the specific areas of the “donor” block. The tissue core biopsies were transferred to the recipient paraffin block at defined array positions. The TMAs contained tissue samples from 92 formalin-fixed, paraffin-embedded cancer specimens with known diagnosis, and correlated noncancerous tissues from the same patients. The block was incubated in an oven at 45 °C for 20 min to allow complete embedding of the grafted tissue cylinders in the paraffin of the recipient block, and then stored at 4 °C until microtome sectioning.

Immunohistochemical staining

Rabbit-derived anti-human HMGB3 antibody (Epitomics, Cat. #2416-1) were used for immunohistochemical (IHC) detection of HMGB3 protein in TMAs. TMA sections were processed for IHC demonstration of HMGB3 protein by the Biotin-Avidin-Peroxidase detection system (Sigma). The anti-HMGB3 antibodies were used at 1:50 dilutions. Endogenous peroxidase was inhibited by incubation with freshly prepared 3% hydrogen peroxide with 0.1% sodium azide. Nonspecific staining was blocked with 0.5% casein and 5% normal goat se-

rum. TMAs were incubated with biotinylated goat anti-rabbit antibodies and ExtrAvidin-conjugated horseradish peroxidase. Staining was developed with diaminobenzidine substrate and sections were counterstained with hematoxylin. Normal mouse serum or phosphate-buffered saline (PBS) replaced anti-HMGB3 antibodies used as negative controls.

The quantification of HMGB3 protein expression

HMGB3 expression was semiquantitatively estimated as the total HMGB3 immunostaining score, which was calculated as the sum of a proportion score and an intensity score. The proportion score reflects the fraction of positive staining cells (score 0, < 5%; score 1, 5%-10%; score 2, 10%-50%; score 3, 50%-75%; score 4, > 75%). The intensity score represents the staining intensity (score 0, no staining signal; score 1, weak positive signal; score 2, moderate positive signal; score 3, strong positive signal). Finally, a total expression score was given ranging from 0 to 12. Based on the analysis in advance, the overexpression of HMGB3 was defined as a total expression score ≥ 9 ^[10].

Cell line and culture conditions

The human gastric cancer cell line BGC823 was obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in Dulbecco's modified Eagle's minimal essential medium media supplemented with 10% fetal bovine serum (Gibco), penicillin (100 IU/mL) and streptomycin (100 mg/mL) and grown in a humidified incubator with a 5% CO₂ atmosphere at 37 °C.

RNA interference

Human HMGB3 small hairpin RNA (shRNA) and control shRNA were obtained from Shanghai R and S Biotechnology Co., Ltd, and were transfected into cells using Lipofectamine 2000 reagent (Life Technologies, Carlsbad, CA, United States) according to the manufacturer's instructions.

Cell proliferation assay

Cells were plated at a density of 5×10^3 cells/well in 96-well plates in 200 mL medium. After siRNA was transfected for 24 h, 48 h and 72 h, 20 μ L MTT was added to each well. The mixture was incubated at 37 °C for 4 h in the dark, washed to remove media and MTT, and 200 μ L dimethyl sulfoxide added to each well. The absorption value at 490 nm was recorded.

Cell cycle analysis

The BGC823/blank, BGC823/small hairpin RNAs-negative control (shRNAs-NC) shRNA-NC and BGC823/shRNA-HMGB3 cells were seeded in six-well plates at a concentration of 5×10^5 cells/mL. Cells were harvested following trypsinization, washed once with cold PBS and fixed in 70% ethanol for 24 h. DNA staining was carried out by resuspending the cells in a solution of PBS containing 20 mg/mL of RNase A, 50 mg/mL of propidium iodide, with subsequent incubation at 37 °C for 30

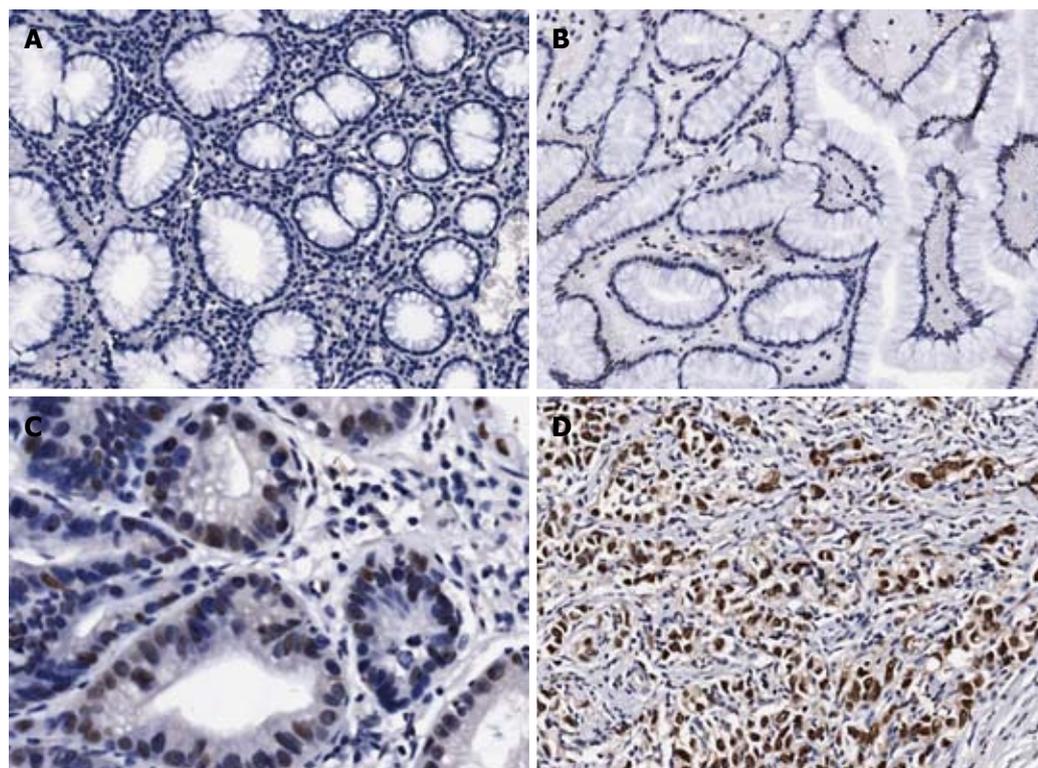


Figure 1 Expression of high mobility group-box 3 in gastric adenocarcinoma tissue and peritumoral tissue. A: Peritumoral tissue, no staining; B: Peritumoral tissue, weak staining; C: Gastric adenocarcinoma tissue, weak staining; D: Gastric adenocarcinoma tissue, highly positive staining.

min. The stained cells were analysed for the FL-2 area using a flow cytometer (Beckman Coulter, Brea, CA), and DNA histograms were analyzed using Modifit software. Experiments were performed in triplicate. Results are presented as percentage of cells in a particular phase.

Statistical analysis

Data was analyzed using the statistical package for the Social Sciences Version 16.0 (SPSS 16.0). For clinical data statistical analysis, the inter-relationship of HMGB3 expression with histology or clinical factors was analyzed using nonparametric tests. The screen of significant factors associated with survival rate in patients with gastric adenocarcinoma used Kaplan-Meier and log-rank test; Cox regression analysis (Forward LR) was used for multivariate analysis and to determine the 95% confidence interval. Cell experiment data were expressed as mean \pm SD of three independent experiments. Significance of differences between groups was determined by one-way analysis of variance. The significance was set at $P < 0.05$.

RESULTS

Expression of HMGB3 protein in the gastric adenocarcinomas

Expression of HMGB3 protein was evaluated by immunohistochemical staining. In gastric adenocarcinoma cells, the expression of HMGB3 protein was mainly found in the nucleus, and weakly detected in cytoplasm (Figure 1). Positive staining was detected at a level of 94.57% (87/92) in gastric adenocarcinoma tissue, and 52.17% (48/92) in peritumoral tissue with significant difference ($P < 0.001$). The rate of HMGB3 overexpression (total expression

score ≥ 9) was elevated in gastric adenocarcinoma tissue compared with corresponding peritumoral tissue (54.35% vs 0.00%, $P < 0.001$). The difference in HMGB3 expression between peritumoral and normal (distant) tissues was not assessed. We also detected the HMGB3 mRNA level in gastric adenocarcinoma tissue and the corresponding peritumoral tissues by using quantitative reverse transcriptase-polymerase chain reaction, and it was found that HMGB3 mRNA expression level was significantly higher in gastric cancer than in peritumoral samples (detailed data not shown).

Relevance between HMGB3 expression and patients' clinical and histopathologic characteristics

We analyzed the correlation between HMGB3 expression and age, gender, tumor localization, histologic grade, extent of wall penetration, lymph node metastasis and tumor stage. The results showed that HMGB3 expression level was much higher in the pT3-4 group compared to the pT1-2 group ($P = 0.005$); it was also much higher in the N1-3 group compared to the N0 group ($P = 0.004$) and in the stage III + IV group compared to the stage I + II group ($P = 0.001$); all the results were statistically significant. But the correlation between HMGB3 expression and the age and gender of the patient and tumor localization, histologic grade had no statistical significance (Table 1).

We used Kaplan-Meier survival analysis to analyze the gender, age, tumor localization, histologic grade, extent of wall penetration, lymph node metastasis, tumor stage and HMGB3 expression correlation with patient's prognosis. It was observed that there was no correlation between gender, tumor localization and histologic grade with prognosis. But age ($P = 0.040$), extent of wall pen-

Table 2 Relationship between high mobility group-box 3 overexpression, clinicopathological characteristics and average survival in gastric adenocarcinoma patients (mean \pm SD)

Clinicopathological characteristics	Samples (n)	Average survival (mo)	95%CI	P value
Sex				
Male	61	41.563 \pm 2.823	36.031-47.096	0.308
Female	31	37.24 \pm 4.027	29.347-45.133	
Age (yr)				
\leq 60	34	47.409 \pm 4.113	39.348-55.470	0.040
> 60	58	35.143 \pm 3.797	27.700-42.585	
Histologic grade				
G1 + G2	22	40.938 \pm 5.238	30.670-51.205	0.323
G3 + G4	70	37.366 \pm 3.366	30.769-43.963	
Lymph node status				
pN0	27	54.800 \pm 1.885	51.085-58.515	0.005
pN1-3	65	33.158 \pm 3.646	26.012-40.304	
Extent of wall penetration				
pT1 + 2	22	48.385 \pm 3.949	40.645-56.124	
pT3 + 4	70	33.447 \pm 3.447	26.692-40.203	0.008
TNM stage				
Stage I + II	37	47.511 \pm 2.927	41.774-53.248	0.007
Stage III + IV	55	32.949 \pm 3.266	26.547-39.352	
HMGB3 expression				
No or low expression	42	49.074 \pm 3.648	41.925-57.311	0.006
Overexpression	50	31.00 \pm 3.773	23.605-38.395	
Location				
Stomach fundus	11	31.455 \pm 7.193	17.356-45.553	0.226 ¹
Gastric body	33	41.395 \pm 3.640	34.226-48.493	0.297 ²
Gastric antrum	48	41.410 \pm 3.211	35.116-47.703	0.922 ³

¹P = 0.226 vs gastric antrum; ²P = 0.297 vs stomach fundus; ³P = 0.922 vs gastric body. HMGB3: High mobility group box 3.

etration ($P = 0.008$), lymph node metastasis ($P = 0.005$), TNM tumor stage ($P = 0.007$) and HMGB3 overexpression ($P = 0.006$) showed a correlation with overall survival (Table 2). This survival analysis revealed that HMGB3 overexpression affected overall survival. There was a significant difference in overall survival between groups with HMGB3 overexpression and with no or low level expression (Table 2 and Figure 2). The expected overall survival time was 31.00 ± 3.773 mo for tumors with HMGB3 overexpression (95%CI = 23.605-38.395) and 49.074 ± 3.648 mo for tumors with HMGB3 no or low level expression (95%CI = 41.925-57.311). Multivariate Cox regression analysis indicate that HMGB3 overexpression was predictive of mortality (hazard ratio = 2.791, 95%CI = 1.233-6.319, $P = 0.019$), and as an independent variable with respect to age, gender, histologic grade, extent of wall penetration, lymph node metastasis, and TNM stage for patients with resectable gastric adenocarcinomas.

Silencing of HMGB3 expression inhibits BGC823 cell proliferation

To determine whether HMGB3 RNAi had an inhibitory effect on gastric cancer cell line BGC823 cell proliferation, we measured cell growth with an MTT assay. Data demonstrated that after HMGB3 was silenced, the cell proliferation rate significantly reduced in 24 h, 48 h, 72 h. Compared to BGC823 cells with shRNA-NC, cell prolifer-

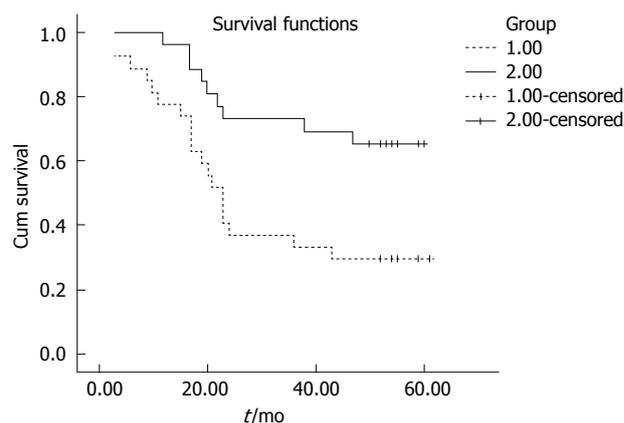


Figure 2 Kaplan-Meier survival curves between high mobility group-box 3 high expression group and low expression group ($P = 0.006$, Mantel-cox). Group 1: High mobility group-box 3 (HMGB3) no or low expression; Group 2: HMGB3 overexpression.

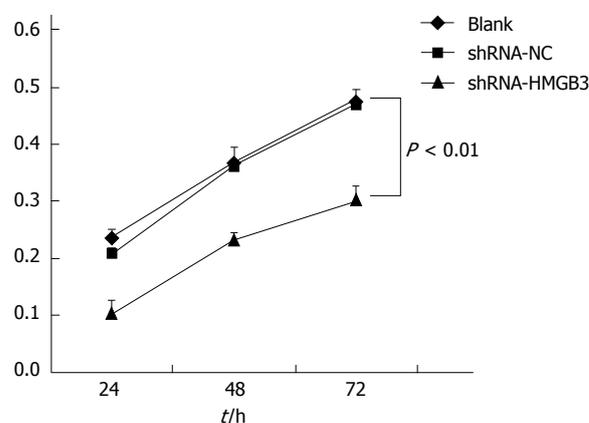


Figure 3 Effects of small hairpin RNAs-high mobility group-box 3 on BGC823 cell proliferation by MTT assay. Data shown as mean \pm SD. Experiment was performed in triplicate, $P < 0.01$ small hairpin RNAs-negative control (shRNAs-NC) vs shRNA-high mobility group-box 3 (HMGB3).

eration rate in cells that have HMGB3 siRNA transfected was decreased significantly ($P < 0.01$; Figure 3). These results indicate that knockdown of HMGB3 inhibits cell proliferation.

Silencing of HMGB3 impacts cell cycle progression

To identify a potential mechanism for HMGB3-specific silencing-mediated reduction of cell proliferation, cell cycle distribution was assessed using flow cytometry. The analysis showed that the percentage of cells in G0/G1 phase increased significantly ($P = 0.001$), whereas cells in the G2/M phases decreased in BGC823/HMGB3 RNAi cells compared with their parental controls. The percentage of cells in G1/G0 phase in BGC823 cells with shRNA-NC and with shRNA-HMGB3 was $46.84\% \pm 1.7\%$ and $73.03\% \pm 3.51\%$ respectively, whereas the percentage of cells in G2/M was $26.51\% \pm 0.83\%$ and $17.8\% \pm 2.26\%$. These data suggest that HMGB3 silencing may induce G0/G1 cell arrest (Figures 4 and 5).

Results above showed that high expression of HMGB3

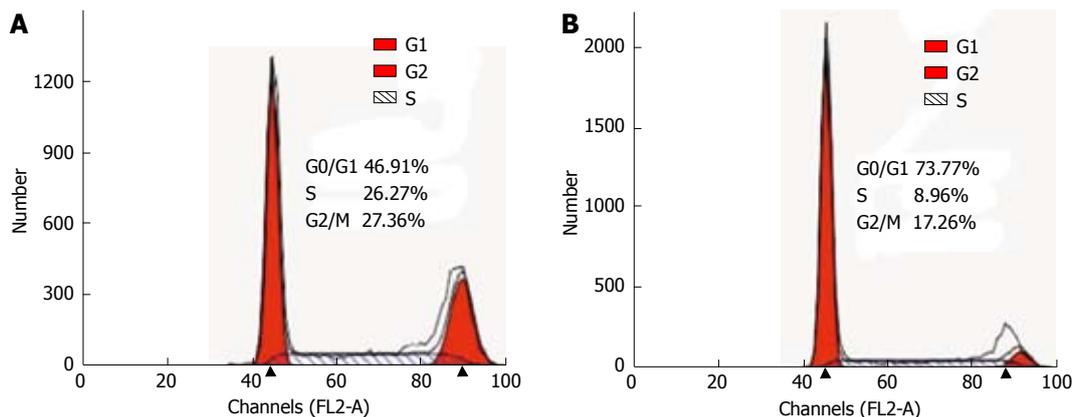


Figure 4 Flow cytometry analysis of cell cycle distribution in BGC823 cells after transfection of high mobility group-box 3 small hairpin RNA for 48 h. A: Small hairpin RNAs (shRNA)-negative control; B: shRNA-high mobility group-box 3 group.

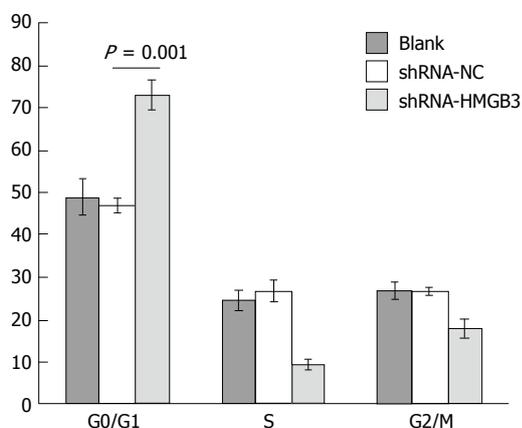


Figure 5 Cell cycle distribution in BGC823 cells after transfection of high mobility group-box 3 small hairpin RNA for 48 h. Data shown as mean \pm SD. Experiment was performed in triplicate, $P = 0.001$ between small hairpin RNAs-negative control (shRNAs-NC) and shRNA-high mobility group-box 3 (HMGB3).

in gastric cancer positively correlated with extensive wall penetration, positive lymph node metastasis, advanced tumor stage, and poor prognosis. Multivariable Cox regression indicated that this protein overexpression can be used as an independent biomarker in prognosis of gastric cancer. It implies that HMGB3 might participate in gastric cancer onset and development. Further silencing of HMGB3 expression in the gastric cancer cell line by shRNA showed that cell proliferation decreased and the cell cycle was blocked in G0/G1 phase. These results indicate that HMGB3 had a role in the proliferation and cell cycle of gastric cancer cells.

DISCUSSION

The onset and progression of gastric cancer correlated with various molecular and genetic incidents. To investigate the significance of the molecular expression in gastric cancer may help us to identify potential treatment targets and (or) predictive markers of prognosis.

The HMG Box subfamily is a family of non-histone chromosomal proteins; these members including HMGB1,

HMGB2 and HMGB3^[2]. HMGB family is thought to play a fundamental role in DNA replication, nucleosome assembly and transcription. There are studies overwhelmingly focusing on HMGB1, since it is widely expressed in all kinds of cell types in adult vertebrates. HMGB1 has been shown to interact with recombination activating gene 1 (RAG1) and RAG2 to play a role in immunology and inflammation, and is associated with proliferation and metastasis of many tumor types^[11,12]. However, little is known about the function of HMGB3.

In this study, we analyzed the role of HMGB3 in gastric cancer. Firstly, we observed HMGB3 expression in gastric adenocarcinoma and found it has a high expression level. The positive expression rate in gastric adenocarcinoma is 94.57% (87/92); the overexpression rate reached 54.35% (50/92). While the positive expression rate in gastric peritumoral tissue is 52.17% (48/92), no overexpression was found in it. Pourhoseingholi *et al.*^[13] reported that HMGB3 also has high expression in progressive breast cancer. A combination of proofs that there is high expression of HMGB3 in recursive leukemia^[8] and the pivotal role of HMGB3 in maintaining the self-renewal capability of leukemia stem cells^[6] imply that HMGB3 is a critical gene participating in cancer progression. Secondly, we further analyzed the correlation of HMGB3 expression with gastric cancer clinicopathologic variables and prognosis. We found high HMGB3 expression correlates with extensive wall penetration, positive lymph node metastasis and advanced tumor stage, which are the important prognostic factors in gastric cancer. Kaplan-Meier survival analysis showed HMGB3 high expression is negatively correlated with the overall survival of patients with resected gastric adenocarcinoma. The expected overall survival time in high HMGB3 expression patients is 31.000 ± 3.773 mo, while in no or low HMGB3 expression patients it is 49.074 ± 3.648 mo. Multivariate analysis shows HMGB3 overexpression can work as an independent variable for poor survival in resectable gastric adenocarcinoma. In analyzing factors affecting gastric cancer prognosis, different research groups reached more or less different conclusions since the methods used and the samples they ana-

lyzed were different. However, there are common factors including age, tumor late stage, small surgical area, large cancer volume, adjuvant chemotherapy *etc.* correlated with poor prognosis in gastric cancer^[14-17]. Many researchers reported that pathological classification is also an important factor affecting prognosis^[14,15,17], though in this study it showed a negative result. This could be due to the number of level II and III patients being large, thus affecting the conclusion. Now the diagnosis and therapy of tumors is entering the “molecular moment”. Molecular biomarkers not only could be prognostic factors, but could also be potential therapeutic targets for clinical therapy. Mitogen-activated protein kinase, Janus kinase/signal transducers and activators of transcription and nuclear factor-kappaB were reported to have high expression levels in gastric cancer^[18-20]; however, none of them can be used as a prognostic biomarker alone. Ki67 and PNCa have been widely used to indicate a high cancer cell proliferation rate, and both of them are highly expressed in gastric cancer^[21], however, neither has been used to determine prognosis^[22,23]. Her2, a therapy target in breast cancer^[24,25], is also found in gastric cancer as a new prognostic factor and a novel therapeutic target^[26,27]. Lastly, we used the gastric cancer cell line BGC823 to investigate cell proliferation and cell cycle changes after silencing of HMGB3. It was observed that the cell proliferation rate was greatly reduced, and the cell cycle was blocked in G1/G0 phase. This indicates that HMGB3 may promote cell proliferation through cell cycle progression. Researchers found that the number of G2/M cells was significantly reduced in HMGB3^{-/-} mouse hematopoietic progenitor cells and this could be attributed to blockage of the cell cycle in G0/G1 phase^[5]. In *Xenopus*, Terada *et al.*^[28] observed that the cell proliferation rate of retinal progenitor cells can be promoted with overexpression of HMGB3. They further discovered that this is accompanied by P27 downregulation^[29]. P27 is a member of the cyclin dependent kinase inhibitor family, CIP/KIP, and can prevent activation of cyclin E-CDK6 and cyclin D-CDK4, which consequently blocks cells in G1 phase^[30]. Thus, we propose that the mechanism under which HMGB3 promotes cancer onset and development is mainly by enhancing cell proliferation.

In summary, high-level expression of HMGB3 protein was detected in gastric adenocarcinoma cells. The overexpression of HMGB3 was correlated with a poor prognosis of gastric cancer patients. HMGB3 may promote gastric cancer cell proliferation by regulating the cell cycle. Therefore, our data encourages further investigations to elucidate the role of HMGB3 and its molecular mechanism.

COMMENTS

Background

Gastric cancer is reported to be one of the common devastating types of cancer that has a high occurrence rate, short survival period and high mortality rate. The mortality rate can be up to 25.2/100 000 in China, which accounts for 25% of deaths in malignant cancer. The occurrence ratio and mortality are ranked as No. 1 and No. 2 in malignant cancer.

Research frontiers

Recent studies showed that high mobility group-box3 (HMGB3) and NPU98 fusion protein form a new oncogenic gene in leukemia. HMGB3 also participates in recurrence of acute lymphoid leukemia and it shows a high expression level in the progression phase of breast cancer. HMGB3 was identified as one of the biomarkers detected in peripheral blood in lung cancer. Though HMGB3 is found in some types of cancers, its role in gastric cancer is still unclear.

Innovations and breakthroughs

The authors observed the expression of HMGB3 in gastric adenocarcinoma tissues by immunohistochemistry, and analyzed its correlation between expression level and clinicopathologic variables and prognosis. Results indicated that the overexpression of HMGB3 is a marker for poor prognosis of gastric adenocarcinomas. HMGB3 may function through affecting gastric cell proliferation and cell cycle.

Peer review

The results are interesting and suggest that HMGB3 is likely to be a useful prognostic marker involved in gastric cancer disease onset and progression by regulating the cell cycle.

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Donor safety and remnant liver volume in living donor liver transplantation

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Abstract

AIM: To evaluate the relationship between donor safety and remnant liver volume in right lobe living donor liver transplantation (LDLT).

METHODS: From July 2001 to January 2009, our liver transplant centers carried out 197 LDLTs. The clinical data from 151 cases of adult right lobe living donors (not including the middle hepatic vein) were analyzed. The conditions of the three groups of donors were well matched in terms of the studied parameters. The donors' preoperative data, intraoperative and postoperative data were calculated for the three groups: Group 1 remnant liver volume (RLV) < 35%, group 2 RLV 36%-40%, and group 3 RLV > 40%. Comparisons included the different remnant liver volumes on postoperative liver function recovery and the impact of systemic conditions. Correlations between remnant liver volume and post-operative complications were also analyzed.

RESULTS: The donors' anthropomatology data, op-

eration time, and preoperative donor blood test indicators were calculated for the three groups. No significant differences were observed between the donors' gender, age, height, weight, and operation time. According to the Chengdu standard liver volume formula, the total liver volume of group 1 was 1072.88 ± 131.06 mL, group 2 was 1043.84 ± 97.11 mL, and group 3 was 1065.33 ± 136.02 mL. The three groups showed no statistically significant differences. When the volume of the remnant liver was less than 35% of the total liver volume, the volume of the remnant had a significant effect on the recovery of liver function and intensive care unit time. In addition, the occurrence of complications was closely related to the remnant liver volume. When the volume of the remnant liver was more than 35% of the total liver volume, the remnant volume change had no significant effect on donor recovery.

CONCLUSION: To ensure donor safety, the remnant liver volume should be greater than the standard liver volume (35%) in right lobe living donor liver transplantation.

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Key words: Donor safety; Remnant liver volume; Living donor; Liver transplantation; Complications grade

Peer reviewer: Christopher Christophi, Professor and Head of The University of Melbourne Department of Surgery, Austin Hospital, Melbourne, 145 Studley Road, Victoria 3084, Australia

Shi ZR, Yan LN, Du CY. Donor safety and remnant liver volume in living donor liver transplantation. *World J Gastroenterol* 2012; 18(48): 7327-7332 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i48/7327.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i48.7327>

INTRODUCTION

To solve the shortage of liver grafts in adult liver trans-

plantation, an increasing number of transplant centers have used right graft living donor liver transplantation. This surgical method can provide a greater proportion of liver grafts to meet the metabolic demands of recipients. However, right lobe graft donors take more risks and have more complications than left graft donors. This has created considerable controversy with respect to donor safety. At the present time, there have been 17 donor deaths reported, and the morbidity was reported to be in the range of 20% to 30%^[1-3].

It has been reported in the literature that donor complications are closely related to remnant liver volume (RLV)^[4,5]. Initial experiences from previous studies have suggested leaving a remnant of not less than 30%^[6]. Other articles have reported that remnant liver volumes less than 35% do not appear to be a contraindication for right liver procurement in living donors^[7].

Considering the controversy regarding safety and remnant liver volume in right-lobe living donor liver transplantation (LDLT), we analyzed our own data. We retrospectively examined the remnant liver volume in our right graft donors and compared those donors with different remnant liver volumes. Thus, the aim of the present study was to assess the relationship between donor recovery, complications and the volume of remnant liver^[7,8].

MATERIALS AND METHODS

Patient population

From July 2001 to January 2009, our transplant centers carried out 197 LDLTs. Inclusion criteria were: (1) a healthy adult donor, age > 18 and < 60 years; (2) a right liver graft without the middle hepatic vein (MHV); (3) adult-to-adult LDLT; (4) single donors; and (5) without a history of long-term drinking. Exclusion criteria: (1) age < 18 or > 60 years; (2) a left hepatic graft or left lateral lobe graft; (3) double donor grafts; (4) adult-to-child transplants; and (5) donors that were hepatitis B virus or hepatitis C virus carriers.

After the above selection criteria eliminated some patients, eligible subjects were identified. In total, we identified 151 cases of right liver adult-to-adult living donors (not including the MHV). Ninety cases were male and 61 were female. The total liver volume was calculated using the Chengdu standard liver volume formula^[9,10]. The volume of actual grafts (excluding the MHV of the right liver) was measured intraoperatively. Remnant liver volume = the total liver volume - the volume of the actual graft. According to the ratio of the remnant liver volume to the total liver volume, the cases within the study group were further subdivided into three groups: Group 1: RLV < 35% ($n = 14$), group 2: RLV 36%-40% ($n = 20$), and group 3: RLV > 40% ($n = 117$).

Written informed consent was obtained from all patients to include their data in this study, which was approved by the HuaXi Ethics Committee and conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Preparation of preoperative clinical data

Indicator variables included: age, gender, body height

(error < 1 cm), and body weight (error < 0.5 kg). Preoperative donor blood test indicators were also obtained. These indicators included: hemoglobin (HGB), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (TBIL). Donor selection was based on a designated radiologist row computerised tomography examination and determination of liver volume. All donors used the same surgery/medical team.

Surgical procedures

An LDLT was completed through a right subcostal incision with an upward midline extension. Intraoperative cholangiography *via* cystic duct cannulation was required to evaluate the anatomy of the bile duct. A right hilar dissection was then performed to isolate the right hepatic artery, right portal vein, and right hepatic duct^[11]. The right lobe of the liver was then rotated towards the left side for division of the ligaments on the right side of the liver, the minute venous branches between the anterior surface of the inferior vena cava and the posterior surface of the paracaval portion of the caudate lobe. To prevent impeding the circulation, the right hepatic vein and the right inferior hepatic veins that were larger than 5 mm were preserved until the time of harvesting.

The transection plane was determined by intraoperative ultrasonography and the temporary occlusion of the right portal vein and right hepatic artery. After identification of the confluence of the left and right hepatic ducts, the right hepatic duct was divided near the confluence of the hepatic ducts using scissors. The divided end was closed transversely using a continuous 5-0 prolene suture. The transection was carried down to the junction of the right hepatic vein and the inferior vena cava. The right hepatic artery was then divided. To accomplish this, the right hepatic vein was clamped at the junction with the inferior vena cava and divided. The stumps of the right portal vein and right hepatic vein were closed with continuous nonabsorbable sutures. The falciform ligament was then sutured to the anterior abdominal wall. A drain was inserted into the right subphrenic cavity prior to wound closure^[12].

Measurement of volume

The weight of the grafted liver was measured using a pan scale, the error was found to be less than 10 g. The volume was measured using the drainage method in a 3 L beaker full of saline; the error was found to be less than 10 mL.

Postoperative procedures

Postoperatively, donors stayed in the intensive care unit (ICU) for monitoring and oxygen, and received parenteral nutrition which was rich in branched-chain amino acids. Donors began parenteral nutrition following intestinal function recovery. When necessary, donors received blood transfusions or plasma and human serum albumin. Postoperative monitoring of HGB, ALT, AST, TBIL, and the international standardization ratio (INR) was performed. ICU time, hospital stay, timely diagnosis, and

Table 1 Characteristics of the study group and volumetric data

Characteristics	Patients			P value		
	Group 1 (RLV < 35%) n = 14	Group 2 (40% > RLV > 35%) n = 20	Group 3 (RLV > 40%) n = 117	P1	P2	P3
Demographic and intraoperative data						
Gender (M/F), 90/61	10/4	12/8	62/46	0.717	1.000	0.395
Age (yr)	37.1 ± 8.7	41.0 ± 12.7	38.0 ± 9.8	0.337	0.156	0.431
Weight (kg)	64.3 ± 11.4	61.7 ± 8.4	63.6 ± 11.8	0.463	0.500	0.844
Height (cm)	164.9 ± 12.3	163.3 ± 9.1	164.5 ± 13.1	0.664	0.679	0.925
Operation time (min)	389.9 ± 87.2	373.4 ± 60.5	363.7 ± 71.9	0.581	0.472	0.765
EBL (mL)	602.8 ± 73.1	582.9 ± 81.6	531.3 ± 50.7	0.740	0.583	0.341
Preoperative laboratory data						
ALT (IU/L)	28.2 ± 16.6	29.8 ± 15.7	27.8 ± 20.9	0.784	0.683	0.937
AST (IU/L)	28.9 ± 17.4	25.6 ± 9.9	23.3 ± 11.1	0.496	0.389	0.105
TBIL (mg/dL)	12.7 ± 3.8	13.9 ± 6.1	15.2 ± 7.0	0.529	0.413	0.189
Hemoglobin (g/L)	147.1 ± 13.2	142.4 ± 17.2	141.5 ± 16.9	0.392	0.822	0.229
Volumetric data of Chengdu standard liver volume formula (mL)						
Whole liver volume of formula	1072.88 ± 131.06	1043.84 ± 97.11	1065.33 ± 136.02	0.463	0.500	
Graft volume	745.00 ± 100.22	653.80 ± 56.55	534.83 ± 89.26	0.006	0.000	
Remnant liver volume of formula	327.88 ± 61.83	390.04 ± 48.46	530.50 ± 125.83	0.002	0.000	
Remnant volume/whole volume of Chengdu formula(%)	30.56 ± 4.17	37.31 ± 2.06	49.63 ± 7.33	0.000	0.000	

P1: Group 1 vs group 2; P2: Group 2 vs group 3; P3: Group 1 vs group 3. RLV: Remnant liver volume; EBL: Estimated blood loss; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin.

treatment of surgical complications were recorded. Donor follow up was 6 to 48 mo, and all follow up information was recorded.

Statistical analysis

The mean ± SD of the data are presented. The SPSS program (version 15.0, SPSS Inc., United States) was used for the statistical analysis. After testing for normal distribution using Kurtosis and Skewness tests, descriptive variables including pre-operative, intra-operative, post-operative and prognostic parameters, were calculated. Fisher's exact test was used to detect the differences among the groups for categorical variables, including gender. Independent-sample *t* tests were calculated to detect differences among the groups for continuous random variables including HGB, ALT, AST, TBIL, volumetric data, postoperative INR, ICU time, hospital stay, and reasonable and customary (R and C). A correlation analysis using the χ^2 test was conducted to determine the incidence of complications. The difference was considered significant if $P < 0.05$.

RESULTS

Baseline data of donors

The donors' anthroposomatology data, operation time, and preoperative blood test indicators were calculated for the three groups: Group 1 RLV < 35%, group 2 RLV 36%-40%, and group 3 RLV > 40%. These data are shown in Table 1. No significant differences were observed between the donors' gender, age, height, weight, and operation time. Preoperative data on ALT, AST, TBIL and HGB were also collected among the three groups of donors. These results suggested that

the conditions of the three groups of donors were well matched in terms of the studied parameters.

Volume-related parameters

Compared to Heinemann, Urata, Vauthey^[13-16], and the Lee formulae^[17], the Chengdu standard liver volume formula was demonstrated to be more reliable in LDLT. In LDLT, this formula can more accurately forecast total liver volume^[10]. Standard liver volumes in the 151 cases were calculated using the Chengdu formula: SLV (mL) = 11.5 × body weight (kg) ± 334. The volumes of the actual grafts (excluding the MHV of the right liver) were measured intraoperatively. Remnant liver volume = the total liver volume - the volume of the actual graft. We determined the ratio of the remnant liver by remnant liver volume/standard liver volume. The liver volume-related parameters are shown in Table 1.

According to the Chengdu standard liver volume formula, the total liver volume in group 1 was 1072.88 ± 131.06 mL, group 2 was 1043.84 ± 97.11 mL, and group 3 was 1065.33 ± 136.02 mL. The three groups showed no statistically significant differences. However, the graft volume in group 1 was 745.00 ± 100.22 mL, group 2 was 653.80 ± 56.55 mL, and group 3 was 534.83 ± 89.26 mL, revealing a statistically significant difference between the groups. Remnant liver volume also showed significant differences.

Postoperative characteristics

Postoperative monitoring of donor ALT peak, AST peak, TBIL peak, INR peak, and HGB value was conducted during their ICU stay and hospital stay. Postoperative characteristics are illustrated in Figure 1. The ALT peak in the smallest remnant liver volume in group 1 was

Table 2 Complications of the 50 donors classified according to the modified Clavien system

Grades, n (%)	Complications	n
Grade 1, 28 (18.5)	Transient bile leak treated conservatively	7
	Superficial wound infection treated without antibiotics	2
	Postoperative voice change	3
	Mild pleural effusion treated conservatively	2
	Mild subphrenic effusion treated conservatively	5
	Hyperbilirubinemia > 1.3 mg/dL 7 d after operation	9
Grade 2, 9 (6.0)	Intra-abdominal bleeding requiring blood transfusion	1
	Bile leak not requiring ERCP or surgical intervention	3
	Dyspepsia	1
	Chyle leak	1
	Wound infection requiring antibiotics	1
	Pneumonia requiring antibiotics	2
Grade 3a, 8 (5.3)	Bile leak needing ERCP	3
	Pleural effusion requiring thoracic cavity puncture	2
	Pleural effusion requiring thoracic drainage	1
	Subphrenic infection requiring abdominal cavity puncture	1
	Chylothorax requiring thoracic cavity puncture	1
	Portal vein thrombosis requiring re-laparotomy	1
Grade 3b, 5 (3.3)	Biliary stricture requiring ERCP with stent placement	2
	Abdominal hematoma requiring intervention	1
	Intra-abdominal bleeding requiring re-laparotomy	1
		0
Grade 4a		0
Grade 4b		0
Grade 5		0

ERCP: Endoscopic retrograde cholangiopancreatography.

325.64 ± 202.33 U/L, this value was significantly higher than that in the other two groups (196.85 ± 130.62 U/L and 200.70 ± 150.94 U/L, respectively). The AST peak of 339.79 ± 172.91 U/L was also significantly higher than that in group 2 and group 3 (*P* value = 0.010 and 0.003, respectively). The ALT peak and AST peak in groups 2 and 3 showed no significant difference (*P* = 0.915 and 0.893, respectively). However, differences in the TBIL peak, INR peak, and HGB value among the three groups of donors were observed, but no statistical differences were found.

The ICU time for group 1 was 6.93 ± 2.13 d, significantly longer than that for group 2 and group 3 (5.10 ± 1.62 d *vs* 5.33 ± 1.63 d, respectively). There was no statistical difference in ICU stay between groups 2 and 3. The three groups of patients exhibited no significant difference in hospitalization time.

Clavien classification system of complications

The Clavien classification system has been increasingly used in the analysis of post-surgical complications^[18,19]. Researchers have also begun to use this classification in the LDLT donor complication category^[20,21]. The donor complications were calculated according to the Clavien classification system of grading.

Donor complication grade

Fifty donors that exhibited a total of 151 complications. According to the Clavien grading system, 28 cases had grade 1 complications, 9 cases had grade 2 complications,

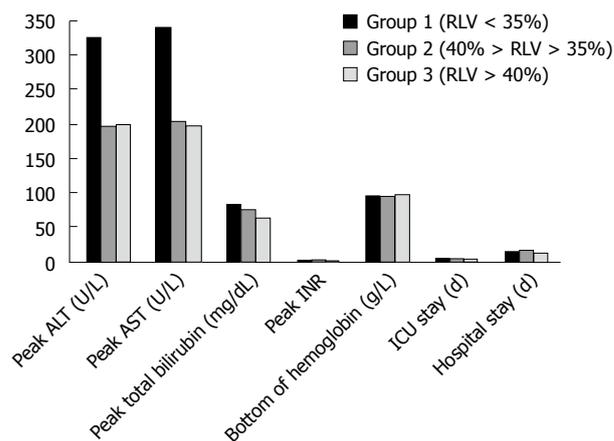


Figure 1 Postoperative characteristics of the donors. RLV: Remnant liver volume; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; INR: International standardization ratio; ICU: Intensive care unit.

8 cases had grade 3a complications, and 5 cases had grade 3b complications. No serious grade 4 or 5 complications were observed. There were no donor deaths (Table 2).

Analysis of donor complications

A correlation analysis of the χ^2 test was used to compare different grades of complications among the three groups of donors (Table 3). R and C correlation analysis revealed that the complication grade had a significant relationship with remnant liver volume.

DISCUSSION

The evaluation of suitable donors is related to both donor and recipient safety. The volume of the graft liver should ensure the absolute safety of the donor, but also meet the needs of the recipient. For example, if the remnant liver volume is too small for the body, it can lead to acute liver failure in the donor. If the graft is too small, it can result in small-for-size graft syndrome^[22,23]. In general, the younger the donor, the better is the liver's regenerative capacity^[24], thus donors aged between 18 and 60 years are required.

In this study, donors age ranged from 18 to 60 years. During the preoperative examination, there were no obvious abnormal liver functions, obvious blood vessels, biliary anatomical abnormalities, or intraoperative liver biopsies with serious fatty degeneration. According to the remnant liver volume, the study group was divided into three groups. Data for each group of donors was recorded, and included preoperative parameters, operative time, and intraoperative blood loss. No statistical differences between the groups were found. The three groups also had homogeneity of the body, excluding other factors of donor recovery.

Postoperative data revealed that the ALT and AST peaks in group 1 donors were significantly higher than those in the other two groups. There were no significant differences (*P* = 0.915 and 0.893, respectively) for the ALT peak or AST peak between groups 2 and 3. The

Table 3 Postoperative complications of donors

Complications	Grades					P value
	No complications (n = 101)	Grade 1 (n = 28)	Grade 2 (n = 9)	Grade 3a (n = 8)	Grade 3b (n = 5)	
Group 1 (RLV < 35%), n = 14	3	5	3	2	1	0.000
Group 2 (40% > RLV > 35%), n = 20	10	4	3	1	2	
Group 3 (RLV > 40%), n = 117	88	19	3	5	2	

RLV: Remnant liver volume.

ICU time in group 1 donors was significantly longer than that in group 2 and 3 donors. No statistical differences in ICU time between groups 2 and 3 were observed.

Postoperative indicators showed that when the remnant liver volume was greater than 35% of standard liver volume, the volume of the remnant liver had no significant effect on the recovery of liver function or the ICU time, however, when the remnant liver volume was less than 35%, this led to a much slower recovery of liver function.

In LDLT, there are three possible types of short-term complications. One type of complication includes bleeding, biliary leakage, embolization, liver failure, metabolic abnormalities caused by cholinesterase, and hypophosphatemia. The second are open surgery-related complications and include intra-abdominal infections, incisional hernias, adhesions, and intestinal obstruction. There are also complications associated with anesthesia.

An international statistical analysis showed that the incidence of complications in donors was 10%-30%, while the case fatality rate was 0.1%-0.3%^[4,25-27]. In this study, 33% of the 151 complications occurred in 50 patients. According to the Clavien grading system, the patients in this study experienced 28 grade 1 complications, 9 grade 2 complications, 8 grade 3a complications, and 5 grade 3b complications. There were no serious grade 4 or grade complications and no donor deaths. R and C correlation analysis showed that complication grades had a significant relationship with remnant liver volume.

In summary, when the volume of a remnant liver was less than 35% of the standard liver volume, the volume of the remnant had a significant effect on the recovery of liver function and ICU time. In addition, the occurrence of complications was closely related to remnant liver volume. Recipients were only available if good results were expected. Therefore, the interests of the donor should be accounted for to minimize their risks during surgery.

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COMMENTS

Background

An increasing number of transplant centers have used right graft living donor liver transplantations. This surgical method could provide a greater number of

liver grafts to meet the metabolic demands of recipients. However, right lobe graft donors take more risks and have more complications than left graft donors. This has created considerable controversy with respect to donor safety. At the present time, 26 donor deaths have been reported, and morbidity was reported to be in the range of 20% to 30%.

Research frontiers

The literature has reported that donor complications are closely related to remnant liver volume (RLV). The authors retrospectively examined the RLV in right graft donors and compared those donors with other different RLVs.

Innovations and breakthroughs

This has created considerable controversy with respect to RLV and donor safety. The aim of the present study was to assess the relationship between donor recovery, complications and the volume of remnant liver.

Applications

The SPSS program (version 15.0, SPSS Inc., United States) was used for the statistical analysis. After testing for normal distributions using Kurtosis and Skewness tests, descriptive variables including pre-operative, intra-operative, post-operative and prognostic parameters were calculated. Fisher's exact test was used to detect the differences among the groups for categorical variables, including gender. Independent-sample *t* tests were used to detect differences among the groups for continuous random variables including hemoglobin, alanine aminotransferase, aspartate aminotransferase, total bilirubin, volumetric data, postoperative International Normalized ratio, intensive care unit time, hospital stay, and reasonable and customary. A correlation analysis using the chi-squared test was conducted to determine the incidence of complications.

Peer review

This study is an analysis of 151 cases of adult right lobe living related donor to determine the effect of volume of the remnant liver on post operative complications. The conclusions reached was that volumes less than 35% of total liver volumes were associated with increased complications and length of recovery. This is a significant study adding further to the knowledge in this area of liver transplantation.

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Incidence of gastroesophageal reflux disease in Uygur and Han Chinese adults in Urumqi

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Abstract

AIM: To investigate the incidence of gastroesophageal reflux disease (GERD) and its related risk factors in Uygur and Han Chinese adult in Urumqi, China.

METHODS: A population-based cross-sectional survey was undertaken in a total of 972 Uygur (684 male and 288 female) aged from 24 to 61 and 1023 Han Chinese (752 male and 271 female) aged from 23 to 63 years. All participants were recruited from the residents who visited hospital for health examination from November 2011 to May 2012. Each participant signed an informed consent and completed a GERD questionnaire (Gerd

Q) and a lifestyle-food frequency questionnaire survey. Participants whose Gerd Q score was ≥ 8 and met one of the following requirements would be enrolled into this research: (1) being diagnosed with erosive esophagitis (EE) or Barrett's esophagus (BE) by endoscopy; (2) negative manifestation under endoscopy (non-erosive reflux disease, NERD) with abnormal acid reflux revealed by 24-h esophageal pH monitoring; and (3) suffering from typical heartburn and regurgitation with positive result of proton pump inhibitor test.

RESULTS: According to Gerd Q scoring criteria, 340 cases of Uygur and 286 cases of Han Chinese were defined as GERD. GERD incidence in Uygur was significantly higher than in Han Chinese (35% vs 28%, $\chi^2 = 11.09$, $P < 0.005$), Gerd Q score in Uygur was higher than in Han Chinese (7.85 ± 3.1 vs 7.15 ± 2.9 , $P < 0.005$), and Gerd Q total score in Uygur male was higher than in female (8.15 ± 2.8 vs 6.85 ± 2.5 , $P < 0.005$). According to normalized methods, 304 (31%) cases of Uygur were diagnosed with GERD, including 89 cases of EE, 185 cases of NERD and 30 cases of BE; 256 (25%) cases of Han Chinese were diagnosed with GERD, including 90 cases of EE, 140 cases of NERD and 26 cases of BE. GERD incidence in Uygur was significantly higher than in Han Chinese (31% vs 25%, $\chi^2 = 9.34$, $P < 0.005$) while the incidences were higher in males of both groups than in females (26% vs 5% in Uygur, $\chi^2 = 35.95$, $P < 0.005$, and 19.8% vs 5.2% in Han, $\chi^2 = 5.48$, $P < 0.025$). GERD incidence in Uygur male was higher than in Han Chinese male (26% vs 19.8%, $\chi^2 = 16.51$, $P < 0.005$), and incidence of NERD in Uygur was higher than in Han Chinese ($\chi^2 = 10.06$, $P < 0.005$). Occupation ($r = 0.623$), gender ($r = 0.839$), smoking ($r = 0.322$), strong tea ($r = 0.658$), alcohol drinking ($r = 0.696$), meat-based diet (mainly meat) ($r = 0.676$) and body mass index (BMI) ($r = 0.567$) were linearly correlated with GERD in Uygur ($r = 0.833$, $P = 0.000$); while gender ($r = 0.957$), age ($r = 0.016$), occupation ($r = 0.482$), strong tea ($r = 1.124$), alcohol drinking ($r = 0.558$), meat diet ($r = 0.591$) and BMI

($r = 0.246$) were linearly correlated with GERD in Han Chinese ($r = 0.786$, $P = 0.01$). There was no significant difference between Gerd Q scoring and three normalized methods for the diagnosis of GERD.

CONCLUSION: GERD is highly prevalent in adult in Urumqi, especially in Uygur. Male, civil servant, smoking, strong tea, alcohol drinking, meat diet and BMI are risk factors correlated to GERD.

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Key words: Gastroesophageal reflux disease; Incidence; Uygur; Han; Risk factors; Urumqi

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is a common acid-related disease worldwide, and severely affects the quality of life of patients. The incidence of GERD is high in Western and developed countries, however, due to changes in lifestyle and dietary habits the incidence of GERD has increased in the Asian-Pacific region in recent years^[1,2], particularly in China. This is due to the introduction of Western fast food into the Chinese dietary structure which has had a significant influence since its introduction ten years ago. The incidence of GERD in China is rising year by year^[3], and is showing a “low in south and high in north” trend^[4]. Xinjiang is located in the Northwest region of China, and Uygur is the main minority group in this autonomous region of China. An important dietary custom in local residents is to prioritize meat (mainly beef and lamb), cooked wheaten food, and alcohol drinking. In addition, strong tasting foods are favored. Together with the development of social and cultural diversity, there appears to have been culture blendings and interactions between Uygur and Han Chinese as time goes by. In addition to these factors, the particular geographic and climatic characteristics of Xinjiang exert an influence on the lifestyle and dietary customs of local inhabitants. Therefore, there are currently both similarities and differences between Uygur and Han Chinese with regard to lifestyle and dietary customs. Furthermore, genetics studies have revealed that the Han Chinese gene contains Oriental Mongoloid characteristics, while the Uygur gene contains both Caucasian and Oriental Mongoloid characteristics. To our knowledge, there has been little research focusing on the incidence of GERD with regard to minority groups^[5],

and large-scale studies focusing on GERD in the Uygur population alone have not been carried out. Based on the above research background, we aimed to elucidate the following: (1) Does lifestyle and dietary customs or dietary structure in Xinjiang inhabitants impact on GERD occurrence; (2) differences in the incidence of GERD in Uygur and Han Chinese; and (3) potential risk factors related to GERD, and provide guidance in clinical practice.

MATERIALS AND METHODS

Participants

This study was a population-based cross-sectional, randomized, single center, open-label trial. A total of 972 Uygur (684 males and 288 females) aged 24 to 61 years and 1023 Han (752 males and 271 females) aged 23 to 63 years living in Urumqi were enrolled into this study. All participants, who were residents of Xinjiang, China (college students, workers and civil servants) were recruited from those who attended the First Affiliated Hospital of Xinjiang Medical University for a health examination from November 2011 to May 2012. All participants signed an informed consent before participating in this research. Each subject completed a GERD questionnaire (Gerd Q) scale and a lifestyle-food frequency questionnaire (FFQ).

Inclusion criteria

Participants whose Gerd Q score was ≥ 8 and who met one of the following requirements were enrolled in this study: (1) diagnosed with erosive esophagitis (EE) or Barrett’s esophagus (BE) on upper gastrointestinal endoscopy; (2) negative manifestations on endoscopy (non-erosive reflux disease, NERD) with abnormal acid reflux revealed by 24-h esophageal pH monitoring; and (3) suffering from typical heartburn and regurgitation with a positive proton pump inhibitor (PPI) test.

Exclusion criteria

Any participant who met one of the following requirements was excluded from this study: (1) receiving a PPI or H₂ receptor blocker; (2) suffered from serious gastrointestinal diseases (esophageal stricture, peptic ulcer, esophagus or stomach tumor) or a history of abdominal surgery; (3) suffered from major organ insufficiency; and (4) pregnant and lactating women.

Assessment of lifestyle and dietary customs

Alcohol consumption in individuals was estimated by multiplying the mean weekly alcohol intake by the concentration of alcohol (12% alcohol for wine or 4% alcohol for beer). Alcohol intake per week of ≥ 140 g for males and ≥ 70 g for females was assessed as alcohol consumption. If a participant smoked more than 10 cigarettes per day then he/she was assessed as a smoker. Tea consumption over the past month was determined using a FFQ^[6]. Nutritional intake was assessed using a validated 110-item FFQ (the Block 98)^[7]. Body mass index (BMI) ≥ 25 kg/m² was defined as overweight.

Table 1 Gastroesophageal reflux disease questionnaire scale

Symptom	Symptoms in the past 7 d	0 d	1 d	2-3 d	4-7 d
Positive symptom	Heart burn	0	1	2	3
	Regurgitation	0	1	2	3
Negative symptom	Upper abdominal pain	3	2	1	0
	Nausea	3	2	1	0
Positive affect	Sleeping disorder	0	1	2	3
	Extra medication	0	1	2	3

Gerd Q and scoring

Total cumulative Gerd Q score was calculated by a gastroenterologist according to Gerd Q after questioning each participant about his or her symptoms during the previous 7 d. Subjects whose Gerd Q score was ≥ 8 were estimated to have pathological acid reflux^[8] (Table 1).

Assessment by upper gastrointestinal endoscopy

Subjects who were willing to undergo endoscopy were instructed to stop all medications that affected alimentary motility and endocrine function, and then fasted for at least 8 h. The presence or absence of reflux esophagitis, endoscopically suspected esophageal metaplasia and hiatal hernia were determined by specialized endoscopists. If EE was present, it was graded according to the Los Angeles classification^[9]. The diagnosis of BE was based on endoscopy and pathology^[10].

Ambulatory 24-h esophageal pH monitoring

Subject who were willing to undergo 24-h esophageal pH monitoring were instructed to stop all medications that affected alimentary motility and endocrine function for at least 2 wk. Ambulatory 24-h esophageal pH monitoring was performed after fasting for at least 8 h. Single-use pH electrodes were placed 5 cm above the proximal margin of the lower esophageal sphincter. The subjects were encouraged to eat regular meals, however, the intake of food and drink with a pH below 4 was restricted. All subjects recorded their meal times (start and end), body position (supine and upright), and any symptoms in a diary. The data were collected using a portable data logger (Digitrapper Mark III, Synetics Medical Co., Stockholm, Sweden) with a sampling rate of 4 s, and were transferred to a computer for analysis. The variables assessed for gastroesophageal reflux in the distal probe were the total percentage of time the pH was < 4 , the percentage of time the pH was < 4 in the supine and upright positions, the number of episodes where the pH was < 4 , the number of episodes the pH was < 4 for ≥ 5 min, the duration of the longest episode where the pH was < 4 and the DeMeester composite score^[11]. A DeMeester score ≥ 14.72 was considered abnormal.

Statistical analysis

All measured data were expressed as mean \pm SD. The χ^2 test, *U* test and analysis of variance were used for comparisons between the groups, linear regression and multi-

variate logistic regression (backward) analyses were used for related factors analysis. Values of $P < 0.05$ (two-tailed) were considered statistically significant. All calculations were performed using SPSS version 20.0 software (IBM SPSS Statistics 20).

RESULTS

Baseline characteristics of the participants

There were no statistically significant differences between the Uygur and Han participants in terms of occupation, gender, age, smoking (long-term and excessive smoking) and alcohol consumption (chronic overdrinking), however, there was a significant difference between the two groups in terms of dietary customs (mainly meat compared with mainly vegetarian) (Table 2).

GERD incidence in Uygur and Han participants

According to Gerd Q scoring criteria, a total score of ≥ 8 was regarded as the assessment point. GERD was detected in 340 Uygur participants (incidence 35%) and in 286 Han participants (incidence 28%). The incidence of GERD in Uygur was significantly higher than that in Han Chinese ($\chi^2 = 11.09$, $P < 0.005$). The Gerd Q score in Uygur (7.85 ± 3.1) was higher than that in Han (7.15 ± 2.9) ($P < 0.005$), and the Gerd Q total score in Uygur males (8.15 ± 2.8) was higher than that in females (6.85 ± 2.5) ($P < 0.005$) (not listed in Table 2).

All participants who had been diagnosed with GERD using the Gerd Q scale underwent further endoscopy. Mucosal lesions were diagnosed as EE or BE, respectively, according to pertinent criteria, while those participants who did not have positive endoscopy findings underwent one of the following examinations: 24-h esophageal pH monitoring or the PPI test, and if any one of these two examinations was positive, the participant was diagnosed with NERD. According to the above three normalized methods (upper gastrointestinal endoscopy, 24-h esophageal pH monitoring and the PPI test), 304 Uygur participants were diagnosed with GERD (incidence 31%), including 89 cases of EE, 185 cases of NERD and 30 cases of BE; 256 Han participants were diagnosed with GERD (incidence 25%), including 90 cases of EE, 140 cases of NERD and 26 cases of BE. The incidence of GERD in Uygur was significantly higher than that in Han, and the incidence of GERD in male Uygur and Han was higher than that in female Uygur and Han, respectively (Table 3).

There was no significant difference between Gerd Q scoring criteria and the three normalized methods regarding the diagnosis of GERD (Uygur: $\chi^2 = 1.48$, $P > 0.5$; Han: $\chi^2 = 0.83$, $P > 0.5$) (not listed in Table 3).

Differences in lifestyle and dietary customs between Uygur and Han participants

We compared the GERD groups and normal groups, and analyzed the relationship between the following factors and GERD using the *U* and χ^2 tests in order to illus-

Table 2 Baseline characteristics of the participants

Ethnic group	<i>n</i>	Occupation (college students, workers/civil servants)	Gender (male/female)	Age (yr)	Smoking (yes/no)	Alcohol drinking (yes/no)	Diet custom (mainly meat/mainly vegetable)
Uygur	972	497/475	684/288	43 ± 8.5	643/329	665/307	952/20
Han	1023	572/451	752/271	44 ± 9.1	728/295	725/298	297/726
<i>P</i> value		NS	NS	NS	NS	NS	0.000

NS: Not significant.

Table 3 Gastroesophageal reflux disease incidence in Uygur and Han

Ethnic group	GERD	GERD			Gender		χ^2	<i>P</i> value
		EE	NERD	BE	Male	Female		
Uygur	304 (31.3) ^a	89	185 ^a	30	254 (26) ^{c,e}	50 (5)	35.95	< 0.005
Han	256 (25)	90	140	26	203 (19.8) ^f	53 (5.2)	5.48	< 0.025
χ^2	9.34		10.06		16.51	0.31		
<i>P</i> value	< 0.005		< 0.005		< 0.005	NS		

^a*P* < 0.05 vs Han; ^c*P* < 0.05 vs female; ^e*P* < 0.05 vs Han male. EE: Erosive esophagitis; BE: Barrett’s esophagus; NERD: Non-erosive reflux disease; NS: Not significant.

Table 4 Risk factors related to gastroesophageal reflux disease in Uygur and Han Chinese by regression analysis

Risk factor	Uygur					Han Chinese				
	B	Std. E	Beta	<i>t</i>	<i>P</i> value	B	Std. E	Beta	<i>t</i>	<i>P</i> value
Occupation	0.623	0.169	0.115	3.079	0.005	0.957	0.218	0.208	4.393	0.000
Gender	0.839	0.271	0.136	3.132	0.002	-0.016	0.005	-0.06	-3.059	0.002
Smoking	0.322	0.123	0.064	2.614	0.009	0.482	0.088	0.113	5.449	0.000
Strong tea	-0.658	0.140	-0.134	-4.797	0.000	-1.124	0.142	-0.233	-7.935	0.000
Drinking	-0.696	0.145	-0.138	-4.805	0.000	-0.558	0.112	-0.128	-4.971	0.000
Mainly meat	-0.676	0.168	-0.132	-4.011	0.000	-0.591	0.165	-0.092	-3.579	0.000
BMI	0.567	0.029	0.638	19.653	0.000	0.246	0.034	0.265	7.183	0.000

BMI: Body mass index.

trate their potential impact on the occurrence of GERD (Figure 1). We found that, occupation (civil servant) and BMI were the collective factors related to GERD shared by the two ethnic groups and both genders; in Uygur participants with GERD, both males and females were correlated with strong tea and alcohol drinking (Figure 1A and B); while for Han participants with GERD, both males and females were correlated with strong tea, alcohol drinking and dietary custom (mainly meat) (Figure 1C and D).

Multivariate logistic regression analysis of risk factors for GERD in Uygur and Han participants

We analyzed the correlation between GERD and these factors further using a multivariate linear statistical method. The relationships between the risk factors for GERD in the two ethnic groups are listed below (Table 4 and Figure 2). Seven factors were found to correlate with GERD in Uygur and Han Chinese, respectively.

After removing age as a risk factor, the backward elimination method revealed that seven factors showed a significant linear correlation with GERD in Uygur participants (Table 4): occupation (*r* = 0.623), gender

(*r* = 0.839), smoking (*r* = 0.322), strong tea (*r* = 0.658), alcohol drinking (*r* = 0.696), mainly meat (*r* = 0.676) and BMI (*r* = 0.567). Accordingly, the regression equation was: y (GERD) = -6.464 + 0.623 × occupation + 0.839 × gender + 0.322 × smoking - 0.658 × strong tea - 0.696 × drinking - 0.676 × mainly meat + 0.567 × BMI (*r* = 0.833, *P* = 0.000) (Figure 2A).

For Han Chinese, seven factors showed a significant linear correlation with GERD: gender (*r* = 0.957), age (*r* = 0.016), occupation (*r* = 0.482), strong tea (*r* = 1.124), alcohol drinking (*r* = 0.558), mainly meat (*r* = 0.591) and BMI (*r* = 0.246), particularly strong tea and BMI (Table 4). Accordingly, the regression equation was: y (GERD) = -4.882 + 0.957 × gender - 0.016 × age + 0.482 × occupation - 1.124 × tea - 0.558 × drinking - 0.591 × meat + 0.246 × BMI (*r* = 0.793, *P* = 0.008) (Figure 2B).

DISCUSSION

GERD is a chronic disorder which has a significant impact on quality of life, and consumes a large number of medical resources worldwide. The incidence of GERD is higher than ever, particularly in Asian countries and

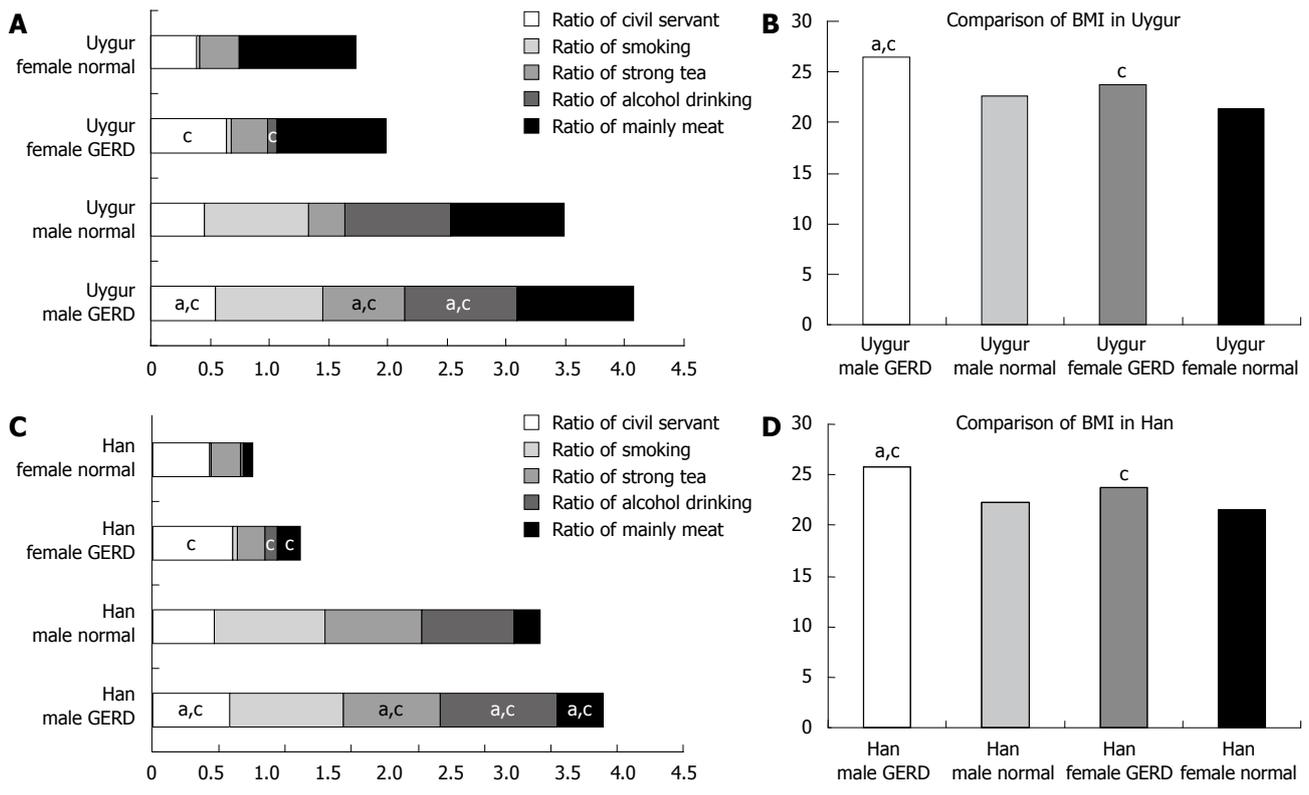


Figure 1 Relationship between occupation (civil servant), smoking, strong tea, alcohol drinking as well as body mass index and gastroesophageal reflux disease in Uygur and Han Chinese adults. A: For gastroesophageal reflux disease (GERD) males, civil servant ratio (OR = 1.49, 95% CI 1.44-1.54), strong tea ratio (OR = 5.36, 95% CI 5.29-5.41), alcohol drinking ratio (OR = 2.33, 95% CI 2.13-2.53); B: Body mass index (BMI) value (26.5 ± 3.1 vs 22.7 ± 3.0 for GERD males vs normal males, $P < 0.005$ and 26.5 ± 3.1 vs 23.8 ± 3.8 for GERD males vs GERD females, $P < 0.01$) were higher than those in normal males and in GERD females, respectively; while for GERD females, civil servant ratio (OR = 2.77, 95% CI 2.56-2.98), alcohol drinking ratio (OR = 6.81, 95% CI 5.62-8) and BMI value (23.8 ± 3.8 vs 21.4 ± 2.9 , $P < 0.05$) were higher than those in normal females; C: For GERD males, civil servant ratio (OR = 1.64, 95% CI 1.59-1.7), strong tea ratio (OR = 2.3, 95% CI 2.23-2.36), alcohol drinking ratio (OR = 3.3, 95% CI 3.19-3.41), mainly meat ratio (OR = 2.35, 95% CI 2.28-2.42); D: BMI value (25.8 ± 3.5 vs 22.3 ± 2.7 for GERD males vs normal males, $P < 0.005$ and 25.8 ± 3.5 vs 23.8 ± 3.0 for GERD males vs GERD females, $P < 0.01$) were higher than those in normal males and in GERD females, respectively; while for GERD females, civil servant ratio (OR = 2.05, 95% CI 1.86-2.24), alcohol drinking ratio (OR = 4.44, 95% CI 3.61-5.27), mainly meat ratio (OR = 2.58, 95% CI 2.19-2.97) and BMI value (22.9 ± 3.8 vs 20.4 ± 2.9 , $P < 0.01$) were higher than those in normal females. ^a $P < 0.05$ vs male of the same ethnic group; ^c $P < 0.05$ vs female of the same ethnic group.

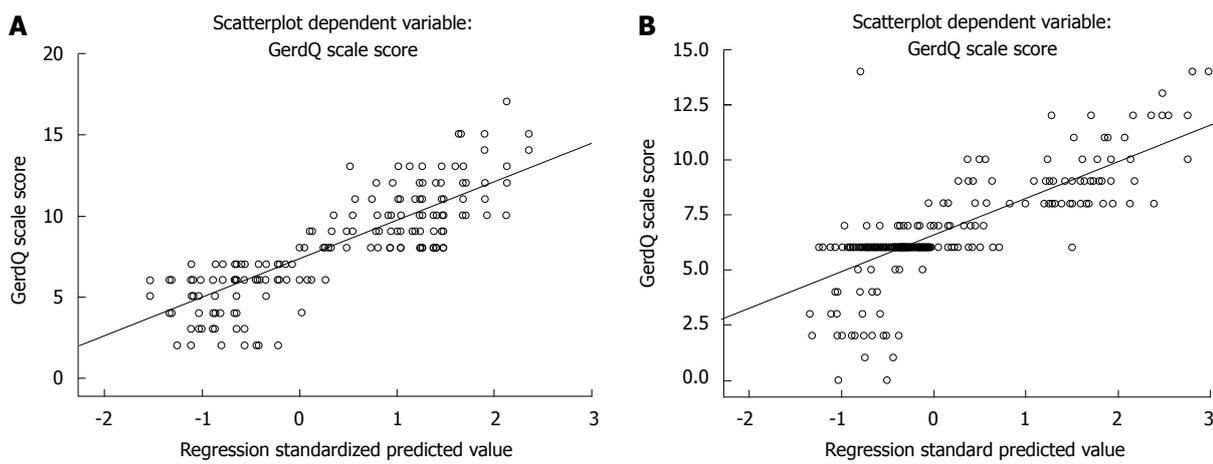


Figure 2 The risk factors linearly correlated with gastroesophageal reflux disease in both Uygur (A) and Han Chinese (B).

China. Many publications on GERD have focused on southern China, however, there are few reports which have focused on northern China or minority groups. Therefore, the purpose of our large-scale, population-based investigation was to determine whether lifestyle

and dietary customs of the local residents in Xinjiang play a role in GERD, whether the incidence of GERD is different between Uygur and Han Chinese, and which risk factors can lead to GERD.

By applying normalized methods (upper gastroin-

testinal endoscopy, 24-h esophageal pH monitoring and the PPI test), a total of 304 Uyghur (31%) and 256 Han (25%) participants were classified as having GERD, 61% had NERD, 29% had EE and 10% had BE in the Uyghur GERD group (254 male and 50 female), and in the Han group (203 male and 53 female), 55% had NERD, 35% had EE and 10% had BE. The incidence of GERD in Uyghur was significantly higher than that in Han Chinese adults. The results of the present investigation also showed that the incidence of GERD in both Uyghur and Han male adults was higher than that in Uyghur and Han female adults, respectively. In addition, the incidence of NERD in Uyghur was higher than that in Han. Using stratification analysis, we found that in Uyghur with GERD, civil servant ratio, strong tea ratio, alcohol drinking ratio and BMI value in males were higher than those in normal males and females, respectively. The civil servant ratio, alcohol drinking ratio and BMI value in Uyghur females with GERD were higher than those in normal females. In Han with GERD, the same results as those described in Uyghur were observed, however, the mainly meat ratio in both males and females with GERD was higher than that in normal males and females. Regression analysis further revealed a correlation between seven factors and GERD in Uyghur and Han participants, respectively. The above results indicated the presence of ethnic and gender differences between Uyghur and Han Chinese with regard to GERD in China. The correlation between occupation, alcohol drinking as well as BMI and GERD was similar to previous studies^[12-14]. A recent study found that the incidence of GERD in Caucasians was higher than that in other ethnic groups, which suggested that Caucasian was a related factor^[15]. Anthropology research has demonstrated that the gene structure in Uyghur subjects contains Caucasian and Mongolian bloodlines^[16,17], thus the gene fusion characteristics in Uyghur may reasonably explain their different phenotype with regard to GERD, which is not only different from but also similar to that of Han Chinese. The mainly meat ratio in both male and female Han participants with GERD was higher than that in normal males and females, respectively, and may be an independent risk factor associated with GERD. Although we cannot explain this result in Han at present, further research is needed in order to determine whether this result is objective, and whether ethnic groups or gene polymorphisms are related to GERD occurrence^[18,19].

It has been shown that there are geographic and population (gender, age, ethnic group) differences in the incidence of GERD^[20]. Related GERD etiological factors include BMI, smoking, alcohol consumption, esophageal hiatus, and inheritance. GERD may also be associated with chronic cough, asthma, pharyngitis, stomatitis and sleep disorder^[12]. In the present study, the incidence of GERD in Han was higher than that found in several previous reports^[3,4], and was possibly due to environmental differences. To date, there have been no published reports on GERD in Uyghur, our findings

showed that the incidence of GERD in Uyghur (including NERD) was significantly higher than that in Han. In addition, the incidence of GERD in Uyghur was higher than that in Israeli subjects, while the GERD ratio in females was lower than those in Israeli and Japanese females^[21,22]. However, our findings were in agreement with those in previous studies carried out in Western populations, which indicated a ethnic group difference^[23], further highlighting ethnic group and geographical differences.

The Gerd Q was designed by Jones *et al*^[8] in 2007, and was used to assess and diagnose GERD, and to assess symptom changes and quality of life in GERD patients. Several studies have demonstrated its effectiveness and reliability^[24]. Receiver operating characteristic curve analysis revealed that when taking a score of 8 as the diagnostic threshold for GERD, the accuracy rating was up to 70%, which was close to the diagnostic level of a gastroenterologist. After comparing the diagnostic rate for GERD, we found that there was no significant difference between the normalized methods and the Gerd Q scale, thus a Gerd Q score of ≥ 8 can be used as a standard to diagnose GERD. Since more than 50% of GERD patients have NERD, it is impracticable to diagnose GERD routinely using endoscopy or 24-h esophageal pH monitoring in China. Therefore, in the case of a young or middle-aged patient intolerant of invasive examination who has typical reflux symptoms, but no warning symptoms, a diagnostic method based on symptoms may be desirable. We suggest that the Gerd Q scale may be used as a primary clinical diagnostic approach^[25,26].

The backward method of logistic regression analysis revealed that gender, occupation, strong tea, alcohol drinking, mainly meat and BMI were the six common related risk factors in Uyghur and Han adults with GERD; for Uyghur adults with GERD, smoking was a related risk factor which was different from that in Han, and for Han, age was a related risk factor which was different from that in Uyghur. Furthermore, all factors correlated with GERD in a linear manner. Because the age of 75% of the participants in this investigation ranged from 35 to 45 years, we did not analyze the relationship between age stratification and GERD, however, the results revealed a gender difference (Table 4). Gender, smoking and alcohol drinking have been demonstrated to be risk factors for GERD^[12]. Smoking was not shown to correlate with GERD in Han participants. The smoking ratio which was lower in Han than in Uyghur and racial difference were the main risk factors, in addition to occupation related to GERD in Han. This may have been due to the nature of work, lifestyle, and less exercise in Han. The above results confirmed the correlation between these risk factors and GERD. We suggest that males and civil servants are susceptible to GERD. A recent study demonstrated that gender was a related factor for GERD in Japan^[27], and our results are consistent with these findings.

Xinjiang is located in Northwest China, and is a multi-

ethnic area, where the day is longer than the night, and cold and dry conditions are the main climatic features which are different from those inland, and dinner-to-bed time is shorter than that inland. A recent report indicated that shorter dinner-to-bed time was significantly associated with an increased OR for GERD^[28]. Uygur is the main minority group in Urumqi, and the ethnic culture, religion, lifestyle and dietary customs are different from those in Han, as mainly meat (beef and lamb) and dairy products are important dietary components. Han Chinese who live in the same region have many similarities to Uygur subjects with the exception of differences such as meat and milky tea (milky tea is a conventional drink in Uygur). The dietary structure of residents in Xinjiang is characterized by high caloric, high fat, spicy, strong flavors, strong tea, smoking and alcohol drinking which are more prevalent in males; in addition, changing lifestyle, obesity prevalence, as well as changing population structure have increasingly led to transition of the disease spectrum, which has become and is becoming common and an important etiology of GERD in Uygur and Han Chinese. Drinking strong tea is a common traditional custom in Uygur and Han subjects in Xinjiang, Fu-Brick tea which the Uygur drink and the various teas which the Han drink are fermentative green teas. Green tea consumption has also been shown to be negatively associated with the risk of chronic atrophic gastritis, as green tea can suppress the proliferation of *Helicobacter pylori* in the stomach and inhibit the progression of chronic atrophic gastritis^[29]. Therefore, green tea, especially strong green tea, can increase gastric acid secretion leading to the development of GERD symptoms. Thus, we consider green tea to be a significant risk factor for GERD^[10].

In conclusion, the present investigation and logistic regression analysis with regard to GERD in a large population clearly demonstrated that GERD is highly prevalent in adults living in Urumqi. The incidence of GERD in Uygur and Han adults, especially Uygur, was similar to that in Western populations. There are ethnic group and gender differences between Uygur and Han, and regional differences between China's Xinjiang residents and other countries. Being male, smoking, alcohol drinking, strong tea, mainly meat and BMI are the main risk factors for GERD in Uygur, while being male, civil servant, alcohol drinking, strong tea, mainly meat and BMI are the main risk factors for GERD in Han. These factors have a significant impact on GERD symptoms and quality of life. Our results also demonstrated that the Gerd Q can be used to diagnose GERD and possibly allow the evaluation of clinical efficacy in GERD patients.

Based on the above results, it would be very advantageous to prevent the development of GERD and cure it by optimizing lifestyle (such as controlling weight^[30], aerobic exercise, avoid overeating, appropriate sleep)^[31], modifying dietary structure (low caloric intake, low fat intake, avoid strong and spicy foods, more vegetables, high cellulose, less stimulating beverages, less smoking, less alcohol drinking), and reducing foods and drugs

that probably lead to a fall in lower esophageal sphincter pressure. When suffering from GERD symptoms, one should consult a doctor and undergo appropriate treatment under the guidance of the doctor.

COMMENTS

Background

The incidence of gastroesophageal reflux disease (GERD) is high in Western and developed countries, however, along with changing lifestyle and dietary habits, the incidence of GERD has also increased in the Asian-Pacific region in recent years, particularly in China, and the incidence in China is rising year by year. Xinjiang is located in the northwest region of China, and Uygur is the main minority group in this autonomous region. The gene for GERD in Uygur is a fusion gene of Caucasian and Mongoloid characteristics. The dietary characteristics of local residents prioritizes meat (mainly beef and lamb), cooked wheaten food, and alcohol drinking. In addition, strong food tastes are favored. To date, there have been few studies on the incidence of GERD with regard to minority groups, and based on this research background, authors designed and performed this study.

Research frontiers

Studies show that there are geographical, and population (gender, age, ethnic group) differences in the incidence of GERD, and etiological factors include body mass index, smoking, alcohol consumption, esophageal hiatus hernia, and inheritance. Authors analyzed the prevalence of GERD in Urumqi, and compared the similarities and differences between Uygur and Han Chinese adults, and then identified risk factors for GERD. This investigation is fundamental for similar studies in the future.

Innovations and breakthroughs

This is a large-scale, epidemiological and cross-sectional, novel investigation focusing on gastroesophageal reflux disease in Uygur and Han subjects residing in Xinjiang, China. To date, there have only been a few reports on GERD in minority groups, and to our knowledge large-scale studies focusing on GERD in Uygur alone have not been carried out. SPSS analysis revealed the risk factors related to GERD, and the results provide further insight into the lifestyle, dietary customs and racial differences relating to GERD between Uygur and Han.

Applications

The particular lifestyle and dietary customs of Uygur prompted us to investigate the similarities and differences between Uygur and Han Chinese living in Xinjiang, China. Their results will go a long way towards research into GERD characteristics in minority groups in China. Several studies have demonstrated that, when taking a score of 8 (using GERD questionnaire, Gerd Q) as the diagnostic threshold for GERD, the accuracy rating was up to 70% which was close to the diagnostic level of a gastroenterologist. They suggest that Gerd Q may be used as a primary clinical diagnostic approach and a standard for estimating GERD.

Peer review

GERD is a world-wide disease that affects the quality of life (reflux may depend only on the sphincter defect and may be caused by bile reflux). GERD reached a high level in Western countries (not only in the so-called developed). The Western life style and diet habits, including fast-food, has been introduced in China, more in the north than in south.

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Transjugular intrahepatic portosystemic shunt vs endoscopic therapy in preventing variceal rebleeding

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Abstract

AIM: To compare early use of transjugular intrahepatic portosystemic shunt (TIPS) with endoscopic treatment (ET) for the prophylaxis of recurrent variceal bleeding.

METHODS: In-patient data were collected from 190 patients between January 2007 and June 2010 who suffered from variceal bleeding. Patients who were older than 75 years; previously received surgical treatment or endoscopic therapy for variceal bleeding; and complicated with hepatic encephalopathy or hepatic cancer, were excluded from this research. Thirty-five cases lost to follow-up were also excluded. Retrospective analysis was done in 126 eligible cases. Among them, 64 patients received TIPS (TIPS group) while 62 patients received endoscopic therapy (ET group). The relevant data were collected by patient review or telephone calls. The occurrence of rebleeding, hepatic encephalopathy or other complications, survival rate

and cost of treatment were compared between the two groups.

RESULTS: During the follow-up period (median, 20.7 and 18.7 mo in TIPS and ET groups, respectively), rebleeding from any source occurred in 11 patients in the TIPS group as compared with 31 patients in the ET group (Kaplan-Meier analysis and log-rank test, $P = 0.000$). Rebleeding rates at any time point (6 wk, 1 year and 2 year) in the TIPS group were lower than in the ET group (Bonferroni correction $\alpha' = \alpha/3$). Eight patients in the TIPS group and 16 in the ET group died with the cumulative survival rates of 80.6% and 64.9% (Kaplan-Meier analysis and log-rank test $\chi^2 = 4.864$, $P = 0.02$), respectively. There was no significant difference between the two groups with respect to 6-wk survival rates (Bonferroni correction $\alpha' = \alpha/3$). However, significant differences were observed between the two groups in the 1-year survival rates (92% and 79%) and the 2-year survival rates (89% and 64.9%) (Bonferroni correction $\alpha' = \alpha/3$). No significant differences were observed between the two treatment groups in the occurrence of hepatic encephalopathy (12 patients in TIPS group and 5 in ET group, Kaplan-Meier analysis and log-rank test, $\chi^2 = 3.103$, $P = 0.08$). The average total cost for the TIPS group was higher than for ET group (Wilcoxon-Mann Whitney test, 52 678 RMB vs 38 844 RMB, $P < 0.05$), but hospitalization frequency and hospital stay during follow-up period were lower (Wilcoxon-Mann Whitney test, 0.4 d vs 1.3 d, $P = 0.01$; 5 d vs 19 d, $P < 0.05$).

CONCLUSION: Early use of TIPS is more effective than endoscopic treatment in preventing variceal rebleeding and improving survival rate, and does not increase occurrence of hepatic encephalopathy.

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Key words: Transjugular intrahepatic portosystemic shunt; Portal hypertension; Rebleeding; Endoscopic

variceal ligation; Cyanoacrylate

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INTRODUCTION

Esophagogastric varices bleeding (EGVB) is prone to be fatal, and often induces deterioration of liver function and may lead to bacterial infection, hepatorenal syndrome, or other complications.

Currently, the first-line treatments in prevention of variceal rebleeding include medication^[1-3] and endoscopic therapy (ET)^[4-6]. Transjugular intrahepatic portosystemic shunt (TIPS) is effective in preventing rebleeding, but due to post-operative stent stenosis and a high incidence of hepatic encephalopathy^[7-11], this procedure is considered not superior to the conventional treatment in survival rate. Consequently, TIPS has been used only as a rescue approach after first-line treatment fails^[12-14]. A large number of studies have shown that although TIPS can effectively control acute bleeding, it has a high mortality rate. Bare stents were used previously, but recently coated stents have been more commonly used, thus the clinical efficacy of TIPS must be re-evaluated^[15-19]. Hence, in this study, through out-patient and telephone communication based follow-up, data of patients with upper gastrointestinal bleeding due to varices over the past three years who were managed in the Department of Gastroenterology, First Affiliated Hospital of Xi'an Jiaotong University, were collected. We compared TIPS with endoscopic surgery in the prevention of gastrointestinal rebleeding, improvement of survival rates and other aspects, in order to assess the feasibility, necessity and long-term efficacy of early implementation of the TIPS treatment.

MATERIALS AND METHODS

Patient data

Patients admitted to our hospital between January 2007 and June 2010 due to upper gastrointestinal bleeding resulting from varices who received TIPS or ET for the first time were included.

Inclusion criteria: (1) Age between 18 and 75 years; (2) liver cirrhosis confirmed by medical history, clinical

manifestations and diagnostic imaging examinations; (3) liver function < 13 points according to Child score; (4) endoscopy confirmed bleeding due to esophageal varices before treatment; and (5) no endoscopic and interventional therapy or surgical treatment prior to admission.

Exclusion criteria: (1) A history of hepatocellular carcinoma (HCC) or malignant tumors of other organs; (2) severe organ dysfunction [heart failure, respiratory failure, moderate to severe jaundice (serum bilirubin > 3-5 mg/dL), hepatorenal syndrome or chronic renal insufficiency]; (3) hepatic encephalopathy stage \geq second stage; and (4) ectopic variceal bleeding. The general information of the patients who were eligible for endoscopic therapy was obtained from the database at our endoscopy room. At the same time, the information of patients eligible for TIPS were obtained from the inpatient database.

Treatment

All patients chose their treatment plans after they were informed of their conditions, and they were required to sign an informed consent for the special treatment they selected. Patients treated with TIPS (TIPS group) received either elective or immediate (in case of acute bleeding phase) interventional treatment. Patients with endoscopic treatment (ET group) were administered with endoscopic band ligation or tissue adhesive agent injection when their acute bleeding was controlled by medication or combined therapy with three-balloon catheters.

TIPS procedure: Catheters commonly used in TIPS included: RUPS-100, balloon dilatation catheter and guide wire, and the metal stent. Stents used in the procedures included bare metal stents (Luminexx, Codis or Zilver stents) of 8 mm and 10 mm in diameter, and coated stent (Fluency coated stent) with an average diameter of 8 mm. The right internal jugular vein was punctured under local anesthesia. Under X-ray monitoring, the guide wire was manipulated to the hepatic vein. A puncture needle was used to establish a pathway to the portal vein, which was then dilated with the balloon catheter. The stent was then put in place to complete the portal systemic shunting. Angiography was performed and if esophageal varice was still present, Corbra 2 catheter was delivered to the varicose vein. Ethanol or stainless steel ring was used to embolize the vein. Postoperative strategies included prevention of hepatic encephalopathy and infection, and heparin treatment for 1 wk, followed by oral intake of aspirin for 1-3 mo after discharge.

Endoscopic treatment: (1) Six-ligation devices (United States Wilson-Cook Company, Model: MBL-6-F) were used in endoscopic variceal ligation. Ligation started at the dentate line near the cardia (varicose vein bulging site) with a high density and a large number of ligating points. All varicose veins should be ligated. For some varicose veins, 2-3 ligations were done at differ-

Table 1 Comparison of early transjugular intrahepatic portosystemic shunt and endoscopic therapy between two groups

Characteristics	TIPS group (64 patients)	ET group (62 patients)	P value
Sex (<i>n</i>)			0.80
Male	42	42	
Female	22	20	
Age (yrs)	51 (± 13)	54 (± 12)	0.96
Cases of disease (<i>n</i>)			0.16
HBV	38	27	
HCV	8	10	
Alcohol	2	7	
Other	16	18	
Endoscopic manifestations (<i>n</i>)			1.00
Varices classification			
Severe	62	61	
Moderate	2	1	
Complicated with peptic ulcer	4	3	1.00
Complicated with gastric disorders due to portal hypertension	23	28	0.29
Child classification (<i>n</i>)			0.40
A	23	17	
B	30	29	
C	11	16	
Child score	7.0 (± 2.0)	8.0 (± 2.0)	0.99
Preoperative manifestations (<i>n</i>)			0.20
Complicated with hepatic encephalopathy	1	5	
Complicated with ascites	39	38	0.97
Compression using Sengstaken-Blakemore tube	2	2	1.00
Pre-operative laboratory parameters			
Bilirubin (μmol/L)	24.03 (± 12.71)	27.94 (± 13.75)	0.19
Albumin (g/L)	32.25 (± 5.03)	30.85 (± 5.31)	0.65
PT (s)	15.80 (± 3.80)	16.30 (± 4.20)	0.40
Creatinine (μmol/L)	72.80 (± 14.8)	81.50 (± 31.00)	0.21

HBV: Hepatitis B virus; HCV: Hepatitis C virus; PT: Prothrombin time; TIPS: Transjugular intrahepatic portosystemic shunt; ET: Endoscopic therapy.

ent locations with bands. The procedure was repeated every two weeks until the varices disappeared or almost disappeared; (2) Endoscopic tissue adhesive injection was administered with the endoscopic sclerosis needle (Germany Ahmed Walker Medical Products Service Co., Ltd; Model: INJ1-A1-10.220), tissue adhesive [α -butyl cyanoacrylate ester; Kang Pat medical glue (embolic)], using sandwich technique.

Postoperative follow-up. All the patients were followed up by the outpatient department through telephone calls. Patients in the TIPS group were scheduled for check-ups at the outpatient department with portal vein Doppler ultrasound at 1 wk, 1 mo and 3 mo, and then every 6-12 mo after the surgery. Patients in the ET group received gastroscopy one mo after treatment, and then once every 3 mo until the 4th one was done. Follow-up continued for 3 years until February 2011. The goal of the follow-up was to check for rebleeding, and check for death, hepatic encephalopathy, and treatment-related complications. Data of repeated treatment, causes and frequency of re-hospitalization, and hospital costs were also evaluated at follow-up.

Statistical analysis

SPSS 13.0 software was used for statistical analysis. Continuous normally distributed variables were analyzed using two independent samples *t* test. Continuous non-normally distributed variables were analyzed using Wilcoxon-Mann Whitney test. χ^2 test or Fisher exact test was used for data measurement. Kaplan-Meier curve analysis was used to analyze the occurrence of rebleeding, hepatic encephalopathy as well as incidence of death. Log-rank test was used to compare the two methods of treatment regarding the differences in prognosis and outcome. Other prognostic variables, after being assigned by grading or quantification, were analyzed by Cox regression to evaluate the impact of covariates on the prognosis. *P* values were two-sided, and *P* < 0.05 was considered as statistically significant.

RESULTS

Comparable study sample

There was no significant difference in gender, age, cause of disease, clinical manifestations, liver function, endoscopic examination and laboratory indicators between the two groups, thus making the sample statistically comparable (Table 1).

Technique comparison

In the TIPS group, 15 patients used 10-mm internal diameter stents, and the rest used 8 mm. Twenty-four patients used the Fluency coated stents, and the rest with bare stents. Thirty-three patients also underwent interventional embolization of esophageal varices during TIPS procedure. In the ET group, 44 patients were subjected to simple routine endoscopic variceal ligation, 13 were treated with endoscopic tissue adhesive injection, and the rest 5 patients were treated with the combined therapy. Ligations were made about 1-3 times per person (on average, 1.2 times per person); and one tissue adhesive injection was used on average.

Rebleeding

The average follow-up was 20.7 ± 1.3 mo for the TIPS group, and 18.7 ± 1.3 mo for the ET group. Eleven cases in the TIPS group and 31 cases in ET group had gastrointestinal rebleeding. Kaplan-Meier analysis showed that there were significant differences in the overall rebleeding rates between the two groups (*P* = 0.000) (Figure 1A). Bonferroni correction showed that at week 6, month 12 and month 24 after operation, the occurrence of rebleeding was significantly lower in TIPS group than in ET group. Cox multivariate regression analysis showed that among the covariates (grouping variables, combined portal hypertensive gastropathy, gastroesophageal varice 2/isolated gastric varice 1, liver function with B/C grade, prothrombin time > 16 s and cirrhosis due to viral hepatitis), only grouping variable with TIPS therapy was a factor affecting the rate of rebleeding [TIPS treatment: B = -1.41; Exp (B) = 0.24, 95% confidence

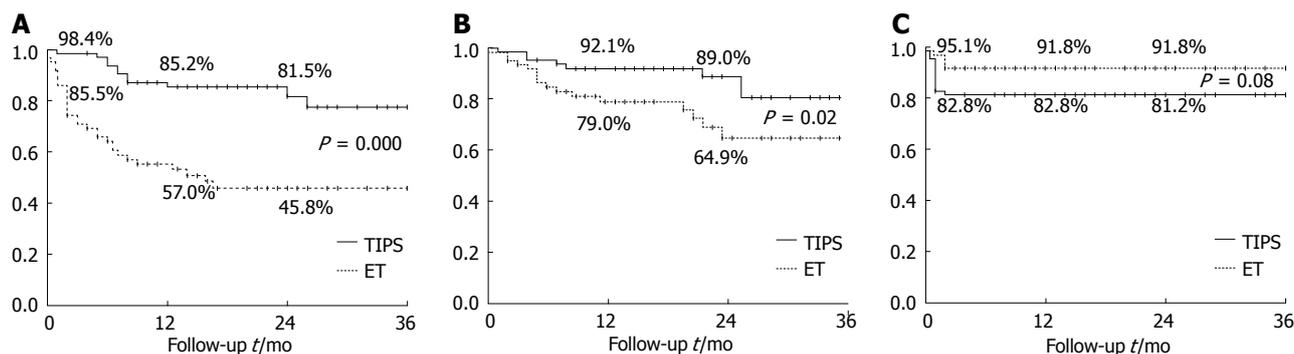


Figure 1 Kaplan-Meier analysis. A: The non-occurrence of rebleeding in the two groups; B: Survival rate; C: The non-occurrence of hepatic encephalopathy. TIPS: Transjugular intrahepatic portosystemic shunt; ET: Endoscopic therapy.

	TIPS group	ET group
Cause of death		
Gastrointestinal bleeding	3	9
Liver failure	4	6
Hepatic encephalopathy	0	1
Infection	1	0
Post-operative severe complications		
Fever	2	2
Spontaneous peritonitis	0	2
Hepatorenal syndrome	0	1
Acute peritonitis	0	1

TIPS: Transjugular intrahepatic portosystemic shunt; ET: Endoscopic therapy.

interval (CI) (0.12, 0.50); $P = 0.001$]. Of the 31 patients in ET group, 8 rebleeding patients received rescue TIPS treatment. Of those 8 patients, one patient, who used a bare stent, had rebleeding 10 mo later. Examination showed stent stenosis, and the patient received conservative treatment. One patient had massive gastrointestinal bleeding, resulting in death 14 mo after operation. No rebleeding occurred in the remaining 6 patients.

Postoperative survival

There were 8 deaths in TIPS group and 16 deaths in ET group. Kaplan-Meier survival analysis showed that the cumulative survival rate was significantly higher in the TIPS group than in the ET group (80.6% vs 64.9%, Kaplan-Meier analysis and log-rank test $\chi^2 = 4.864$, $P = 0.02$) (Figure 1B). Individual time point comparison showed that there was no significant difference 6 wk after operation between the two groups in survival rate; after 12 mo and 24 mo, the survival rate was significantly higher in the TIPS group than in the ET group (92% vs 79%; 89% vs 64.9%). Cox regression curves showed that the covariates [age > 53 years, with ascites, cirrhosis due to hepatitis, liver function with total bilirubin > 34 $\mu\text{mol/L}$, creatinine (CRE) > 77 $\mu\text{mol/L}$] age > 53 years, viral hepatitis (hepatitis B virus and hepatitis C virus) resulting in cirrhosis, as well as CRE > 77 $\mu\text{mol/L}$ were predictable factors affecting survival. Wald test revealed

that after adjusting these risk factors, TIPS therapy was more beneficial for improving survival [B = -0.89; Exp (B) = 0.41, 95%CI (0.16, 1.00); $P = 0.04$]. Comparison in the cause of death between the two groups is shown in Table 2.

Hepatic encephalopathy and other complications

Twelve of the postoperative TIPS patients had newly onset of hepatic encephalopathy (Child A grade 4, Child B grade 8), and 5 in ET (Child A grade 2, Child B grade 3). Kaplan-Meier curve analysis showed that there was no statistically significant difference between the two groups in the non-occurrence of hepatic encephalopathy (TIPS group 81.2% vs ET group 91.8%, $P = 0.08$) (Figure 1C). Individual time point comparison showed that the non-occurrence of hepatic encephalopathy 6 wk after operation in the ET group was higher than in the TIPS group, the difference being statistically significant (TIPS group 82.8% vs ET group 95.1%), and after 12 mo and 24 mo, the difference was no longer statistically significant (TIPS group 82.8% vs ET group 91.8%, TIPS group 81.2% vs ET group 91.8%). Cox regression analysis showed that all selected covariates (age > 53 years, ascites, cirrhosis due to hepatitis, liver function grade with Tbil > 34 $\mu\text{mol/L}$, CRE > 77 $\mu\text{mol/L}$) had no significant effect on the occurrence of hepatic encephalopathy [grouping variable for TIPS treatment: B = 0.94; Exp (B) = 2.56, 95%CI (0.86, 7.62); $P = 0.09$]. Other complications are shown in Table 2.

Hospitalization costs

Hospitalization cost was compared between the two groups (Table 3). The cumulative total cost of hospitalization was 52 678 RMB/person in TIPS group and 38 844 RMB/person in ET group; the cost in the TIPS group was significantly higher than in the ET group ($P < 0.05$). In the follow-up period, the average hospitalization frequency for the TIPS group was 0.4 times/person and 1.3 times/person for the ET group; ET group had a significantly higher frequency of hospitalization than the TIPS group ($P = 0.01$). The average length of hospital stay during the follow-up for TIPS group was 5 d/person and 19 d/person for ET group; the ET group had

Table 3 Comparison of length of hospital stay and expenses between two groups

	Initial TIPS (64 cases)	Rescue TIPS (8 cases)	Rest of ET (54 cases)
Average total expense (RMB)	52 678	63 003	35 298
Average length of hospital stay during follow-up (d)	5	20	18
Average number of hospitalization during follow-up	0.4	2.1	1.2

TIPS: Transjugular intrahepatic portosystemic shunt; ET: Endoscopic therapy.

significantly longer hospital stay than the TIPS group ($P < 0.05$). The patients in the ET group who received TIPS treatment after the failure of endoscopic treatment were included into rescue TIPS group, and comparative results are shown in Table 3.

DISCUSSION

Currently, the endoscopic variceal treatment remains a predominant method for prevention and treatment of recurrent gastrointestinal bleeding^[19]. However, endoscopic therapy cannot fundamentally solve the problem of portal hypertension, and patients often cannot tolerate repeated therapies, therefore leading to a high rate of rebleeding. The TIPS procedure, through reducing portal pressure, serves to prevent and control esophageal and gastric variceal bleeding, and alleviate ascites as well. Therefore, with respect to preventing recurrent gastroesophageal bleeding, TIPS is better than endoscopic therapy.

For the TIPS procedure, stenosis of the stent is an important cause of postoperative recurrent bleeding. Previous studies have shown that the rate of stent stenosis is about 30%-70%^[20] with the use of bare stent in TIPS whereas the postoperative patency rate is up to 100% with the use of coated stents and Viatorr stent grafts^[21-23], and compared with bare stents, they did not increase the incidence of hepatic encephalopathy. This study showed that in the TIPS group, five cases had postoperative stenosis. Of the five cases, only one used the coated stent, and this patient did not follow doctor's orders to take aspirin for anticoagulation after being discharged from hospital. This further confirms that the use of coated stent can significantly lower the incidence of postoperative stent stenosis, and anticoagulation therapy can further reduce postoperative stent stenosis.

It has been confirmed that with respect to prevention of recurrent bleeding, TIPS is better than medication therapy^[13] and endoscopic therapy^[14]. The postoperative rebleeding rate was 12%-22% in TIPS, and it is even lower with the use of coated stent. For endoscopic therapy, however, the rebleeding rate is much higher (20%-43%)^[15]. Recently, a study by García-Pagán *et al.*^[24], comparing the use of Viatorr stent graft TIPS with drug combined endoscopic variceal ligation treatment, showed that in the 16 mo follow-up, only one case of re-

current bleeding occurred in the TIPS group as opposed to 14 cases in the other groups (3.1% vs 45.2%, $P = 0.001$). In this study, 17.2% of patients in the TIPS group had recurrent bleeding. Cases of rebleeding caused by stenosis of the stent were mostly seen in patients with bare stents. In the ET group, 50% of the patients had recurrent bleeding. This further confirmed that TIPS is superior to endoscopic therapy in prevention of recurrent bleeding and coated stents can further reduce the incidence of recurrent bleeding by lowering the rate of stent stenosis.

It has been shown that TIPS can effectively reduce refractory ascites (RA). It can also lower the incidences of hepatorenal syndrome (HRS), hepatic hydrothorax and other portal hypertension syndromes that are closely related to refractory ascites^[24]. In this study, serious postoperative complications associated with portal hypertension, such as RA, HRS and spontaneous bacterial peritonitis, mainly occurred in the ET group, possibly due to increased portal pressure acting as a common mechanism resulting in the above syndromes, whereas TIPS can effectively reduce portal pressure^[25].

A large number of clinical studies^[13,14] and meta-analyses^[15,25,26] indicate that TIPS procedure is not superior to endoscopic therapy with respect to improvement of survival time. This is the main reason why TIPS is used as a rescue option after the failure of the traditional therapeutic method. However, although rescue TIPS procedure can effectively control acute bleeding, the postoperative one-year survival rate is only 27%-55%^[27]. Most of the previous studies on TIPS procedure were based on the use of bare stents, and most patients chose TIPS 2-3 years after traditional treatment, thus making TIPS appear to be not superior to ET in survival rates.

In the study by García-Pagán *et al.*^[24], the patients in the TIPS group received TIPS with coated stent at the first incidence of bleeding during the early stages. Results showed that early and middle stage survival rates were much higher in TIPS group than in drug combined endoscopic group (post-operative 6 wk rate for TIPS group was 97% and combined therapy group 67%; post-operative 1 year rate for TIPS group was 86% and combined therapy group 61%), and the middle to long-term efficacy was similar to this study. As for the combined endoscopic group, of the 7 patients who received rescue TIPS due to recurrent bleeding, 4 died within 36 d. Another five patients died due to recurrent bleeding that led to liver failure and incapability to undergo remedy TIPS. Therefore, early implementation of TIPS after the first bleeding may raise the long-term survival rate better than endoscopic therapy. To use TIPS only after the failure of the traditional method may cause the patient to lose the opportunity for the TIPS procedure, thus delaying the illness and lowering survival rate.

Previous studies indicate that the incidence rate of hepatic encephalopathy, 1 year after TIPS was 30%-55%^[8,9]. Most cases occur in the early post-operative stage, and are transient in nature. As the brain adapts and adjusts to the

increase in ammonia toxins brought by the redistribution of intestinal blood flow, the symptoms disappear^[8]. The incidence rate of persistent hepatic encephalopathy that respond poorly to medication was low, 3%-7%^[10] for bare stent and 8%^[9] for coated stent. García-Pagán *et al.*^[24] showed that early TIPS treatment did not increase the incidence rate of hepatic encephalopathy (TIPS 28% and combined endoscopic therapy 40%, $P = 0.13$). This study demonstrated similar findings. Although in the early post-operative stage, more cases of hepatic encephalopathy occurred in TIPS group than in the ET group, these were mostly transient, and can quickly be controlled through timely administration of anti-hepatic encephalopathy medication.

In this study, although in the early stage, the average overall time spent in the TIPS group is longer than in the ET group, it significantly reduced re-hospitalization and the length of hospital stay. It also decreased incidences of RA, HRS and other portal hypertension related complications. In the ET group in this study, patients who underwent EBL had an average of 1.2 ligations per person. If following the regular course of treatment, the average total cost in the ET group may not be lower than that in the TIPS group. Also, after the failure of the endoscopic therapy, rescue TIPS increases the total cost. This does not comply with the principles of health economics and may cause the patient to lose the opportunity for the TIPS procedure, thus delaying their treatment of illness and lowering the survival rate.

COMMENTS

Background

Variceal bleeding is a severe complication of portal hypertension and a major cause of death in patients with cirrhosis. Combined treatment with vasoactive drugs and endoscopic techniques is the recommended standard of care for patients with acute variceal bleeding. However, treatment failure occurs in about 10% - 20% of patients requiring treatment with a transjugular intrahepatic portosystemic shunt (TIPS) as rescue therapy. TIPS is highly effective in controlling bleeding in such patients, but mortality is still very high. The authors conducted a study to determine whether early treatment with TIPS can improve outcomes in patients with cirrhosis and variceal bleeding compared with endoscopic therapy.

Research frontiers

TIPS has been used for more than 20 years to treat the complications of portal hypertension. Previous studies evaluating the role of TIPS in the prevention of recurrent variceal bleeding showed that TIPS reduces the rebleeding rate but increases hepatic encephalopathy without improving survival. TIPS is currently recommended only as a rescue therapy. Current practice guidelines for treating patients with acute variceal bleeding recommend fluid resuscitation, antibiotic prophylaxis, and vasoactive drugs such as glypressin or somatostatin analogues, followed by early endoscopy and either ligation or sclerosis of the varices.

Innovations and breakthroughs

In this study, early treatment with TIPS, as compared with medical treatment, was associated with an improved prognosis among patients at high risk for uncontrolled bleeding or rebleeding on the basis of a hepatic venous pressure gradient of 20 mmHg or higher. The results of a randomized, multi-center study that compared early TIPS with optimal medical therapy (endoscopic therapy plus vasoactive drugs) in patients at high risk for rebleeding who were either in Child-Pugh class B with active bleeding at endoscopy or in Child-Pugh class C. This study shows the benefit of early TIPS in patients with Child-Pugh class B or C disease who are at high risk for uncontrolled bleeding with standard therapy.

Applications

The study re-evaluated how the authors approach variceal bleeding in patients with Child-Pugh class B or C disease. Physicians should consider the early

use of TIPS with an e-PFTE-covered stent as first-line therapy rather than as rescue treatment if rebleeding occurs in high-risk patients with Child-Pugh B or C disease.

Terminology

TIPS is a technique in which a stent is placed between the portal vein and hepatic vein in the liver to provide a portosystemic shunt to reduce portal hypertension. The procedure is carried out under radiological control through the internal jugular vein. Successful shunt placement will stop/prevent bleeding. Endoscopic variceal ligation is a technique with less trauma and fewer side effects, in which varices were sucked into an endoscope allowing them to be occluded with a tight rubber band.

Peer review

The main drawbacks of the present study are that it is a retrospective analysis, and the study groups were not randomized. However, this study provides another evidence that early TIPS is associated with significant advantages over endoscopic therapy in terms of rebleeding rates, survival and length of hospital stay.

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c-Jun N-terminal kinase is required for thermotherapy-induced apoptosis in human gastric cancer cells

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Abstract

AIM: To investigate the role of c-Jun N-terminal kinase (JNK) in thermotherapy-induced apoptosis in human gastric cancer SGC-7901 cells.

METHODS: Human gastric cancer SGC-7901 cells were cultured *in vitro*. Following thermotherapy at 43 °C for 0, 0.5, 1, 2 or 3 h, the cells were cultured for a further 24 h with or without the JNK specific inhibitor, SP600125 for 2 h. Apoptosis was evaluated by immunohistochemistry [terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)] and flow cytometry (Annexin *vs* propidium iodide). Cell proliferation was determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. The production of p-JNK, Bcl-2, Bax and caspase-3 proteins was evaluated by Western blotting. The expression of JNK at mRNA level was determined by reverse transcription polymerase chain reaction.

RESULTS: The proliferation of gastric carcinoma SGC-7901 cells was significantly inhibited following thermotherapy,

and was 32.7%, 30.6%, 43.8% and 52.9% at 0.5, 1, 2 and 3 h post-thermotherapy, respectively. Flow cytometry analysis revealed an increased population of SGC-7901 cells in G0/G1 phase, but a reduced population in S phase following thermotherapy for 1 or 2 h, compared to untreated cells ($P < 0.05$). The increased number of SGC-7901 cells in G0/G1 phase was consistent with induced apoptosis (flow cytometry) following thermotherapy for 0.5, 1, 2 or 3 h, compared to the untreated group ($46.5\% \pm 0.23\%$, $39.9\% \pm 0.53\%$, $56.6\% \pm 0.35\%$ and $50.4\% \pm 0.29\%$ *vs* $7.3\% \pm 0.10\%$, $P < 0.01$), respectively. This was supported by the TUNEL assay ($48.2\% \pm 0.4\%$, $40.1\% \pm 0.2\%$, $61.2\% \pm 0.29\%$ and $52.0\% \pm 0.42\%$ *vs* $12.2\% \pm 0.22\%$, $P < 0.01$) respectively. More importantly, the expression of p-JNK protein and JNK mRNA levels were significantly higher at 0.5 h than at 0 h post-treatment ($P < 0.01$), and peaked at 2 h. A similar pattern was detected for Bax and caspase-3 proteins. Bcl-2 increased at 0.5 h, peaked at 1 h, and then decreased. Furthermore, the JNK specific inhibitor, SP600125, suppressed p-JNK, Bax and caspase-3 at the protein level in SGC7901 cells following thermotherapy, compared to mock-inhibitor treatment, which was in line with the decreased rate of apoptosis. The expression of Bcl-2 was consistent with thermotherapy alone.

CONCLUSION: Thermotherapy induced apoptosis in gastric cancer cells by promoting p-JNK at the mRNA and protein levels, and up-regulated the expression of Bax and caspase-3 proteins. Bcl-2 may play a protective role during thermotherapy. Activation of JNK *via* the Bax-caspase-3 pathway may be important in thermotherapy-induced apoptosis in gastric cancer cells.

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Key words: Thermotherapy; Gastric cancer; Apoptosis; c-Jun N-terminal kinase; Apoptosis-related protein

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INTRODUCTION

Gastric cancer is the second leading cause of cancer death worldwide and may remain one of the leading causes of all deaths in the near future^[1-3]. At present, the majority (about 80%) of patients diagnosed with gastric cancer are in an advanced stage with limited surgical options^[4]. Chemotherapy is an alternative treatment for advanced gastric cancer, however, patient outcome following chemotherapy is still very poor, with a median overall survival time of less than 1 year^[5]. Chemotherapy with cytotoxic drugs usually leads to severe toxicity which lowers the quality of life of patients. Thermotherapy is a new cancer treatment which is used following surgical, radiotherapy, chemotherapy and biological treatments. Thermotherapy induces malignant cell apoptosis and has a synergistic effect with chemotherapy resulting in improved outcomes and reduced side effects of chemotherapy, particularly in the later stages of malignancy or in tumors resistant to other treatments^[6,7]. A few studies have reported that induction of cellular apoptosis is important in thermotherapy, but the underlying mechanism remains to be explored.

c-Jun N-terminal kinase (JNK) is a member of the mitogen-activated protein kinase family. Activation of JNK by phosphorylation has been implicated in a variety of processes, including embryonic development, cellular transformation and initiation of apoptosis following various stresses^[8-11]. It has been reported that overexpression of JNK may cause cellular apoptosis in transfected cells^[12-14]. However, the function of JNK is complicated during cell stress, because JNK also has anti-apoptotic action^[15]. Thus, it is believed that JNK has either anti- or pro-apoptotic activity depending on cell type, apoptotic stimuli and other signalling pathways^[16]. JNK can be activated by thermotherapy, and ultimately leads to cellular apoptosis^[17]. It is unclear whether JNK involves or regulates apoptosis of gastric cancer cells in response to thermotherapy. In the current study, the human gastric cancer cell line, SGC-7901, was chosen as a model for thermotherapy-induced apoptosis *in vitro*. The role of JNK was determined to further investigate the underlying mechanism of thermotherapy-induced apoptosis in human gastric cancer. This may provide useful information for both clinical therapy and basic scientific research in gastric cancer.

MATERIALS AND METHODS

Reagents and equipment

Annexin V-fluoresceine isothiocyanate (V-FITC), propidium iodine (PI), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphen-

yltetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO, United States). Mouse monoclonal antibodies against human p-SAPK/JNK (Thr183/Tyr185), Caspase-3, Bcl-2, Bax and the JNK-specific inhibitor, SP600125, were also purchased from Sigma (St. Louis, MO, United States). The M-MLV reverse transcription polymerase chain reaction (RT-PCR) kit was purchased from Promega (Beijing, China). Taq DNA polymerase was purchased from Takara (Dalian, China). Fetal bovine serum was purchased from Sijichun Bioengineering Materials, Inc. (Hangzhou, Zhejiang, China). Dulbecco's Modified Eagle Media (DMEM) culture media and 0.25% trypsin were purchased from Invitrogen-GIBCO (Carlsbad, CA, United States). dNTP and TRIzol were purchased from Invitrogen (Invitrogen, United States). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining kits was purchased from KGI (Nanjing, China). The gastric cancer cell line, SGC-7901^[18], was obtained from the Jiangxi Province Digestive Institute (Jiangxi, Nanchang, China).

Gastric cancer cell thermotherapy and JNK blockage

Gastric cancer SGC-7901 cells were cultured in DMEM media (high glucose) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were maintained at 37 °C and 5% CO₂ in an incubator, and the culture medium was changed every 2-3 d. After thermotherapy (43 °C) treatment for 0, 0.5, 1, 2 or 3 h, SGC-7901 cells were cultured for a further 24 h. In addition, the JNK-specific inhibitor, SP600125, was added to SGC-7901 cells 2 h before thermotherapy treatment to determine whether the JNK pathway was involved in thermotherapy-induced apoptosis.

Proliferation of SGC-7901 cells determined by MTT assay

The cells (2×10^5 cell/mL) were seeded onto 96-well plates with 200 µL in six wells for each thermotherapy treatment for 0, 0.5, 1, 2 and 3 h. Twenty µL of MTT solution (5 mg/mL) was added to each well and incubated at 37 °C for 4 h, and the reaction was terminated with a detergent solution to lyse the cells and solubilize the colored formazan crystals. The supernatant was centrifuged at 3000 g for 10 min to obtain a formazan pellet. The supernatant was removed, and the pellet was dissolved completely with 150 µL dimethyl sulfoxide and observed at a wavelength of 570 nm using an enzyme-linked immunosorbent assay plate reader.

The relative inhibition rate was calculated as a percentage, as follows: $(1 - A_{\text{experiment}}/A_{\text{control}}) \times 100\%$. Three independent experiments were performed.

Cell cycle analysis and apoptosis assays

The effect of thermotherapy on the cell cycle and apoptosis in SGC-7901 cells was analyzed by flow cytometry. Cells floating in medium combined with the adherent layer were collected by trypsinization and fixation with 2 mL citrate buffer for 1 h. For detection of the cell cycle,

Table 1 Primers for reverse transcription polymerase chain reaction

Gene	Primer sequence	Size (bp)	Annealing temperature
<i>β-actin</i>	F: TCAGGTCATCACTATCGGCAAT	432	57 °C
	R: AAAGAAAGGGTGTAAAAGGCA		
<i>JNK</i>	F: CACAGTCCTAAAACGATACC	354	57 °C
	R: CCACACAGCATTGATAGAG		

JNK: c-Jun N-terminal kinase; F: Forward primer; R: Reverse primer.

the cells were stained with propidium iodide (1500 μL) after RNase A (1500 μL) treatment. The labeled cells were immediately analyzed by flow cytometry to evaluate the cell cycle and apoptosis. TUNEL assay, in which the residue of digoxigenin-labeled dUTP was catalytically incorporated into the DNA by terminal deoxynucleotidyl transferase II, was performed according to the manufacturer's instructions. The positive particles of diaminobenzidine staining were viewed under an optical microscope. The number of apoptotic cells was counted under a microscope (400×) and expressed as the apoptotic index (the number of apoptotic bodies/1000 cells).

Western blotting

Protein concentrations from isolated SGC-7901 cells were determined. The proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis gels (polyacrylamide concentration 100 g/L) and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were blocked with 5% skimmed milk at 37 °C for 1 h and probed with the primary antibody: mouse anti-human JNK (1:2000), caspase-3, Bcl-2, Bax (1:1000), or β-actin (1:1000) monoclonal antibody overnight at 4 °C. The labeled antibody was visualized by horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (1:5000) and enhanced chemiluminescence. The blots were washed with 1 × Tris-buffered saline and Tween buffer for 10 min, 3 times between each step. The density of the targeted bands was quantified using the Quantity One 4.6.2 Imaging Analysis System.

RT-PCR analysis

Total RNA was extracted from the SGC-7901 cells with TRIzol following the manufacturer's instructions. cDNA was synthesized from 2 μg total RNA using the M-MLV RT-PCR kit in a 20 μL volume, according to the manufacturer's instructions. Two μL of cDNA, 2 μL each primer (50 pmol/L), 1 μL dNTP mix (10 mmol/L) and 1 μL Taq DNA polymerase were used for the PCR analysis. The PCR amplification cycles consisted of denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 60 s, annealing for 60 s, extension at 72 °C for 60 s, and final elongation at 72 °C for 10 min. The PCR products were separated on a 1.5% agarose gel, stained with 0.5 mg/mL ethidium bromide, and visualized by ultraviolet

Table 2 Cell cycle of SGC-7901 cells following thermotherapy for various time periods (%)

Group	SGC-7901 cells		
	G0-G1	S	G2-M
0 h	27.22	62.47	10.31
1 h	71.15 ^a	22.52 ^a	6.33
2 h	70.89 ^a	17.11 ^a	12

^a*P* < 0.05 vs without thermotherapy treatment.

light. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase and shown as the ratio of absorbance values. The primer sequences and annealing temperature are listed in Table 1.

Statistical analysis

Statistical analysis was performed using statistical software (SPSS version 13.0). Data are expressed as the mean ± SD. The means of 2 groups were compared using the *t* test. The means of more than 2 groups were compared using one-way analysis of variance, and *P* < 0.05 was regarded as statistically significant.

RESULTS

Inhibitory effect of thermotherapy on gastric cancer cells

To evaluate the effects of thermotherapy on cell viability, cultured gastric cancer SGC-7901 cells underwent thermotherapy for different time periods. Viability inhibitory rates of gastric carcinoma SGC-7901 cells were about 30%, 30%, 43% and 53% after 0.5, 1, 2 and 3 h of thermotherapy, respectively. These data suggest that proliferation of gastric carcinoma SGC-7901 cells was significantly inhibited in a time-dependent manner.

Effect of thermotherapy on cell cycle distribution in gastric cancer cells

The effects of thermotherapy on the cell cycle of SGC-7901 cells were determined using flow cytometry. Our results showed that thermotherapy increased the number of SGC-7901 cells in G0/G1 phase, but reduced the number in S phase (*P* < 0.05) at 1 h and 2 h, compared with that at 0 h. These results suggest that thermotherapy arrested gastric cancer cells in G0/G1 phase, inhibiting mitosis, and subsequent proliferation (Figure 1 and Table 2).

Induction of apoptosis by thermotherapy in gastric cancer cells

As arrest of cell cycle progression in tumor cells is usually associated with concomitant activation of cell apoptosis pathways, we investigated the effect of thermotherapy on induction of apoptosis in SGC-7901 cells by flow cytometry and TUNEL assay. Flow cytometry showed that the proportion of apoptotic cells was significantly increased after thermotherapy for 0.5, 1, 2 and 3 h, compared to untreated SGC-7901 cells, which was further confirmed

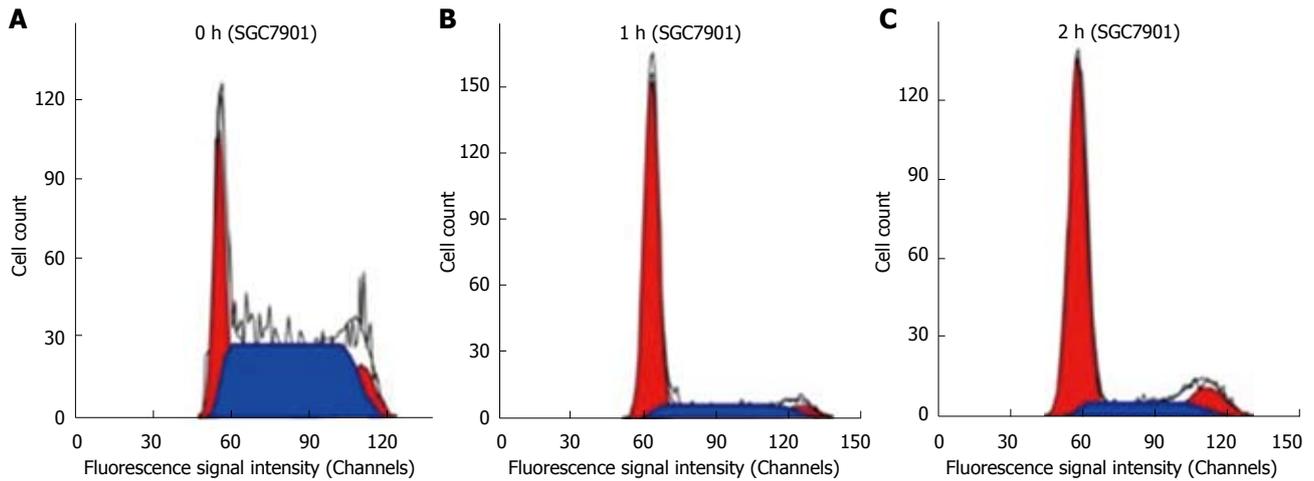


Figure 1 Cell cycle change in SGC-7901 cells following thermotherapy for various time periods. A-C: Cell cycle distribution in SGC-7901 cells following thermotherapy for 0 h, 1 h, 2 h respectively. Thermotherapy increased the number of SGC-7901 cells in G0/G1 phase, but reduced the number in S phase ($P < 0.05$) at 1 h and 2 h, compared with that at 0 h.

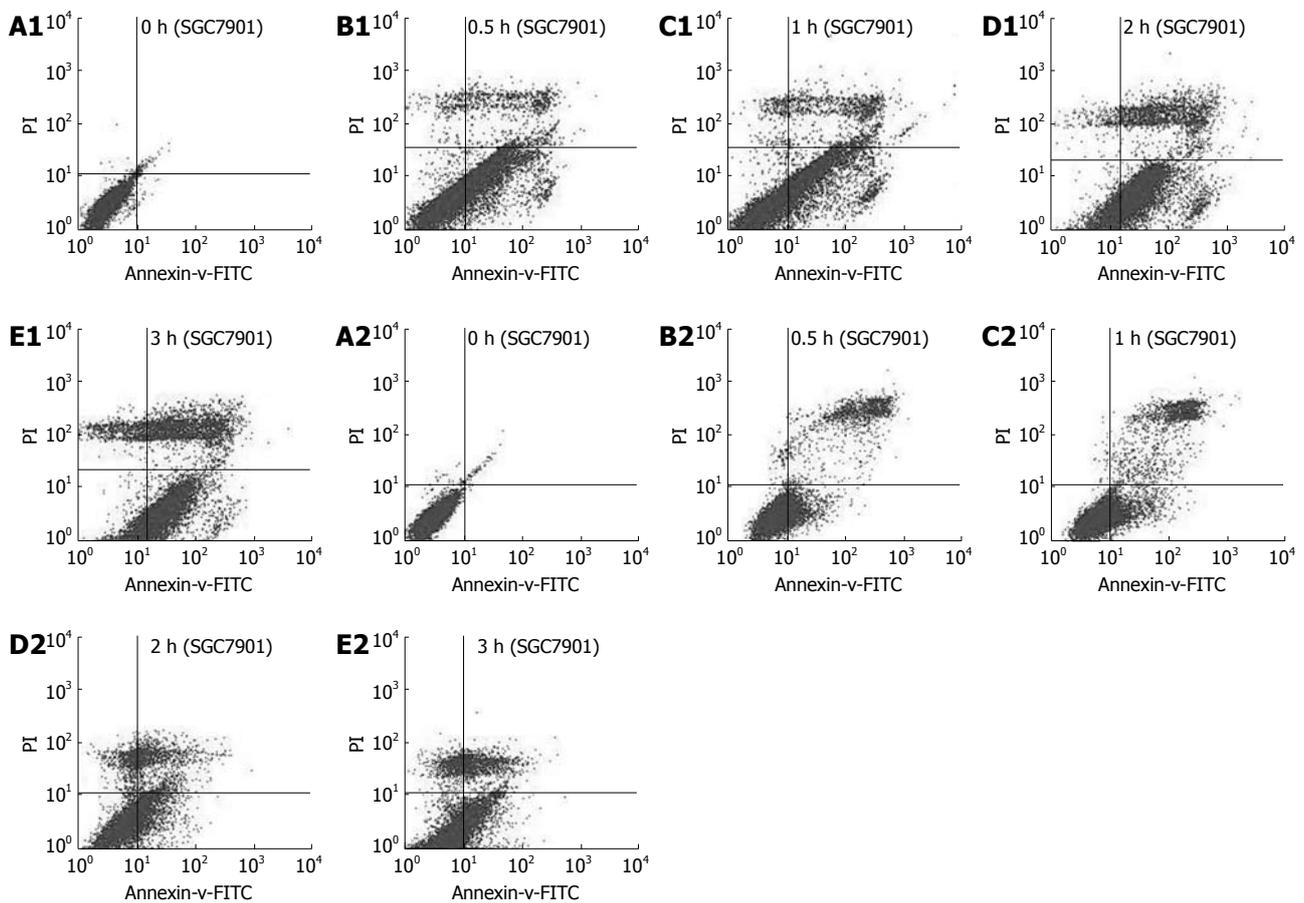


Figure 2 Apoptotic rate of SGC7901 cells with or without SP600125 treatment following thermotherapy for various time periods. A1, B1, C1, D1, E1: Apoptotic rate of SGC-7901 cells without SP600125 treatment, significantly increased after thermotherapy for 0.5, 1, 2 and 3 h, compared to that at 0 h ($P < 0.01$); A2, B2, C2, D2, E2: Apoptotic rate of SGC-7901 cells with SP600125 treatment, significantly inhibited cellular apoptosis induced by thermotherapy at 0.5, 1, 2 and 3 h ($P < 0.01$), compared to that at the same time points in the control groups. PI: Propidium iodide; FITC: Fluoresceine isothiocyanate.

by TUNEL assay (Table 3). To further address the role of JNK in thermotherapy-induced apoptosis, pretreatment of cells with the JNK-specific inhibitor, SP600125, significantly inhibited cellular apoptosis induced by thermo-

therapy at 0.5, 1, 2 and 3 h ($P < 0.01$), compared to that at the same time points in the control groups. The apoptotic rates at 0 h ($P > 0.05$) in the experimental group and the control group were similar (Figures 2 and 3, Table 3).

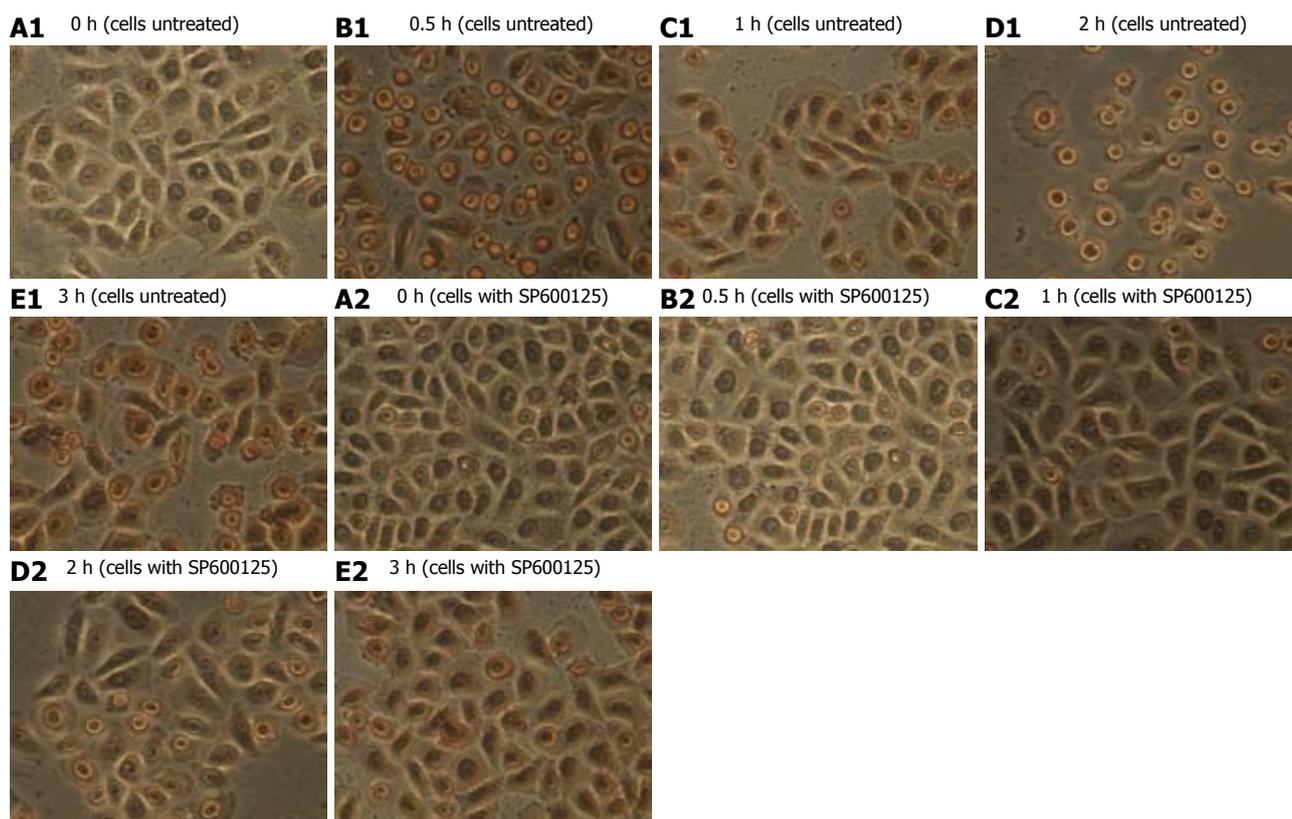


Figure 3 Apoptosis of SGC-7901 cells with or without SP600125 treatment following thermotherapy for various time periods ($\times 400$). Apoptotic cells showing yellow fluorescence. A1, B1, C1, D1, E1: Apoptosis of SGC-7901 cells without SP600125 treatment, significantly increased after thermotherapy for 0.5, 1, 2 and 3 h, compared to that at 0 h ($P < 0.01$); A2, B2, C2, D2, E2: Apoptosis with SP600125 treatment, significantly inhibited cellular apoptosis induced by thermotherapy at 0.5, 1, 2 and 3 h ($P < 0.01$), compared to that at the same time points in the control groups.

These results suggested that the JNK-specific inhibitor, SP600125, significantly inhibited thermotherapy-induced apoptosis in gastric cancer cells.

Stimulation of p-JNK protein expression by thermotherapy

To determine whether thermotherapy activates p-JNK expression in gastric cancer cells, the expression level of p-JNK protein was determined in SGC-7901 cells following thermotherapy for 0.5, 1, 2 and 3 h using Western blotting analysis. The protein production of p-JNK was significantly higher at 0.5 h than that at 0 h post-treatment (Figure 4) ($P < 0.01$), peaked at 2 h, and then decreased. Interestingly, p-JNK protein was slightly lower at 1 h than that at 0.5 h post-treatment. Thus, these results clearly indicate that JNK in gastric cancer cells was activated by thermotherapy.

Effect of thermotherapy on JNK mRNA expression

Since activation of JNK is known to induce JNK mRNA expression in several cell systems, we examined whether the expression of JNK mRNA was also induced by thermotherapy in gastric cancer cells. The expression of JNK mRNA in SGC-7901 cells was determined by RT-PCR analysis, which showed that the induction patterns of the expression of p-JNK protein and JNK mRNA were very similar in thermotherapy-induced apoptosis. Namely, the

Table 3 Apoptotic rate of SGC-7901 cells with or without SP600125 treatment following thermotherapy for various time periods as determined by flow cytometry and terminal deoxynucleotidyl transferase dUTP nick end labeling assay

Group (h)	Apoptotic rate(%)	
	Control group	SP600125 group
Flow cytometry		
0	7.3 \pm 0.10	3.2 \pm 0.08
0.5	46.5 \pm 0.23 ^b	21.8 \pm 0.15 ^{b,d}
1	39.9 \pm 0.53 ^b	17.9 \pm 0.26 ^{b,d}
2	56.6 \pm 0.35 ^b	22.4 \pm 0.36 ^{b,d}
3	50.4 \pm 0.29 ^b	24.5 \pm 0.72 ^{b,d}
TUNEL assay		
0	12.2 \pm 0.22	11.3 \pm 0.13
0.5	48.2 \pm 0.40 ^b	25.8 \pm 0.19 ^{b,d}
1	40.1 \pm 0.20 ^b	19.2 \pm 0.09 ^{a,d}
2	61.2 \pm 0.29 ^b	26.3 \pm 0.23 ^{a,d}
3	52.0 \pm 0.42 ^b	23.4 \pm 0.36 ^{a,d}

^a $P < 0.05$, ^b $P < 0.01$ vs without thermotherapy; ^d $P < 0.01$ vs without SP600125 treatment. TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling.

expression of JNK mRNA was significantly higher at 0.5 h than that at 0 h post-treatment ($P < 0.01$), reached a maximum at 2 h, followed by a decrease, and was slightly lower at 1 h than at 0.5 h post-treatment. Taken together, the datas in Figure 5 demonstrate that JNK plays a role in

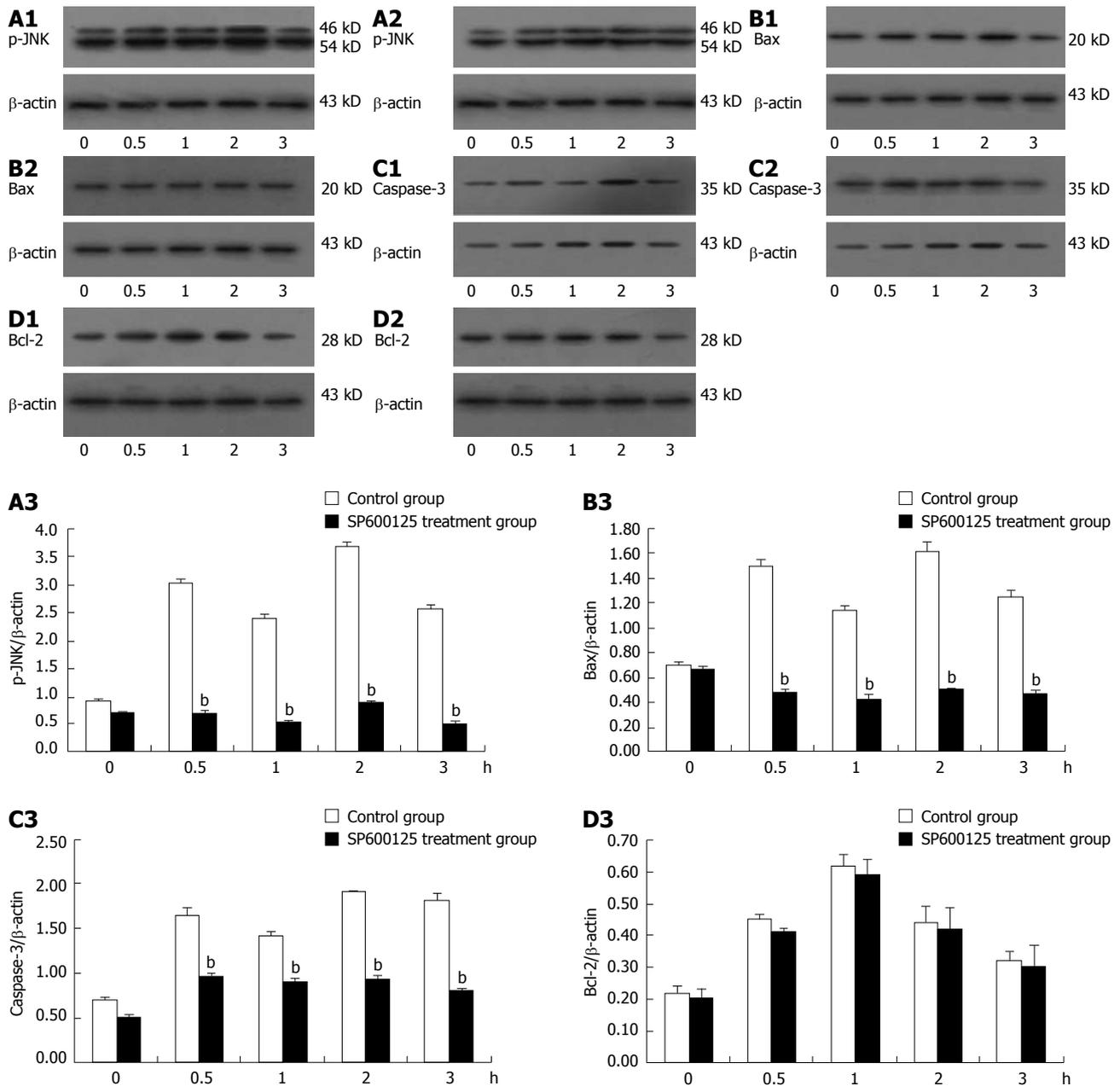


Figure 4 Expression of p-c-Jun N-terminal kinase, Bax, caspase-3 and Bcl-2 proteins in SGC-7901 cells with or without SP600125 treatment following thermotherapy for various time periods. A1, B2, C1, D1: Expression of p-JNK, Bax, caspase-3 and Bcl-2 proteins in SGC-7901 cells without SP600125 treatment; A2, B2, C2, D2: Expression of p-JNK, Bax, caspase-3 and Bcl-2 proteins in SGC-7901 cells with SP600125 treatment; A3, B3, C3, D3: Comparison of p-JNK, Bax, caspase-3 and Bcl-2 proteins in SGC-7901 cells with or without SP600125 treatment; Expression of p-JNK, Bax, caspase-3 proteins in SGC-7901 cells with SP600125 treatment was significantly inhibited compared with that in SGC-7901 cells without SP600125 treatment ($P < 0.01$); Bcl-2 protein did not show an obvious change in SGC-7901 cells with or without SP600125 treatment ($P > 0.05$). JNK: c-Jun N-terminal kinase.

the effect of thermotherapy on gastric cancer cells.

Effect of JNK on Bax, Bcl-2 and caspase-3 proteins expression in thermotherapy-induced apoptosis

Apoptosis-related proteins, such as Bax, Bcl-2 and caspase-3 are known to cause cell apoptosis. Thus, the activation of JNK may affect the expression of Bax, Bcl-2 and caspase-3 proteins in thermotherapy-induced apoptosis. To determine whether the expression of Bax, Bcl-2 and caspase-3 proteins was regulated by activation of JNK protein, we measured the expression of Bax, Bcl-2 and

caspase-3 proteins in SGC-7901 cells treated with thermotherapy at various time points using Western blot analysis. As shown in Figure 4, the expression of Bax and caspase-3 proteins was significantly higher at 0.5 h than at 0 h post-treatment ($P < 0.01$), reached a maximum at 2 h, followed by a decrease, and was slightly lower at 1 h than at 0.5 h post-treatment. Bcl-2 was increased at 0.5 h, peaked at 1 h, and decreased thereafter.

To further understand how the activation of JNK regulates the expression of Bax, Bcl-2 and Caspase-3 proteins in thermotherapy-induced apoptosis in SGC-7901 cells we

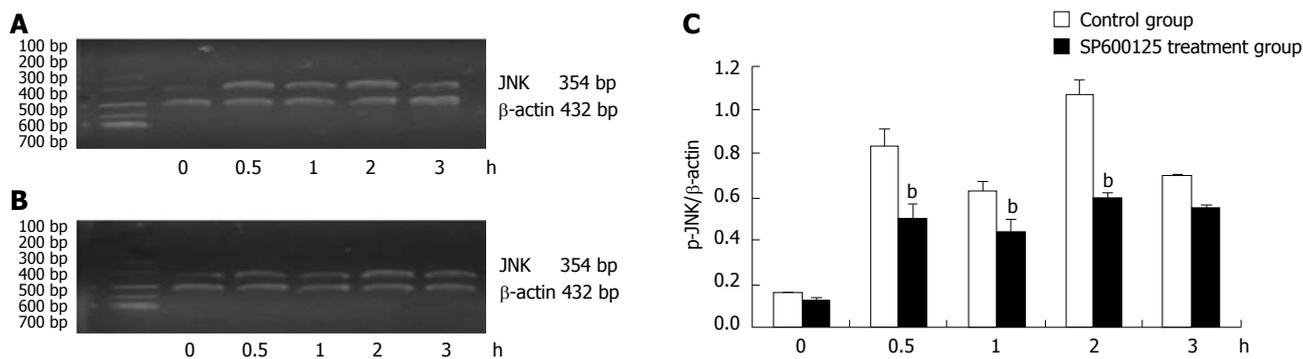


Figure 5 Expression of c-Jun N-terminal kinase at the mRNA level in SGC-7901 cells with or without SP600125 treatment following thermotherapy for various time periods. A: Expression of c-Jun N-terminal kinase (JNK) at the mRNA level in SGC-7901 cells without SP600125 treatment; B: Expression of JNK at the mRNA level in SGC-7901 cells with SP600125 treatment; C: Comparison of JNK at the mRNA level in SGC-7901 cells with or without SP600125 treatment; Expression of JNK at the mRNA level in SGC-7901 cells with SP600125 treatment was significantly inhibited compared with that in SGC-7901 cells without SP600125 treatment ($P < 0.01$).

used the JNK-specific inhibitor, SP600125, which exhibits significant selectivity for JNKs leading to inhibition of phosphorylation of both c-Jun and JNKs^[19]. Therefore, we treated SGC-7901 cells with thermotherapy in the absence or presence of SP600125 and analyzed the expression of p-JNK, Bax, Bcl-2 and caspase-3 proteins (Figure 4). We found that the presence of SP600125 significantly reduced the p-JNK level at various time points ($P < 0.01$), while the p-JNK level did not change markedly at 0 h ($P > 0.05$), compared to cells in the absence of SP600125. Concurrently, the expression of Bax and caspase-3 protein was also inhibited in the presence of SP600125, as well as that of p-JNK. However, there was no significant difference in the expression of Bcl-2 in SGC-7901 cells at any of the time points ($P > 0.05$) between the SP600125 treatment and mock treatment (Figure 4). These results collectively indicate that thermotherapy induced-apoptosis may be mediated by activation of JNK and the up-regulated expression of Bax and caspase-3 proteins, causing cellular apoptosis in gastric carcinoma cells. Bcl-2 protein did not seem to be involved and played a protective role in this process. These data suggest that JNK plays an important role in thermotherapy-induced apoptosis in gastric carcinoma cells.

DISCUSSION

Thermotherapy is a new cancer therapy which has re-emerged in the last 20 years, and plays an important role in comprehensive tumor treatment. Combined thermo- and chemo-therapy (green therapy) has become one of the important auxiliary therapies, as it improves the efficacy of cancer treatment^[20,21]. Heat-induced cell apoptosis was reported in the 1990s. More recently, JNK was activated in apoptotic U937 cells by radiotherapy^[22], and similar results were obtained using thermotherapy in human colon cancer^[16]. Thus, the expression of phospho-JNK at the protein level and JNK at the mRNA level were determined in SGC-7901 cells treated with thermotherapy. Thermotherapy increased the expression of p-JNK at different time points, and the protein produc-

tion of p-JNK was significantly higher at 0.5 h than that at 0 h post-treatment ($P < 0.01$), peaked at 2 h, and then decreased. The reason for this decline may be that some SGC-7901 cells were killed directly, not by induced apoptosis at 3 h, and a few SGC-7901 cells restarted proliferation after a long period of thermotherapy. It is interesting that p-JNK protein was slightly lower at 1 h than that at 0.5 h post-treatment, this indicates that SGC-7901 cells may have the ability to respond to thermotherapy. Similarly, the RT-PCR data showed that the induction patterns of the expression of p-JNK protein and JNK mRNA level were very similar in thermotherapy-induced apoptosis (Figures 4 and 5). Namely, the expression of JNK mRNA was significantly higher at 0.5 h than that at 0 h post-treatment ($P < 0.01$), reached a maximum at 2 h, followed by a decrease, and was slightly lower at 1 h than at 0.5 h post-treatment. Taken together, these results demonstrate that JNK plays a role in the effects of thermotherapy in gastric cancer cells. However, the mechanism of JNK activation in thermotherapy-induced apoptosis is still largely unknown in gastric cancer cells.

JNK is thought to induce mitochondria-dependent apoptosis mainly through direct or indirect activation of Bax and down-regulation of Bcl-2^[23-27], a pro-apoptotic Bcl-2 family member, which plays an essential role in inducing apoptosis^[28]. It is well known that most cell apoptosis-inducing factors eventually cause cell apoptosis through the caspase-mediated signal transduction pathway^[29,30], and caspase3 is a main effector in cell apoptosis. Therefore, to investigate the roles of Bax, Bcl-2 and caspase3 proteins during thermotherapy-induced apoptosis in human gastric cancer cells, we determined the expression of Bax, Bcl-2 and caspase3 proteins in thermotherapy-stimulated SGC-7901 cells. The data showed that thermotherapy increased the expression of Bax protein at different time points, which peaked at 2 h, and then decreased. These findings were similar to those obtained for the expression of p-JNK and caspase-3 proteins after thermotherapy in SGC-7901 cells. Bcl-2 was increased at 0.5 h and peaked at 1 h. These results suggest that thermotherapy-induced apoptosis was associated with activation

of JNK and increased expression of Bax and caspase-3 proteins. The JNK-specific inhibitor, SP600125, substantially inhibited thermotherapy-induced activation of JNK expression and the expression of Bax and caspase-3 proteins. No significant change in the expression of Bcl-2 was observed. Apoptosis was significantly decreased compared to that in thermotherapy-treated cells alone at different time points (Figure 4). These findings proved that thermotherapy-induced apoptosis is associated with JNK activation through up-regulation of the expression of Bax and caspase-3 proteins. These results suggest that the JNK cascade is required for apoptosis induction following thermotherapy in human gastric cancer SGC-7901 cells, which will be investigated in the future.

In summary, our study demonstrates that thermotherapy promoted phospho-JNK production, induced cell apoptosis and inhibited SGC-7901 cell viability. JNK was phosphorylated and activated, and the expression of Bax and caspase-3 was upregulated which subsequently caused cellular apoptosis in gastric cancer cells during thermotherapy. Bcl-2 protein, an anti-apoptotic protein, was not involved and played a protective role in this process. Therefore, JNK plays a critical role in thermotherapy-induced apoptosis in human gastric cancer.

COMMENTS

Background

Gastric carcinoma is one of the most common human cancers worldwide and is likely to remain a leading cause of death in the near future. New approaches in the treatment of gastric cancer are required. Recent studies have shown that c-Jun N-terminal kinase (JNK) is involved in thermotherapy-induced apoptosis in colon cancer cells, and is involved in vitamin E succinate-induced apoptosis in gastric carcinoma cells. However, it is unknown whether JNK is involved in thermotherapy-induced gastric carcinoma cell apoptosis, and which pathway is affected. These problems need to be explored in further studies. Thermotherapy is a novel approach in the treatment of gastric cancer.

Research frontiers

JNK, a member of the mitogen-activated protein kinase (MAPK) family, is activated through phosphorylation of JNK and has been implicated in a variety of processes in response to various stresses. It is believed that JNK has anti- or pro-apoptotic activity depending on cell type, apoptotic stimuli and other signalling pathways. However, the role of p-JNK during thermotherapy-induced apoptosis has not yet been elucidated. In this study, the authors demonstrate that activation of JNK via the Bax-caspase-3 pathway may represent an important mechanism in thermotherapy-induced apoptosis in gastric cancer cells.

Innovations and breakthroughs

Recent reports have highlighted that JNK is involved in thermotherapy-induced apoptosis in colon cancer cells. This is the first study to report that JNK is also involved in thermotherapy-induced apoptosis in gastric carcinoma cells. Furthermore, the studies show that activation of JNK via the Bax-caspase-3 pathway may represent an important mechanism in thermotherapy-induced apoptosis in gastric cancer cells.

Applications

The results of this study indicate the role of JNK in thermotherapy-induced apoptosis in gastric carcinoma cells, and are the basis for further investigations on the mechanism of thermotherapy-induced apoptosis which may be used in clinical applications.

Terminology

JNK is a member of the MAPK family. Many studies have shown that JNK activation has anti- or pro-apoptotic activity depending on cell type, apoptotic stimuli and other signalling pathways. Unsurprisingly, activation of JNK via the Bax-caspase-3 pathway may cause cell apoptosis following thermotherapy in gastric cancer cells.

Peer review

The paper describes the effects of thermotherapy on apoptosis and cell cycle progression in human gastric cancer. Further, the role of JNK was determined to investigate the underlying mechanism of thermotherapy-induced apoptosis in human gastric cancer. It would be interesting to have some pieces of information in the field of gastric cancer therapy.

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Serum pepsinogen II is a better diagnostic marker in gastric cancer

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Abstract

AIM: To investigate screening makers for gastric cancer, we assessed the association between gastric cancer and serum pepsinogens (PGs).

METHODS: The subjects comprised 450 patients with gastric cancer, 111 individuals with gastric atrophy, and 961 healthy controls. Serum anti-*Helicobacter pylori* (*H. pylori*) immunoglobulin G (IgG), PG I and PG II were detected by enzyme-linked immunosorbent assay. Gastric atrophy and gastric cancer were diagnosed by endoscopy and histopathological examinations. Odds ratios and 95% CIs were calculated using multivariate logistic regression.

RESULTS: Rates of *H. pylori* infection remained high in Northeastern China. Rates of *H. pylori* IgG positivity were greater in the gastric cancer and gastric atrophy groups compared to the control group (69.1% and 75.7% vs 49.7%, $P < 0.001$). Higher levels of PG II (15.9 $\mu\text{g/L}$ and 13.9 $\mu\text{g/L}$ vs 11.5 $\mu\text{g/L}$, $P < 0.001$) and lower PG I / PG II ratio (5.4 and 4.6 vs 8.4, $P < 0.001$) were found in patients with gastric cancer or gastric atrophy compared to healthy controls, whereas no correlation was found between the plasma PG I concentration and risk of gastric cancer ($P = 0.537$). In addition, multivariate logistic analysis indicated that *H. pylori* infection and atrophic gastritis were independent risk factors for gastric cancer. Lower plasma PG I / PG II ratio was associated with higher risks of atrophy and gastric cancer. Furthermore, plasma PG II level significantly correlated with *H. pylori*-infected gastric cancer.

CONCLUSION: Serum PG II concentration and PG I / PG II ratio are potential biomarkers for *H. pylori*-infected gastric disease. PG II is independently associated with risk of gastric cancer.

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Key words: Gastric cancer; Pepsinogens; *Helicobacter pylori*; Gastric atrophy; Screening

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INTRODUCTION

Gastric cancer remains the third leading cause of cancer death in China^[1]. *Helicobacter pylori* (*H. pylori*) and gastric atrophy have both been identified as etiological factors for gastric cancer. However, screening and eradication of *H. pylori* in the general population is not advised because the cost of programs outweighs the modest effect on reduced incidences of gastric cancer. Therefore, identification of patients with the early stage gastric cancer could improve treatment and survival. Gastric atrophy, the precancerous lesion of gastric cancer, can be diagnosed by histological examination, and measurement of serum concentration of pepsinogens (PGs). Levels of two biochemically and immunologically distinct types, PG I and PG II, indicate different status of gastric mucosa. Serum PG level screening can be carried out at low cost in countries with high and moderate incidences of gastric cancer, such as Japan and China^[2].

Most studies have demonstrated that low concentrations of PG I and low PG I /PG II ratios in the serum or plasma are indicators of atrophic gastritis, which are linked with elevated gastric cancer risk^[3-5]. However, few studies have assessed correlations between PG II and gastric cancer risk^[6,7]. It is well known that *H. pylori* infection leads to development of both atrophic gastritis and gastric cancer. Detection of serum anti-*H. pylori* immunoglobulin G (IgG) antibodies and screening for gastric atrophy and gastric cancer at an early stage are important for clinical assessments and interventions^[8,9]. Absence of data on *H. pylori* infection and serum PG levels are available in large epidemiological surveys. To identify relationships between gastric mucosal lesions and serum PG levels, we carried out an assessment of PG I and PG II levels, PG I /PG II ratio and risk of gastric cancer in a cross-sectional study.

MATERIALS AND METHODS

Study participants

Four hundred and fifty patients with gastric cancer were selected from the Department of Gastric and Colorectal Surgery, and Department of Gastroenterology, First Hospital of Jilin University, from 2008 to 2010. All gastric cancer patients underwent tumor resection with histologically confirmed diagnosis of gastric adenocarcinoma. From 2009 to 2010, gastric atrophy cases and healthy controls were recruited from the check-up center of the same hospital. The subjects were of Han descent from the Changchun region. A total 1109 subjects (644 male and 465 female, aged 35-80 years) participated in the study. Gastric atrophy was diagnosed by endoscopy and histopathological examinations. Written informed consent was obtained from all subjects and the study protocol was approved by the Ethics Committee of the First Hospital, Jilin University.

Sampling and determination of serum

Fasting blood was taken for all participants and serum

was collected and stored at -80 °C. In the gastric cancer group, the samples were collected before surgery. Serum PG I and PG II levels were measured by enzyme-linked immunosorbent assay (ELISA) (Biohit ELISA kit, Biohit, Helsinki, Finland). Serum IgG antibodies to *H. pylori* were detected by ELISA using an *H. pylori*-IgG ELISA kit (Biohit). The antibody titers were defined by optical density values according to the manufacturer's protocol and titers higher than the cutoff value of 30 EIU were considered as positive for *H. pylori* infection. The quality control sample showed a coefficient of variation (CV) of 6.4%. For the pooled plasma samples the CV was 4.5%. According to the Chinese guidelines for diagnosis, patients are considered to have atrophic gastritis if PG I is $\leq 82.3 \mu\text{g/L}$ and PG I /PG II ratio is ≤ 6.05 ^[10]. The quality control samples showed CVs of 4.5%, 4.3% and 4.7% for *H. pylori*, PG I and PG II, respectively. All suspected cases with gastric atrophy by serum screening were re-determined by endoscopic biopsy and histological examinations.

Statistical analysis

The data did not fit a normal distribution, therefore, we used the Wilcoxon rank sum test to compare medians (quartiles) between the two groups. The χ^2 test was used to compare multiple sample rates. Non-conditional logistic regression analysis was used to calculate the odds ratio of risk-related factors for gastric cancer and its 95%CI. Two tailed *P* values < 0.05 were considered as statistically significant. All statistical analysis were carried out by SPSS version 18 software.

RESULTS

There were 450 patients with gastric cancer (324 male and 126 female, aged 35-80 years). Sixty-four cases were categorized as tumor-node-metastasis stage I (14.2%), 182 as stage II (40.4%), 145 as stage III (32.2%), and 59 as stage IV (13.1%). Among the 1109 participants, 148 individuals were screened for gastric atrophy using serum PG examination and 111 patients were confirmed with gastric atrophy by biopsy and histopathological examinations. Seventeen subjects were diagnosed with pseudopositive gastric atrophy and excluded from the study; 20 participants who rejected endoscopic examinations were also excluded. The remaining 961 individuals were included in the control group. The mean age was older in the gastric cancer group than in the gastric atrophy and control groups. The subject characteristics are summarized in Table 1.

H. pylori infection

H. pylori-positive rates in the gastric cancer and gastric atrophy groups were significantly higher compared to those in the control group (69.1% and 75.7% *vs* 49.7%, *P* < 0.001). The *H. pylori* infection rates were higher in the 45-65 years group compared to those aged < 45 years or > 65 years. Among the 450 gastric cancer patients, *H. pylori* infection rate was higher in those with stage I and

Table 1 Comparison of *Helicobacter pylori* infection between gastric cancer and control groups

	Gastric cancer group (%)	Gastric atrophy group (%)	Control group (%)	<i>P</i> value
No.	450	111	961	
<i>H. pylori</i> infection				
Negative	139 (30.9)	27 (24.3)	483 (50.3)	< 0.001
Positive	311 (69.1)	84 (75.7)	478 (49.7)	
Sex				
Male	324 (72.0)	66 (59.5)	564 (58.7)	< 0.001
Female	126 (28.0)	45 (40.5)	397 (41.3)	
Age				
< 45 yr	38 (8.4)	20 (18.0)	285 (29.7)	< 0.001
45-65 yr	240 (53.3)	81 (73.0)	607 (63.2)	
> 65 yr	172 (38.2)	10 (9.0)	69 (7.2)	

H. pylori: *Helicobacter pylori*.

stage II disease, compared to those with stage III and IV disease (73.6% *vs* 63.7%, *P* = 0.02). There were no correlations found between *H. pylori* infection and pathological type, tumor site, and differentiation of tumors (well-differentiated 50%, mild differentiation 69.8%, and poor differentiation 72.9%, *P* = 0.47).

PG levels

The median (interquartile range; IQR) PG I and PG II levels, and the ratio of PG I /PG II in the controls were 92.6 µg/L (75.0-116.1), 11.5 µg/L (7.1-18.4), and 8.4 µg/L (6.1-11.1), respectively. PG II level in the gastric cancer group was significantly higher than that in the control group (*P* < 0.001), whereas the PG I /PG II ratio showed a marked decline (*P* < 0.001) (Table 2). However, no statistically significant difference was found in PG I level between the two groups (*P* = 0.532). In the control group, a higher PG I level was detected in patients with *H. pylori* infection compared to *H. pylori*-negative patients (median: 99 *vs* 78.9 µg/L, *P* < 0.001).

Median (IQR) levels of PG I and PG II, and PG I /PG II ratio were 94.4 µg/L (38.9-148.8), 13.3 µg/L (7.7-22.5) and 6.2 (3.5-10.0) µg/L for 139 *H. pylori*-negative gastric cancer patients, respectively. Median (IQR) levels of PG I and PG II, and PG I /PG II ratio were 92.2 µg/L (53.0-145.3), 18.4 µg/L (10.4-30.0) and 5.1 µg/L (3.5-7.2), respectively, for 311 *H. pylori*-positive gastric cancer patients. The difference in PG II level and PG I /PG II ratio was significant (*P* = 0.01) between *H. pylori* (-) and *H. pylori* (+) subjects. However, no significant difference in PG I concentrations was found between *H. pylori* (-) and *H. pylori* (+) cases in the gastric cancer group (92.2 µg/L *vs* 94.4 µg/L, *P* = 0.96) (Table 3). In addition, serum PG I concentration was significantly lower in patients with stage III and IV tumors compared to stage I and II (Table 4).

DISCUSSION

Serum PG I is produced by chief cells and mucous neck cells of gastric fundic glands, while PG II is produced

by those cells and also by pyloric glands and Brunner's glands^[11]. Atrophic gastritis, characterized by loss of the specialized cells and glands in the stomach, is considered as a gastric cancer precursor lesion^[12]. Serum PG levels, pepsin precursors, reflect the functional and morphological status of the gastric mucosa. It is widely accepted in gastric *H. pylori* infection that the fundic gland mucosa and PG I levels gradually decrease, whereas PG II levels remain constant^[13,14]. Consequently, a stepwise reduction of the PG I /PG II ratio is closely linked to progression of atrophic gastritis. However, PG II is considered to be a subordinate marker in clinical diagnosis. In fact, PG II, a mature marker of gastric epithelia, reflects the physiological or pathophysiological functions of the gastric system^[15,16].

Gastritis is more prevalent and severe in Japan, with more corpus predominant atrophy and intestinal metaplasia^[17-19]. PG I ≤ 70 ng/mL and PG I /PG II ratio ≤ 3.0 have been used for the diagnosis of extensive atrophic gastritis with sensitivity of 80% and specificity of 70%^[20]. In contrast, our study showed a low incidence of gastric mucosal atrophy in the Chinese population. Inconsistency between studies may be due to different ethnic groups with different genetic backgrounds, different ELISA kits, and different cutoff values used for assessment of gastric mucosal atrophy^[21]. We used PG I ≤ 82.3 µg/L and PG I /PG II ratio ≤ 6.05 according to the study for diagnosis of atrophic gastritis in the Chinese population^[10]. For reference, the cut-off points of PG I and PG I /PG II ratio were calculated using receiver operator characteristic (ROC) curves. For patients with atrophic gastritis, the area under the ROC for PG I was 0.878 (95%CI: 0.837-0.919) and the best cutoff value was 82.30 µg/L (sensitivity 85.9%, specificity 75.1%). The area under the ROC for PG I /PG II ratio was 0.819 (95%CI: 0.767-0.871) and the best cutoff value was 6.05 (sensitivity 78.3%, specificity 71.6%)^[10]. The limitation of our study was that it could not determine the area under the curve *via* numerical integration of ROC curves, because we did not administer a further validity study. In our study, we found that serum PG I levels were significantly decreased in the gastric atrophy group, but no difference was found between the gastric cancer and control groups. Thus, it is considered that serum PG I levels represent gastric atrophy but not gastric cancer. The reason was that the prevalence of gastric atrophy was relatively low in our gastric cancer group compared with that in Japan. In Japan, the incidence of gastritis was higher than in other countries, and it was characterized by heavy predominant atrophy and intestinal metaplasia in the corpus of the stomach. Compared to the findings in Japan, our study showed a lower incidence of gastric mucosal atrophy. This may be due to differences in the research populations and their genetic backgrounds, even though the same *H. pylori* infection can lead to differences in severity of gastric atrophy^[22-24]. Other factors such as the use of proton pump inhibitors (PPIs) may also influence the incidence of gastric atrophy. However, a history of using PPIs and lifestyle

Table 2 Comparison of pepsinogen levels between gastric cancer, gastric atrophy and control groups

	Gastric cancer group	Gastric atrophy group	Control group	P value (cancer vs control)	P value (atrophy vs control)
PG I (μg/L)	93.2 (49.8-147.3)	64.0 (52.3-75.3)	92.6 (75.0-116.1)	0.537	< 0.001
PG II (μg/L)	15.9 (9.0-28.0)	13.9 (11.9-16.9)	11.5 (7.1-18.4)	< 0.001	< 0.001
PG I /PG II	5.4 (3.5-8.1)	4.6 (3.6-5.3)	8.4 (6.1-11.1)	< 0.001	< 0.001

Data are expressed as median (interquartile range). PG: Pepsinogen.

Table 3 *Helicobacter pylori* infection and pepsinogen levels of serum in gastric cancer patients

	<i>H. pylori</i> (+) (n = 311)	<i>H. pylori</i> (-) (n = 139)	P value
PG I (μg/L)	92.2 (53.0-145.3)	94.4 (38.9-148.8)	0.96
PG II (μg/L)	18.4 (10.4-30.0)	13.3 (7.7-22.5)	0.01
PG I /PG II	5.1 (3.5-7.2)	6.2 (3.5-10.0)	0.01

Data are expressed as median (interquartile range). PG: Pepsinogen; *H. pylori*: *Helicobacter pylori*.

were not investigated in this study. A further prospective control study is needed. PG II levels were significantly increased in patients with gastric atrophy and gastric cancer, especially in *H. pylori* (+) gastric cancer, resulting in a reduction in PG I /PG II ratio, which was consistent with a previous study^[6].

A few studies have investigated PG II serum level as an independent biomarker with potential clinical applications^[23]. Higher PG II expression has been demonstrated in patients with gastric ulcers, suggesting that PG II level reflects chronic inflammation in *H. pylori*-related chronic gastritis^[26-28]. Another study has shown that insertion/deletion polymorphisms of the PG II gene are highly associated with genetic predisposition to gastric cancer in the carriers^[116]. Our study demonstrated that chronic *H. pylori* infection is at a high level in the Chinese population. The overall *H. pylori* (+) rate in patients with gastric carcinoma was 78.6% in young patients (< 45 years), which was significantly higher than that in the control group (51%). Active gastritis caused by *H. pylori* infection and chronic inflammation resulted in elevated PG II levels in the gastric cancer group.

We also found that serum PG I level was significantly lower in patients with stage III and IV tumors, compared to those with stage I and II tumors. The mechanism is unclear but it may be associated with gastric mucosal atrophy gradually increasing with advancing gastric cancer.

We demonstrated that serum PG II level significantly increased in Chinese patients with *H. pylori*-infected gastric cancer, indicating that PG II can be used as an independent diagnostic marker for gastric cancer. It is cost-effective to screen PG II in countries with a high incidence of gastric cancer, compared with the high cost of endoscopy. Further studies on normal ranges of PG II and its changes in gastric diseases may elucidate its physiological and pathological functions.

In conclusion, PG II can be used as an independent diagnostic marker for gastrointestinal cancer^[29-31].

Table 4 Gastric cancer stages and serum pepsinogen levels

TNM stage (n)	PG I (μg/L)	PG II (μg/L)	PG I /PG II
I (64)	101.4 (76.3-147.3)	18.4 (12.1-27.2)	6.3 (4.5-8.4)
II (182)	96.3 (44.7-167.2)	16.3 (9.3-27.1)	5.5 (4.0-8.2)
III (145)	85.8 (43.4-133.3)	15.4 (8.8-28.7)	4.9 (2.8-7.5)
IV (59)	71.5 (35.7-137.0)	13.1 (6.7-13.1)	5.1 (3.1-8.1)
P value	0.02	0.44	0.09

PG: Pepsinogen; TNM: Tumor-node-metastasis.

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COMMENTS

Background

Early detection is an important way to reduce mortality of gastric cancer. Measurement of serum pepsinogen (PG) is a popular non-invasive serological screening test for gastric cancer.

Research frontiers

Research has shown that serum-PG testing, based on the combination of the measurement of serum PG I concentration and the PG I /PG II ratio, is a good predictor of atrophic gastritis and gastric cancer development.

Innovations and breakthroughs

PG II has been as considered a subordinate marker in clinical diagnosis. In this study, we demonstrated that the serum PG II level significantly increased in *Helicobacter pylori*-infected gastric cancer in the Chinese population, indicating that PG II can be used as an independent diagnostic marker for gastric cancer.

Applications

Serum PG II level measure is a good method for screening patients with gastric cancer. It is very cost-effective to screen PG II in countries with high incidences of gastric cancer, compared with the high cost of endoscopy.

Terminology

PGs are inactive proenzymes for the specific digestive enzyme, pepsin, originating from the gastric mucosa, and can be classified into PG I and PG II.

Peer review

The authors performed a clinical epidemiological study and evaluated the possibility and significance of PG II in serum and PG I /PG II ratio as the diagnostic markers for Chinese patients with gastric cancer and gastric atrophy. They concluded that serum level of PG II could be a useful diagnostic marker for patients with gastric cancer.

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A meta-analysis of the effects of energy intake on risk of digestive cancers

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Abstract

AIM: To quantitatively assess the relationship between energy intake and the incidence of digestive cancers in a meta-analysis of cohort studies.

METHODS: We searched MEDLINE, EMBASE, Science Citation Index Expanded, and the bibliographies of retrieved articles. Studies were included if they reported relative risks (RRs) and corresponding 95% CIs of digestive cancers with respect to total energy intake. When RRs were not available in the published article, they were computed from the exposure distributions. Data were extracted independently by two investigators and discrepancies were resolved by discussion with a third investigator. We performed fixed-effects meta-analyses and meta-regressions to compute the summary RR for highest versus lowest category of energy intake and for per unit energy intake and digestive cancer incidence by giving each study-specific RR a weight that was proportional to its precision.

RESULTS: Nineteen studies consisting of 13 independent cohorts met the inclusion criteria. The studies

included 995 577 participants and 5620 incident cases of digestive cancer with an average follow-up of 11.1 years. A significant inverse association was observed between energy intake and the incidence of digestive cancers. The RR of digestive cancers for the highest compared to the lowest caloric intake category was 0.90 (95% CI 0.81-0.98, $P < 0.05$). The RR for an increment of 239 kcal/d energy intake was 0.97 (95% CI 0.95-0.99, $P < 0.05$) in the fixed model. In subgroup analyses, we noted that energy intake was associated with a reduced risk of colorectal cancer (RR 0.90, 95% CI 0.81-0.99, $P < 0.05$) and an increased risk of gastric cancer (RR 1.19, 95% CI 1.08-1.31, $P < 0.01$). There appeared to be no association with esophageal (RR 0.96, 95% CI 0.86-1.07, $P > 0.05$) or pancreatic (RR 0.79, 95% CI 0.49-1.09, $P > 0.05$) cancer. Associations were also similar in studies from North America and Europe. The RR was 1.02 (95% CI 0.79-1.25, $P > 0.05$) when considering the six studies conducted in North America and 0.87 (95% CI 0.77-0.98, $P < 0.05$) for the five studies from Europe.

CONCLUSION: Our findings suggest that high energy intake may reduce the total digestive cancer incidence and has a preventive effect on colorectal cancer.

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Key words: Diet; Cancer prevention; Energy intake; Digestive cancer

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Yu XF, Wang YQ, Zou J, Dong J. A meta-analysis of the effects of energy intake on risk of digestive cancers. *World J Gastroenterol* 2012; 18(48): 7362-7370 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i48/7362.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i48.7362>

INTRODUCTION

An important discovery in recent years is that lifestyle and environmental factors affect cancer initiation, promotion, and progression. Epidemiological studies strongly suggest that the majority of cancer deaths can be attributed to factors such as unhealthy diets, tobacco, alcoholism, infections, and occupational exposure. In particular, data from several observational studies support the theory that diet plays an important role in the initiation of many common cancers^[1]. Calorie restriction (CR) is an experimental mode in which test animals receive a lower-calorie diet than ad libitum-fed controls. It has emerged as the most potent, broadly acting dietary intervention for preventing carcinogenesis in rodent models of cancer^[2]. Recent reports of extended life span and delayed cancer development in response to CR in rhesus monkeys^[3] and observations that CR during the premenopausal years decreases postmenopausal breast cancer risk in women^[4] suggest that the anticancer effects of CR reported in rodent models extend to primates, including humans.

Although animal models have clearly demonstrated a protective effect of CR on cancer risk, it is less clear and there is little direct evidence that such a protective effect exists in humans. A study of normal-weight humans found that a 20% energy restriction for 10 wk did not reduce oxidative DNA damage^[5]. In free-living populations, it is difficult to answer the important question of whether such an effect exists within the range of energy intake by humans. In human populations, energy intake is determined by physical activity, body size, and metabolic efficiency, and all these factors may be related to cancer risk, which makes the relationship between energy intake and cancer in humans complex.

Very few studies have assessed the relationship between CR and the risk of various cancer sites because of ethical issues. One study of the 1944-1945 Dutch famine and subsequent overall cancer incidence^[6] found no evidence that the short famine affected overall cancer risk. However, higher energy intake in childhood may increase the risk of developing cancer in adulthood^[7]. Data from case-control studies may be subject to recall bias with respect to energy intake and to selection bias with respect to the control group. Additional prospective cohort studies excluding those biases would be more useful for observing energy-cancer associations. We therefore systematically reviewed and performed a meta-analysis of prospective cohort studies to quantitatively assess the association between energy intake and digestive cancer risk in free-living human populations.

MATERIALS AND METHODS

Literature search

We searched the electronic databases MEDLINE (1966 to May, 2012), EMBASE (1985 to May, 2012), and Science Citation Index Expanded (1945 to May, 2012),

using the Medical Subject Heading term energy intake combined with digestive system neoplasms. Furthermore, we reviewed reference lists of retrieved articles to search for additional studies. Only studies published as full-length articles in English were considered.

Inclusion and exclusion criteria

For inclusion, studies had to fulfill the following criteria: have a prospective cohort design, report relative risks (RR) or hazard ratios and their corresponding 95% CIs (or data to calculate them) of digestive cancers relating to every category of energy intake, and provide the categories or total intake of calories. Studies were excluded if a case-control design was used, the experimental participants were children or adolescents, energy intake from special food was reported in which the total intake of calories could not be calculated, or adequate classification of intake could not be determined because categories of energy intake were not reported. If multiple published reports from the same study cohort were available, we included only the one with the most detailed information for both outcome and energy intake. If there were multiple articles on different types of digestive cancers in the same cohort, we combined the outcomes to calculate the summary RR and its corresponding 95% CIs.

Data extraction

Data were extracted independently by two investigators (Yu XF and Dong J) according to the meta-analysis of observation studies in epidemiology guidelines^[8], and discrepancies were resolved by discussion with a third investigator (Zou J). For each study, the following information was extracted: first author's last name, year of publication, country of origin, follow-up period, number of patients and cases, digestive cancer sites, category amounts of energy intake, outcome assessment, RR or hazard ratios of cancer and the corresponding 95% CIs for every category of energy intake, and covariates adjusted for in the statistical analysis.

Statistical analysis

The measures of interest were the RR and the corresponding 95% CIs for included cohort studies. When RRs were not available in the published article, they were computed from the exposure distributions. We computed the summary RR for highest versus lowest category of energy intake and for per unit energy intake and digestive cancer incidence by giving each study-specific RR a weight that was proportional to its precision (i.e., the inverse of the variance was derived, when necessary, from the reported 95% CIs).

Statistical heterogeneity among studies was estimated using Q and I^2 statistics. For the Q statistic, heterogeneity was considered present for $P < 0.1$. We pooled the study-specific estimates using both the fixed-effect model and the random-effect model proposed by DerSimonian and Laird; when a significant heterogeneity was

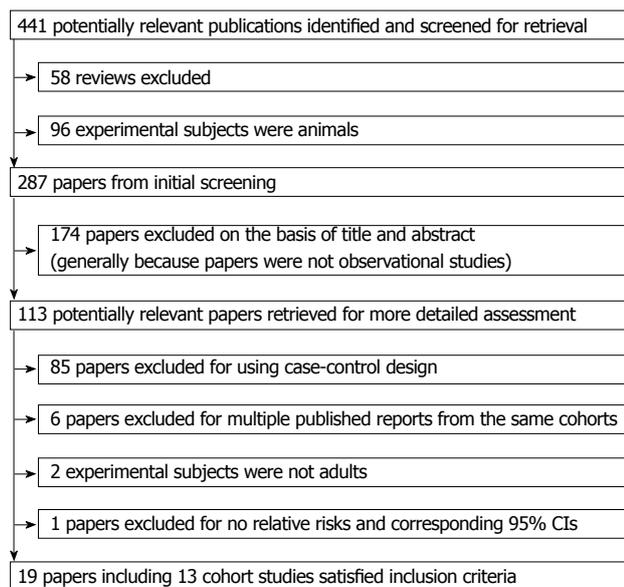


Figure 1 Flow diagram of the search strategy and study selection.

found, the random-effect model results were presented. A sensitivity analysis was also conducted, in which one study at a time was removed and the rest were analyzed to estimate whether the results could have been markedly affected by a single study.

Finally, publication bias was evaluated with funnel plot visual analysis and with the Begg's and Egger's tests. $P < 0.05$ was considered statistically significant. All statistical analyses were performed with STATA (Version 9.0; Stata Corp., College Station, TX).

RESULTS

Using the predefined search strategy, we identified 19 publications and 13 prospective cohort studies (Figure 1), including 995 577 participants and 5620 incident cases of digestive cancer with an average follow-up of 11.1 years, which were eligible for inclusion in the meta-analysis^[9-27]. The characteristics of the included cohorts are summarized in Table 1. Initial agreement between the two reviewers on whether a study was eligible for inclusion occurred for 108/113 manuscripts (95.6%; $\kappa = 0.912$). Of the 13 cohorts included in the meta-analysis, 6 were conducted in Europe, 6 in North America (United States), and 1 in Asia (Singapore).

From the 13 cohorts reporting energy intake, 11 cohorts could be used for the qualitative meta-analyses for the highest versus the lowest category of exposure and digestive cancer incidence. Figure 2A shows the estimated RRs for the highest versus lowest category of energy intake from cohort studies. The summary RR of digestive cancers from all combined studies was 0.90 (95%CI 0.81-0.98). There was no significant heterogeneity across the studies ($Q = 14.6, P = 0.148, I^2 = 31.4\%$).

From the included cohorts, nine studies could be used in the per unit energy intake meta-analysis. The

summary RR of digestive cancers for an increment of 239 kcal/d energy intake was 0.97 (95%CI 0.95-0.99), and no significant heterogeneity between studies was present ($Q = 11.7, P = 0.167, I^2 = 31.4\%$) (Figure 2B).

When stratified by the site of digestive cancer, we noted that energy intake was associated with a reduced risk of colorectal cancer (RR 0.90, 95%CI 0.81-0.99) and an increased risk of gastric cancer (RR 1.19, 95%CI 1.08-1.31). There appeared to be no association with esophageal (RR 0.96, 95%CI 0.86-1.07) or pancreatic (RR 0.79, 95%CI 0.49-1.09) cancer. Associations were also similar in studies from North America and Europe. The RR was 1.02 (95%CI 0.79-1.25) when considering the six studies conducted in North America and 0.87 (95%CI 0.77-0.98) for the five studies from Europe.

There was no indication of publication bias from either visualization of the funnel plot or Egger's ($P = 0.661$) and Begg's ($P = 0.533$) (Figure 3) tests. A sensitivity analysis, in which one study was removed at a time, was performed to evaluate the stability of the results. This analysis confirmed the stability of our results.

DISCUSSION

Over the past 30 years, CR has emerged as the most potent, broadly acting dietary intervention for preventing carcinogenesis in rodent models of cancer. Some observational studies further support the hypothesis that CR has beneficial effects on longevity and cancer risk in humans^[28]. However, physical activity and body size are highly related to total energy intake, and it is difficult to assess the independent effect of energy intake on cancer risk. In addition, energy intake is also difficult to assess in large-scale epidemiologic studies. Animal experimental studies have suggested the importance of energy balance as a determinant for cancer risk^[28,29]. Although very few studies have assessed the relationship between CR and the risk of various cancer sites in humans because of ethical issues, we quantitatively assessed the relationship between energy intake and the incidence of digestive cancers in a meta-analysis of cohort studies. Our meta-analysis yielded an inconsistent result in former studies and showed that energy intake was inversely associated with the risk of digestive cancers. The summary RR of digestive cancers was 0.97 (95%CI 0.95-0.99) for an increment of 239 kcal calorie intake per day.

Data from countries that experienced varying degrees of energy restriction during World War II may support our results. For example, a cohort of Norwegians showed reduced breast cancer risk when exposed to acute (< 1 year) energy restriction (50% reduction in caloric intake without significant changes in diet quality)^[30]. In contrast, survivors of the Dutch famine of 1944, during which energy restriction (70% reduction in rations for adults; 50% reduction in rations for children) was more severe than in the Norwegian study, experienced higher breast cancer rates but no apparent change in risk of any other cancer^[6]. Cohorts exposed to even

Table 1 Summary characteristics of cohorts included in the meta-analysis

Ref.	Country	Follow-up period (yr)	Age (yr)	Cohort size	Cases	Exposure details (kcal/d)	Outcome	Contrast between groups (kcal)	Relative risk (95%CI)	Adjustments
Giovannucci <i>et al</i> ^[11]	United States	6	40-75	47 949	205	Caloric intake	Colon cancer incidence	1229 1586 1884 2308 2820	1 1.92 (1.28-2.90) 1.33 (0.85-2.08) 1.12 (0.70-1.80) 0.94 (0.57-1.55)	Age
Goldbohm <i>et al</i> ^[12]	Netherlands	3.3	55-69	120 852	215	Caloric intake	Colon cancer incidence	1510 (M); 1163 (F) 1836 (M); 1435 (F) 2096 (M); 1626 (F) 2364 (M); 1848 (F) 2791 (M); 2200 (F)	1 0.88 (0.57-1.69) 1.12 (0.75-1.70) 0.84 (0.54-1.31) 0.74 (0.47-1.18)	
Chyou <i>et al</i> ^[13]	United States	24	45-68	7903	695	Caloric intake	Upper digestive Tract, colorectal Cancer incidence	< 2000 2000-2499.9 ≥ 2500	1 0.91 (0.60-1.22) 0.94 (0.64-1.24)	Age, alcohol, number of cigarettes day, number of years smoked
Gaard <i>et al</i> ^[15]	Norway	11.4	20-54	50 535	143	Energy intake kJ/d	Colon cancer incidence	≥ 9999: highest quintile ≥ 6654 (F) ≤ 6857: lowest quintile ≤ 4453 (F)	1.24 (0.56-1.92)	Age, height, BMI, attained age, smoking status
Martínez <i>et al</i> ^[16]	United States	12	30-55	89 448	501	Caloric intake	Colon cancer incidence	5th: highest quintile 1st: lowest quintile	1.18 (0.89-1.57)	Age
Harnack <i>et al</i> ^[17]	United States	9	55-69	33 976	355	Caloric intake	Esophageal, gastric, Pancreatic, colon Cancer incidence	≤ 1450 1451-1900 > 1900	1 0.69 (0.49-0.88) 0.73 (0.53-0.92)	Age, alcohol use, pack-years of smoking, yellow/orange vegetables, grains intake
Kato <i>et al</i> ^[18]	United States	7.1	34-65	14 727	100	Energy intake	Colorectal cancer incidence	Quintile 1 Quintile 2 Quintile 3 Quintile 4	1.0 1.16 (0.67-2.00) 0.85 (0.47-1.53) 1.20 (0.69-2.08)	Age, educational level, place at enrollment
Järvinen <i>et al</i> ^[21]	Finland	24	≥ 15	9959	109	Energy intake	Colorectal cancer incidence	4th: highest quintile 1st: lowest quintile	0.78 (0.42-1.44)	Age, sex, BMI, smoking, occupational group, geographical area
Stolzenberg-Solomon <i>et al</i> ^[23]	Finland	13	50-69	27 111	459	Energy intake	Gastric, pancreatic, colorectal cancer incidence	≤ 2155 > 2155 and ≤ 2541 > 2541 and ≤ 2917 > 2917 and ≤ 3410 > 3410	1 1.18 (0.78-1.58) 1.19 (0.76-1.85) 0.99 (0.62-1.35) 0.75 (0.42-1.09)	Age, BMI, educational level, calcium intake, smoking years, alcohol consumption, physical activity at work
Tiemersm <i>et al</i> ^[24]	Netherlands	8.5	20-59	> 36 000	102	Energy intake kJ/d	Colorectal cancer incidence	mean cases: 6895 mean controls: 6773		
Wong <i>et al</i> ^[25]	Singapore	7	45-74	63 257	482	Energy intake	Colorectal cancer incidence	mean cases: 1511 mean controls: 1492		
Friedenreich <i>et al</i> ^[26]	10 European countries	6.4	35-70	413 044	1693	Energy intake	Colorectal cancer incidence	< 1827 1827-2351 > 2351	1 0.91 (0.78-1.05) 0.90 (0.78-1.02)	Age, center, education, smoking, fiber
Prentice <i>et al</i> ^[27]	United States	12	50-79	80 816	561	Energy intake	Pancreatic, colorectal cancer incidence	Quartile 1 Quartile 2 Quartile 3 Quartile 4	1 1.19 (0.81-1.57) 1.18 (0.82-1.54) 1.47 (0.99-1.94)	

BMI: Body mass index; F: Female; M: Male.

longer and more severe (> 80% reduction in normal energy intake) energy restriction, such as European Jewish survivors exposed to the Holocaust^[31] or Russian survivors of the Siege of Leningrad^[32], show increased

risk of some cancers. The confounding effects of severe physical and psychosocial stress, malnutrition, infection, and other factors associated with war conditions make these studies challenging to interpret. However, based

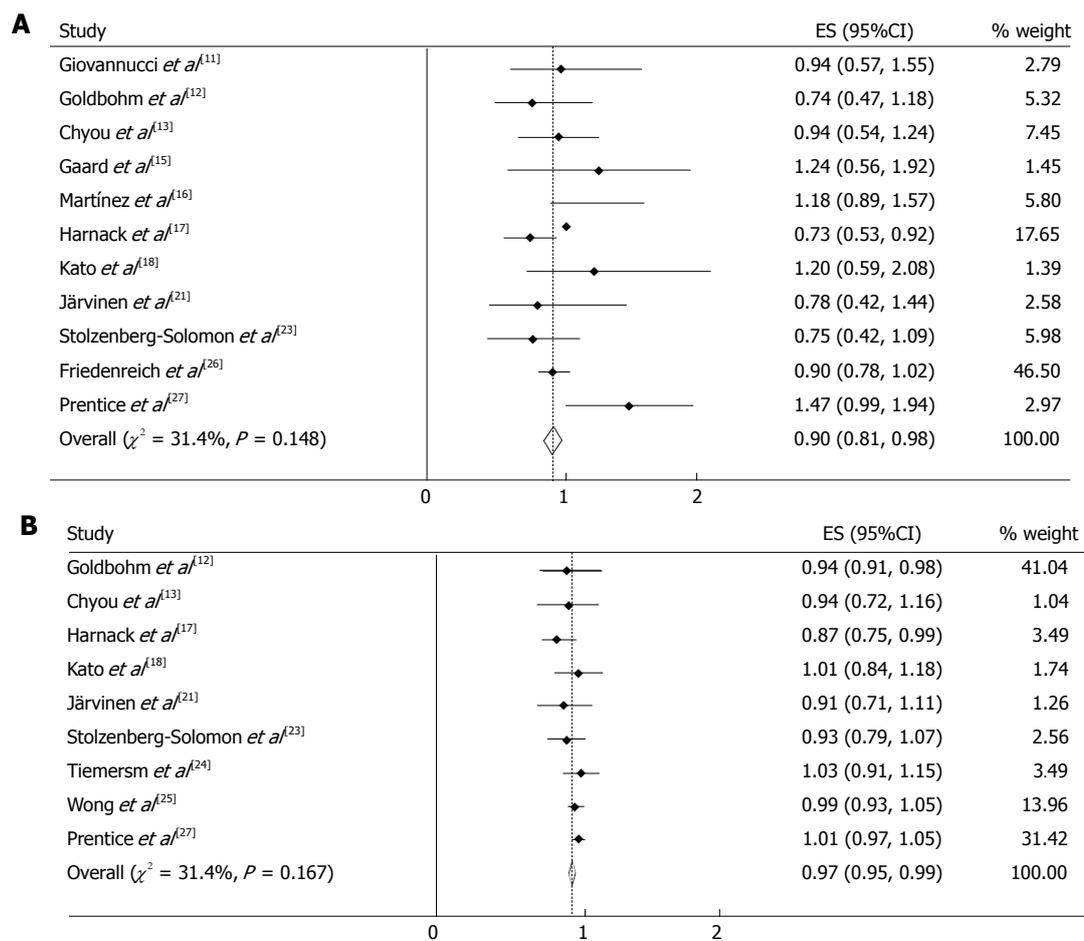


Figure 2 Summary relative risks of digestive cancers. A: The highest vs lowest category of energy intake from included cohorts; B: An increment of 1 MJ/day energy intake from included cohorts. Squares represent study-specific relative risk (RR) estimates (size of the square reflects the study-specific statistical weight, that is, the inverse of the variance); horizontal lines represent 95% CIs; diamonds represent summary RR estimates with corresponding 95% CIs.

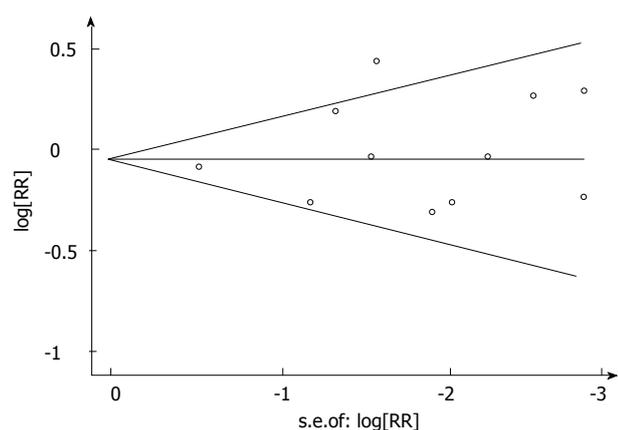


Figure 3 Publication bias in the studies. Begg's funnel plot indicating no publication bias in the studies included in this meta-analysis. No indication of publication bias was noted from both visualization of the funnel plot and Egger's test.

on data from animal and human studies, it seems clear that although CR typically decreases cancer risk, the anti-cancer effects associated with reduced energy intake can be neutralized or overcome in the presence of extreme stressors, such as what occurred during World War II.

The mechanisms responsible for CR-mediated beneficial effects on cancer are thought to involve metabolic

adaptations to CR itself, including (1) decreased production of growth factors and anabolic hormones^[33,34]; (2) decreased production of reactive oxygen species and modulation of the endogenous antioxidant systems that decrease oxidative stress and free radical-induced DNA damage^[35,36]; (3) decreased plasma concentrations of inflammatory cytokines and an increase in circulating corticosteroids, ghrelin, and adiponectin that results in reduced inflammation^[37-40]; and (4) protection against aging-associated deterioration in immunosurveillance^[41]. In addition, CR simultaneously affects multiple processes that are involved in cancer pathogenesis, including DNA repair processes, removal of damaged cells through apoptosis, autophagy, and protection from the effects of damaging agents (e.g., toxic and genotoxic compounds)^[42,43]. Many of the effects of CR are probably mediated by regulation of gene expression, including upregulation of tumor suppressor genes and genes promoting DNA and cellular repair, protein turnover, stress resistance and antioxidant genes, downregulation of proinflammatory genes, and modulation of energy metabolism pathways^[44,45]. Whether CR with adequate nutrition reduces the cancer incidence in humans is unknown, but data from studies of long-term CR suggest that the metabolic and physiological responses to CR in

humans are similar to those in rodents and monkeys^[46-49].

To further elucidate the relationship between energy intake and risk of digestive cancer at various sites, we performed subgroup analysis and noted that energy intake was associated with a reduced risk of colorectal cancer and an increased risk of gastric cancer. There appeared to be no association with esophageal or pancreatic cancer. Colorectal cancer is one of the most common cancers worldwide. Only one study assessed the association between energy restriction and colorectal cancer risk^[50]. This study observed no significant relationship between energy restriction early in life and subsequent colon carcinoma risk in men and women who had lived in a western city in 1944-1945 (hunger winter) in the Netherlands. Interestingly, of studies that have examined the relationship between energy intake and colon cancer, many prospective investigations have similarly found inverse associations with greater energy intake, whereas case-control studies have observed positive associations^[51-55].

A combined analysis of 13 case-control studies demonstrated a positive association with total energy intake in 11 of the 13 studies. The association was similar between men and women, between younger (< 50 years old) and older (> 50 years old) people, between colon and rectal cancer, and between right and left colon cancer sites^[54]. On the other hand, cohort studies have usually reported a weak or null association^[15,18]. In the study by Bostick *et al.*^[10], a decreasing risk of colon cancer with increasing total energy intake was seen following age-adjusted analysis. The RR comparing the highest quintile (> 2.238 kcal/d) with the lowest quintile (< 1.301 kcal/d) was 0.60 (95%CI 0.39-0.92). Martínez *et al.*^[16] reported a weak positive association between energy intake and colorectal cancer. The age-adjusted RR for the highest compared with the lowest quintile of energy intake was 1.18 (95%CI 0.89-1.57). Among 63 257 Asian participants followed for an average of 7 year in which 310 incident cases of colorectal cancer were identified, no significant difference was found between the median total caloric intake of patients with colorectal cancer (1494.0 kcal/d) and controls (1483.5 kcal/d)^[56].

In our meta-analysis, 10 cohorts were identified from Finland, the Netherlands, Norway, and the United States. The summary RR of colorectal cancer was 0.90 (95%CI 0.81-0.99) for the highest versus lowest category of energy intake. We observed an inverse association between energy intake and the risk of colorectal cancer. The inverse association observed in some of these prospective studies may be explained by the greater energy intake associated with energy expenditure from greater physical activity, which is protective against colon cancer^[57].

There were over 20 case-control studies concerning the relationship between total energy intake and the risk of gastric cancer. In all studies, total energy consumed during adulthood was assessed. Some studies reported a positive association between total energy intake and the risk of gastric cancer^[58-62]. Regarding cohort studies, Ahn *et al.*^[63] reported a approximately 60% decreased

risk of gastric cancer with increased total energy intake in Korea. Kasum *et al.*^[22] studied the association between energy intake and the risk of gastric cancer in postmenopausal women in the United States. Among 34 651 participants followed for an average of 14 years in which 56 incident cases of gastric cancer were identified, the summary RR of stomach cancer was 1.10 for an increment of 250 kcal/d energy intake. Our meta-analysis including two cohort studies suggested a significant positive relationship between energy intake and gastric cancer (RR = 1.19; 95%CI 1.08-1.31).

Over the past three decades, many studies have been conducted to examine the relationship between energy intake and pancreatic cancer. Harnack *et al.*^[17] found that in 33 976 postmenopausal women in the United States, the summary RR of pancreatic cancer for individuals with an energy intake of > 1900 kcal/d was 1.20 (95%CI 0.67-2.15) compared with those having an intake of < 1450 kcal/d. In 27, 111 male smokers in Finland, Stolzenberg-Solomon *et al.*^[23] studied the association between energy intake and the risk of pancreatic cancer. After following participants for an average of 10.2 years, 163 incident cases of exocrine cancer of the pancreas were identified. The RR comparing the highest quintile (> 3410 kcal/d) with the lowest quintile (< 2155 kcal/d) was 0.62 (95%CI 0.36-1.07). After a pooled analysis of three cohort studies, we found that the summary RR of pancreatic cancer was 0.79 (95%CI 0.49-1.09) for the highest versus lowest category of energy intake. Thus, there appears to be no obvious association between energy intake and pancreatic cancer risk.

Some limitations of this meta-analysis should be acknowledged. First, as in all observational studies of diet and disease, the possibility of bias and confounding factors cannot be excluded. However, cohort studies, which are less susceptible to bias because of the prospective design, also showed an inverse association between energy intake and risk of digestive cancers, suggesting that the finding is not likely attributable to recall and selection bias. Individual studies may have failed to adjust for potential known or unknown confounders. Second, energy intake in our study may be a marker for greater nutrient intake and better nutritional status, because energy is correlated with many nutrients, and their combined effect may also explain the protective association that we observed. Dietary data do not necessarily reflect absorbed or biologically active doses and may contain measurement error from nutritional assessment techniques and nutrient databases, and participants may have changed their diets since baseline. All these parameters may have attenuated risk estimates. Third, we extracted the risk estimates that reflected the greatest degree of the control potential confounders because it was difficult to obtain raw data from each study to conduct standardized adjustments. Therefore, the results based on adjustment for different confounders were likely different from those based on standardized adjustments. Finally, only published studies were included in our meta-analysis. Therefore, publication bias may have occurred,

although no publication bias was indicated from both visualization of the funnel plot and Egger's test.

This meta-analysis presents epidemiologic evidence about the relationship between energy intake and risk of digestive cancers. In summary, we observed an inverse association between energy intake and the risk of digestive cancers. High energy intake may increase the risk of gastric cancer and decrease that of colorectal cancer. However, because physical activity, body size, and metabolic efficiency are highly related to total energy intake and expenditure, it is difficult to assess a possible independent effect of energy intake on digestive cancer risk. More investigations are needed to determine the biological mechanism of the inverse relationship between energy intake and the incidence of digestive cancers.

COMMENTS

Background

An important discovery in recent years is that lifestyle and environmental factors affect cancer initiation, promotion and progression. Epidemiological studies strongly suggest that the majority of cancer deaths can be attributed to factors such as unhealthy diets, tobacco, alcoholism, infections, and occupational exposure. Recent reports of extended life span and delayed cancer development in response to calorie restriction (CR) in rhesus monkeys and observations that CR during the premenopausal years decreases postmenopausal breast cancer risk in women suggest that the anticancer effects of CR reported in rodent models extend to primates, including humans.

Research frontiers

Very few studies have assessed the relationship between CR and the risk of various cancer sites because of ethical issues. One Dutch famine study and subsequent overall cancer incidence found no evidence that the short famine affected overall cancer risk. However, higher energy intake in childhood may increase the risk of developing cancer in adulthood. Data from case-control studies may be subject to recall bias with respect to energy intake and to selection bias with respect to the control group. Prospective cohort studies excluding those biases would be more useful for observing energy-cancer associations.

Innovations and breakthroughs

This meta-analysis presents epidemiologic evidence about the relationship between energy intake and risk of digestive cancers. They observed an inverse association between energy intake and the risk of digestive cancers. High energy intake may increase the risk of gastric cancer and decrease that of colorectal cancer. However, because physical activity, body size, and metabolic efficiency are highly related to total energy intake and expenditure, it is difficult to assess a possible independent effect of energy intake on digestive cancer risk. More investigations are needed to determine the biological mechanism of the inverse relationship between energy intake and the incidence of digestive cancers.

Applications

The study results suggest that an inverse association between energy intake and the risk of digestive cancers. High energy intake may increase the risk of gastric cancer and decrease that of colorectal cancer. They could prevent digestive cancers by controlling energy intake.

Terminology

CR is an experimental mode in which test animals receive a lower-calorie diet than ad libitum-fed controls. It has emerged as the most potent, broadly acting dietary intervention for preventing carcinogenesis in rodent models of cancer.

Peer review

This manuscript presents a meta-analysis of selected studies about the effects of energy intake on risk of digestive cancer. It is well designed with the use of only prospective studies. Appropriate statistical methods are used for each reported meta-analysis for the assessment of effects on outcome.

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Itopride therapy for functional dyspepsia: A meta-analysis

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Abstract

AIM: To evaluate the therapeutic effects of itopride vs other drugs (placebo, domperidone, mosapride) for functional dyspepsia (FD).

METHODS: Randomized controlled trials (RCTs) of itopride for FD were retrieved from databases. Relevant information was extracted and analyzed, using the relative risk (RR) and weighted mean deviation, as appropriate. A random or fixed effect model was used, based on the heterogeneity of the included articles, and visual inspection of funnel plots was used to evaluate publication bias.

RESULTS: Nine RCTs enrolling 2620 FD cases were included; 1372 cases received itopride treatment and 1248 cases received placebo or other drugs (control groups). Compared with control groups, itopride had superior RR values of 1.11 [95%CI: (1.03, 1.19), $P = 0.006$], 1.21 [95%CI: (1.03, 1.44), $P = 0.02$], and

1.24 [95%CI: (1.01, 1.53), $P = 0.04$] for global patient assessment, postprandial fullness, and early satiety, respectively. For the Leeds Dyspepsia Questionnaire score, the weighted mean deviation was -1.38 [95%CI: (-1.75, -1.01), $P < 0.01$]. The incidence of adverse effects was similar in the itopride and control groups. The funnel plots for all indicators showed no evidence of publication bias.

CONCLUSION: Itopride has good efficacy in terms of global patients assessment, postprandial fullness, and early satiety in the treatment of patients with FD and shows a low rate of adverse reactions. Itopride can greatly improve FD syndromes-score.

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Key words: Itopride; Functional dyspepsia; Meta-analysis; Randomized controlled trials; Prokinetic agents

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INTRODUCTION

Functional dyspepsia (FD) is a common, functional gastrointestinal disorder^[1]. In a multi-centre Asian study of 1115 patients with uninvestigated dyspepsia (UD) (Rome II criteria) from nine countries, 43% turned out to have FD after investigation^[2]. FD places a heavy financial burden on society^[3,4]. Globally the majority of patients suffering from dyspepsia, which account for approximately

5% of primary care, fall into the category of FD^[5]. FD is a complex problem resulting from the interaction of gastric dysmotility^[6,7], visceral hypersensitivity, and psychological factors, and causes delayed gastric emptying, abnormal gastric regulation, and aberrant myoelectricity. As many as 60% of FD patients have gastric dysmotility. Outcomes of drug therapy [including Chinese herbal medicines, antidepressant drugs, proton pump inhibitors (PPI), and *Helicobacter pylori* (*H. pylori*) eradication] for FD patients have not been satisfactory^[8-10] compared with placebos. Although prokinetic agents have been proven to improve symptoms in FD patients by reducing gastroesophageal reflux, promoting gastric emptying, and improving gastric regulation, metoclopramide is associated with a high incidence of central nervous system (CNS)-related adverse drug reactions (ADRs), domperidone can elevate serum prolactin levels and cause gynecomastia and galactorrhea, and cisapride has been withdrawn because of safety concerns including high risk of prolonging the QT interval and severe arrhythmias^[11].

Itopride, a novel prokinetic agent, works by antagonizing dopamine D2-receptors and inhibiting acetylcholinesterase^[12]. It does not cause any CNS-related ADRs because its high polarity does not allow it to cross the blood-brain barrier, it barely elevates prolactin levels and does not prolong the Q-T interval^[5]. In a multicentre, randomised, double-blind, placebo-controlled trial, itopride significantly improved symptoms in patients with FD, and showed a greater response rate than placebo^[13]. However, it was recently reported that itopride was no more effective in showing a difference in symptom response from placebo in FD^[5]. Therefore, given the conflicting results for efficacy in some study reports and the possible serious adverse reactions (SARs) of itopride, a meta-analysis of randomised controlled trial (RCT) data published prior to December 2011 was conducted, with a view to evaluating more objectively the efficacy and safety of itopride in the treatment of FD.

MATERIALS AND METHODS

Inclusion and exclusion criteria

Inclusion criteria for the studies used in the analysis required that they: (1) contained inclusion and exclusion criteria, and the study design was an RCT with a quality level above B; (2) were designed to study FD as the target population; (3) had a study group that was given itopride and a control group that was given placebo, domperidone, or mosapride, *etc.*; and (4) included one or more of the following indicators for comparison of efficacy between itopride and other therapy: Global patient assessment (GPA), postprandial fullness, early satiation, epigastric discomfort, adverse reaction, and the Leeds Dyspepsia Questionnaire (LDQ) score. Studies were excluded that had: (1) incomplete data; (2) been re-published (only those with credible data were chosen); (3) a control group that used itopride together with other drugs; (4) patients with obvious organic diseases such as

gastritis, peptic ulcer, and cholecystitis, *etc.*; and (5) baseline data that were not similar.

Literature search and data collection

Databases searched included the Cochrane Library, PubMed, Elsevier, EMBASE, ISI, CNKI, VIP Chinese Scientific and Technological Periodical Database and Wanfang Data, prior to December 2011. Search terms and search strategy included: “itopride”, “functional dyspepsia”, “randomized or random or randomly or randomised”, “controlled trial”, “Yi Tuo Bi Li” (the Chinese character for “itopride”), “Gong Nen Xing Xiao Hua Bu Liang” (the Chinese character for “functional dyspepsia”), “sui ji dui zhao” (the Chinese character for “randomized control”), excluding studies involving children or pregnant women, as well as review papers. Meanwhile, articles published in core journals in China and abroad this year, such as *Chinese Journal of Digestion*, *Chinese Journal of Internal Medicine*, *Chinese Journal of Gastroenterology*, *Gastroenterology*, and *Gut* were searched manually. Conference papers published this year were also consulted, along with the references of the included articles, so as to include studies that may have been omitted. Extracted data included outcome measures, risk of bias and characteristics of trials, patients, and interventions. Authors of included trials were approached for additional information when necessary. The articles were screened by two reviewers independently, according to the steps for preliminary screening and full-text screening, and any differences were settled through discussions by the reviewers themselves or with assistance from a third party.

Quality evaluation

Study quality was evaluated according to the quality evaluation criteria recommended in the Cochrane Reviewers' Handbook 4.2.2. Briefly, the quality of a study was rated A, B, or C based on its randomization method, allocation concealment, double-blind method, missing follow-up, and withdrawal from observation. Grade A completely conforms to the four quality standards and has the lowest possibility of bias. Grade B partially conforms to one or more quality standards and shows moderate possibility of bias. Grade C does not conform to any of the four quality standards and has a high possibility of bias.

Data analysis

Revman 5.0 (the Cochrane collaboration; <http://www.cochrane.org/>) was used for statistical analysis of the data. Relative risk (RR) was used to test the heterogeneity of such numerical data as GPA, epigastric fullness, early satiation, epigastric discomfort, and adverse reactions between the two groups of each study. Weighted mean deviation (WMD) was used for statistical analysis of the LDQ scores, and the effect variables were expressed by 95% confidence intervals. Statistical assessment was then performed using a χ^2 test of homogeneity and evaluation of the inconsistency index (I^2) statistic. The I^2 statistic is defined as the percentage of variability caused by hetero-

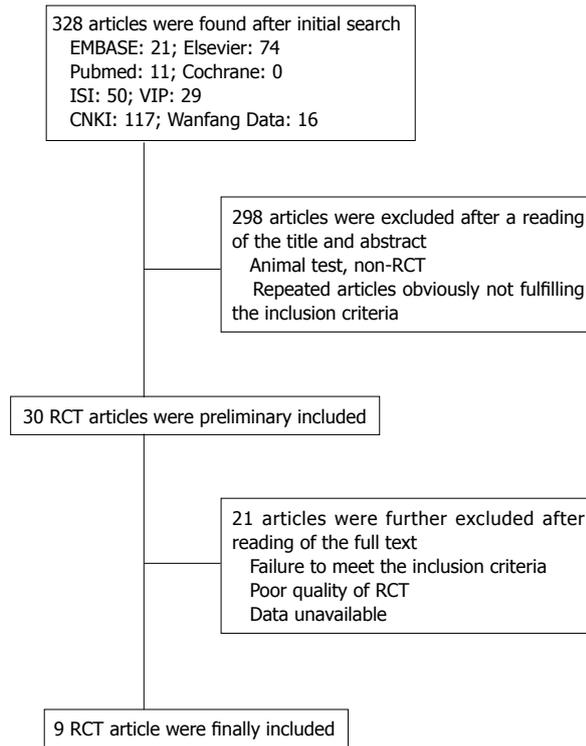


Figure 1 Flow chart of article inclusion and screening. RCT: Randomized controlled trial.

genity rather than chance with values $> 50\%$ representing the possibility for substantial heterogeneity. A fixed effect model was used to estimate the overall effect if RR was homogenous; if RR was non-homogenous, a random effect model was used.

Publication bias

Funnel plots were drawn using the RR values of each of GPA, epigastric fullness, early satiation, epigastric discomfort, and adverse reactions of the two groups included in the meta-analysis as the X coordinate and the standard error (SE) (log RR) as the Y coordinate, as well as using the mean deviation (MD) of LDQ scores as the X coordinate and the SE (MD) as the Y coordinate, after which the symmetry of the plots was observed to evaluate the impacts of publication bias. Subgroup analyses were performed to evaluate intervention effects in trials comparing itopride *vs* placebo or other prokinetic agents, trials with adequate bias control (assessed through randomization methods) and publication status.

RESULTS

Results of the literature search and information on included studies

328 articles were collected; 319 were excluded for not meeting the inclusion criteria, nine RCT articles^[5,13-20] were finally included, as shown in Figure 1. Of the included RCT articles, seven were graded as grade B and two as grade A. Included studies contained a total of 2620 patients, 1372 of whom received itopride, and 1248

received placebo or other control drugs. Table 1 shows the basic characteristics of the studies included.

Analysis results of efficacy indicators

GPA: Six RCT articles^[5,13,14,18-20] reported the GPA of itopride in FD patients, of which three were domperidone-controlled, one was mosapride-controlled, and the other two were placebo-controlled. The chi-square value of the test for heterogeneity was 13.69, with an I^2 value of 49%, indicating that there was homogeneity of effects among the trials. Therefore, a fixed effect model was used, and the calculated RR value was 1.11 [95%CI: (1.03, 1.19), $P = 0.006$], as shown in Figure 2A. Itopride improved the GPA of FD patients more significantly than control groups.

Postprandial fullness: Four RCT articles^[15-17,20] reported the efficacy of itopride with respect to postprandial fullness of FD patients, all of which were domperidone-controlled. The chi-square value of the test for heterogeneity was 6.09, with an $I^2 = 51\%$, indicating that there was heterogeneity of effects among the trials. Therefore, a random effect model was used, and the calculated RR value was 1.21 [95%CI: (1.03, 1.44), $P = 0.02$], as shown in Figure 2B. Itopride improved the postprandial fullness of FD patients more significantly than domperidone.

Early satiation: Four RCT articles^[15-17,20] reported the efficacy of itopride with respect to early satiation of FD patients, all of which were domperidone-controlled; the chi-square value of the test for heterogeneity was 9.18, with a $I^2 = 67\%$, indicating that there was heterogeneity of effects among the trials. Therefore, a random effect model was used, and the calculated RR value was 1.24 [95%CI: (1.01, 1.53), $P = 0.04$]. Compared with domperidone, itopride improved the early satiation of FD patients more significantly.

Epigastric discomfort: Three RCT articles^[15,16,20] reported the efficacy of itopride with respect to epigastric discomfort of FD patients, all of which were domperidone-controlled; the chi-square value of the test for heterogeneity was 2.67, with a $I^2 = 25\%$, indicating that there was homogeneity of effects among the trials. Therefore, a fixed effect model was used, and the calculated RR value was 1.00 [95%CI: (0.88, 1.14), $P = 0.98$]. Itopride and domperidone had similar efficacy on epigastric discomfort of FD patients.

LDQ: Two RCT articles^[5,13] reported that itopride improved the LDQ scores of FD patients, both of which were placebo-controlled; the chi-square value of the test for heterogeneity was 18.53, and $I^2 = 84\%$, indicating that there was heterogeneity of effects between the trials. Therefore, a random effect model was used, and the calculated WMD value was -1.38 [95%CI: (-1.75, -1.01), $P < 0.01$]. Thus, itopride improved the LDQ scores of FD patients more significantly than placebo.

Table 1 Clinical data of included articles

Ref.	Year	Quality grade	Total cases	Duration of therapy (wk)	Treatment group			Control group		
					Cases (male/female)	Average age (yr)	Itopride dosing regimen	Cases (male/female)	Average age (yr)	Dosing regimen
Zhou <i>et al</i> ^[14]	2000	B	208	2	105	43	50 mg <i>tid</i>	103	46	Domperidone 10 mg <i>tid</i>
Sun <i>et al</i> ^[15]	2003	B	232	2	115	-	50 mg <i>tid</i>	117	-	Domperidone 10 mg <i>tid</i>
Mo <i>et al</i> ^[16]	2003	B	80	2	40	-	50 mg <i>tid</i>	40	-	Domperidone 10 mg <i>tid</i>
Chen <i>et al</i> ^[17]	2004	B	42	4	21	35	50 mg <i>tid</i>	21	36	Domperidone 10 mg <i>tid</i>
Amarapurkar <i>et al</i> ^[18]	2004	B	60	2	30 (19/11)	45	50 mg <i>tid</i>	30 (11/19)	40	Mosapride 5 mg <i>tid</i>
Zhu <i>et al</i> ^[19]	2005	B	236	4	119	-	50 mg <i>tid</i>	117	-	Domperidone 10 mg <i>tid</i>
Li <i>et al</i> ^[20]	2005	B	200	4	100 (47/53)	38	50 mg <i>tid</i>	100 (47/53)	38	Domperidone 10 mg <i>tid</i>
Holtmann <i>et al</i> ^[13]	2006	A	412	8	50 mg: 135 (48/87) 100 mg: 135 (57/78)	47	50 mg <i>tid</i> 100 mg <i>tid</i>	142 (53/89)	49	Placebo
Talley <i>et al</i> ^[5] , INT	2008	A	524	8	264 (86/178)	43	100 mg <i>tid</i>	260 (99/161)	43	Placebo
Talley <i>et al</i> ^[5] , NOR	2008	A	626	8	308 (109/199)	43	100 mg <i>tid</i>	318 (96/222)	43	Placebo

Incidence of ADRs: Eight RCT articles^[13-20] reported the ADRs of itopride in the treatment of FD patients, of which six were domperidone-controlled, one was mosapride-controlled, and the other one was placebo-controlled; the chi-square value of the test for heterogeneity was 4.51, with a $I^2 = 0\%$, indicating that there was homogeneity of effects among the trials. Therefore, a fixed effect model was used, and the calculated RR value was 0.96 [95%CI: (0.78, 1.17), $P = 0.67$], as shown in Figure 2C. Analysis of the sub-groups showed that itopride did not have a higher incidence of ADRs than domperidone, mosapride, or placebo.

Analysis of publication bias

As compared with the control groups, itopride's funnel plots of GPA, postprandial fullness, early satiation, epigastric discomfort, and ADR all showed a symmetrical shape that was narrow at the top and wide at the bottom, indicating that there was no publication bias.

DISCUSSION

The pathogenesis of FD is far from fully understood, but gastrointestinal motility and visceral sensitivity are proven to play very important roles^[21,22] in the occurrence of FD symptoms. Clinically, prokinetic agents, such as domperidone, cisapride, and mosapride, are often used to treat these patients. Recently a meta-analysis by Hiyama^[23] showed a significant treatment benefit in favour of prokinetic agents in patients with FD. However, in that study, itopride is rarely involved. Given the concern for safety and efficacy of the existing prokinetic agents, a novel agent that is safer and more effective is urgently needed. Itopride is a prokinetic agent that has a completely different mechanism of action from existing ones; it works by both antagonizing dopamine receptors and inhibiting the activity of acetylcholinesterase. It not only stimulates release of acetylcholine, but also inhibits its degradation, thus promoting gastrointestinal motility. There are a few well-designed RCTs on the efficacy of itopride in

the treatment of FD, and the reported efficacy was controversial. Therefore, a meta-analysis of previously published high quality RCTs was conducted.

In the present study, when compared with the control groups, the RRs of itopride for GPA, postprandial fullness, and early satiation of FD patients indicate that this drug could significantly improve the GPA scores, postprandial fullness, and early satiation in FD patients. However, it did not improve epigastric discomfort more significantly than the comparator, which could be a result of itopride's action of increasing postprandial gastric receptive relaxation^[24] and gastrointestinal motility^[20]. To further evaluate the efficacy of itopride in improving the symptoms of FD patients, the LDQ was used to evaluate FD patients' symptoms at baseline and after treatment, and the calculated WMD was -1.38 [95%CI: (-1.75, -1.01), $P < 0.01$], suggesting that the drug could significantly reduce the LDQ scores of FD patients, which made the results more convincing. As for safety, it showed that the incidence of ADRs was no higher for itopride than for domperidone, mosapride, or placebo. The ADRs attributed to itopride were mainly abdominal pain and diarrhoea, which were all mild to moderate, without clinically related changes in the electrocardiogram, particularly prolongation of QT intervals. This appears to be different from other prokinetic agents, possibly because the polarity of itopride largely prevents it from entering the brain or the CNS^[25]. In addition, as compared with other dopamine receptor antagonists, itopride caused a much lower incidence of CNS-related ADRs and hyperprolactinaemia while keeping dopamine active. Meanwhile, there were fewer drug interactions of itopride compared with other prokinetic agents^[26], probably because itopride is metabolized by a monooxygenase, while mosapride and other prokinetics are metabolized by cytochrome P450, as reported by Mushiroda^[26].

Considering the discrepancy in contradictory trial results^[12,20], study design issues are important. There were several probable reasons, including heterogeneity of the conditions and differences in patient selection.

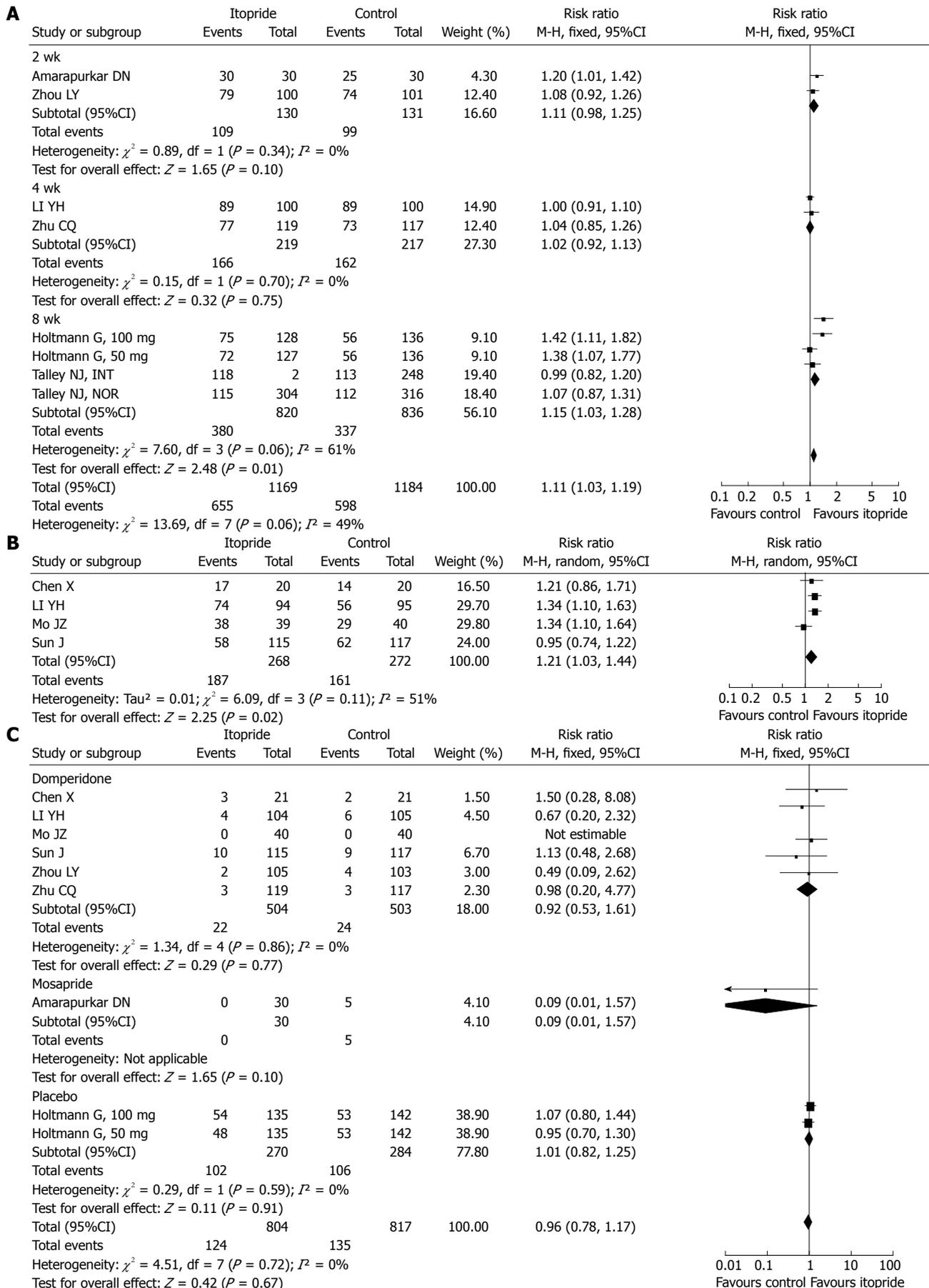


Figure 2 Forest plot for global patient assessment (A), postprandial fullness (B) and adverse reactions (C) with itopride treatment for functional dyspepsia.

In the Tally's trial, the requirement that all patients had to be *H. pylori* negative, exclusion of heartburn and that the LDQ score needed to be > 9 at baseline meant high intensity scores for the typical symptoms of pain and fullness were needed for LDQ, all of which might contribute to the high placebo response rate^[12]. On the other hand, the majority of dyspeptic subjects overlap with heartburn symptoms as well as *H. pylori* infection, and heartburn also is a predictor of response, so the exclusion criteria were much stricter in the Tally's study, as Veldhuyzen mentioned^[27].

This meta-analysis covered a wide range of high-quality articles, and all studies included were randomized controlled trials RCTs. In addition, the diagnostic criteria for inclusion of articles were uniform. Considering publication bias, that is, the disproportionate publication of research articles with a positive result than of those with a negative result, an effort was made to collect as full a range of related literature as possible through many different approaches (including computer search, manual search, and literature tracing), and repeated publications were excluded. All nine studies included in this analysis had definitive inclusion criteria and baseline descriptions of sex, age, disease severity, and concomitant medications of the population included, and the ratios of the population in the study groups and the control groups were reasonable.

However, the present study did have some limitations. Firstly, the ethnic groups of the populations in the articles were varied. Race and/or western lifestyle are important risk factors^[28,29]. Secondly, because of differences in trial design, comparators used for the control group, and follow-up, there was a large degree of heterogeneity among the studies included, as well as in the GPA, early satiation, and LDQ scores. For this reason, a random effect model was used for the meta-analysis, which probably affected the results of the evaluation. Thirdly, *Helicobacter pylori* may play a role in pathogenesis of functional dyspepsia^[1]. However, seven of the FD trials included in this meta-analysis were from Asia, which has a higher prevalence of Hp, and this probably affected the results.

In summary, the results of this meta-analysis suggest that itopride has therapeutic benefits with respect to GPA, postprandial fullness, early satiation, and the LDQ of FD patients, with a lower incidence of ADRs. However, because of the existence of heterogeneity, further studies of more high-quality RCTs with consistent indicators are probably warranted to validate the safety and efficacy of itopride.

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COMMENTS

Background

Functional dyspepsia (FD) is a complex problem resulting from the interac-

tion of gastric dysmotility, visceral hypersensitivity, and psychological factors, which causes delayed gastric emptying, abnormal gastric regulation, and aberrant myoelectricity. As many as 60% of FD patients have gastric dysmotility. Itopride works by antagonizing dopamine D2-receptors and inhibiting acetylcholinesterase. In a multicentre, randomised, double-blind, placebo-controlled trial, itopride significantly improved symptoms in patients with FD, and showed a greater rate of response than placebo. However, it was recently reported that itopride was not more effective in showing a difference in symptom response from placebo in FD. Therefore, it is necessary to perform a comprehensive meta-analysis to evaluate more objectively the efficacy and safety of itopride in the treatment of FD.

Research frontiers

Although prokinetic agents are proven to improve symptoms in FD patients, metoclopramide is associated with a high incidence of central nervous system-related adverse drug reactions, domperidone can elevate serum prolactin levels and cause gynecomastia and galactorrhea, and cisapride has been withdrawn due to safety concerns including high risk of prolonging the QT interval and severe arrhythmias. It is essential to search for more effective and safe drugs.

Innovations and breakthroughs

The study comprehensively searched for all randomised controlled trials involving itopride in the treatment of FD, and used meta-analysis to analyze the effects and safety of itopride.

Applications

The results indicate that itopride has therapeutic benefits with respect to Global Patient Assessment, postprandial fullness, early satiation, and the Leeds Dyspepsia Questionnaire of FD patients, with a lower incidence of adverse drug reactions.

Peer review

The author investigated the efficacy of itopride for functional dyspepsia in a meta-analysis. The article is overall easy to understand, and the method of meta-analysis is correct. The results are interesting and suggest that itopride shows good efficacy for the treatment of global patients assessment, postprandial fullness, and early satiety in patients with FD.

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Effect of dietary fiber on constipation: A meta analysis

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Abstract

AIM: To investigate the effect of dietary fiber intake on constipation by a meta-analysis of randomized controlled trials (RCTs).

METHODS: We searched Ovid MEDLINE (from 1946 to October 2011), Cochrane Library (2011), PubMed for articles on dietary fiber intake and constipation using the terms: constipation, fiber, cellulose, plant extracts, cereals, bran, psyllium, or plantago. References of important articles were searched manually for relevant studies. Articles were eligible for the meta-analysis if they were high-quality RCTs and reported data on stool frequency, stool consistency, treatment success, laxative use and gastrointestinal symptoms. The data were extracted independently by two researchers (Yang J and Wang HP) according to the described selection criteria. Review manager version 5 software was used for analysis and test. Weighted mean difference with 95%CI was used for quantitative data, odds ratio (OR)

with 95%CI was used for dichotomous data. Both I^2 statistic with a cut-off of $\geq 50\%$ and the χ^2 test with a P value < 0.10 were used to define a significant degree of heterogeneity.

RESULTS: We searched 1322 potential relevant articles, 19 of which were retrieved for further assessment, 14 studies were excluded for various reasons, five studies were included in the analysis. Dietary fiber showed significant advantage over placebo in stool frequency (OR = 1.19; 95%CI: 0.58-1.80, $P < 0.05$). There was no significant difference in stool consistency, treatment success, laxative use and painful defecation between the two groups. Stool frequency were reported by five RCTs, all results showed either a trend or a significant difference in favor of the treatment group, number of stools per week increased in treatment group than in placebo group (OR = 1.19; 95%CI: 0.58-1.80, $P < 0.05$), with no significant heterogeneity among studies ($I^2 = 0$, $P = 0.77$). Four studies evaluated stool consistency, one of them presented outcome in terms of percentage of hard stool, which was different from others, so we included the other three studies for analysis. Two studies reported treatment success. There was significant heterogeneity between the studies ($P < 0.1$, $I^2 > 50\%$). Three studies reported laxative use, quantitative data was shown in one study, and the pooled analysis of the other two studies showed no significant difference between treatment and placebo groups in laxative use (OR = 1.07; 95%CI 0.51-2.25), and no heterogeneity was found ($P = 0.84$, $I^2 = 0$). Three studies evaluated painful defecation: one study presented both quantitative and dichotomous data, the other two studies reported quantitative and dichotomous data separately. We used dichotomous data for analysis.

CONCLUSION: Dietary fiber intake can obviously increase stool frequency in patients with constipation. It does not obviously improve stool consistency, treatment success, laxative use and painful defecation.

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Key words: Dietary fiber; Constipation; Meta-analysis; Stool frequency; Stool consistency

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Yang J, Wang HP, Zhou L, Xu CF. Effect of dietary fiber on constipation: A meta analysis. *World J Gastroenterol* 2012; 18(48): 7378-7383 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i48/7378.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i48.7378>

INTRODUCTION

Constipation is a health problem that influences almost 20% of the world's population^[1]. It is a bothersome disorder which negatively affect the quality of life and increase the risk of colon cancer^[2]. There are a wide-range of treatment methods. Life-style modification, such as increased fluid intake or exercise, are usually recommended as first-line treatment, but data on the effectiveness of these measures are limited^[3]. Laxatives are most commonly used for treatment of constipation, but frequent use of these drugs may lead to some adverse effects^[4,5], alternative treatment measure is, therefore, needed. Soluble fiber absorbs water to become a gelatinous, viscous substance and is fermented by bacteria in the digestive tract. Insoluble fiber has a bulking action^[6]. Dietary fiber is the product of healthful compounds and has demonstrated some beneficial effect. Increase of dietary fiber intake has been recommended to treat constipation in children and adults^[7-9]. In a large-population case-control study, Rome found that dietary fiber intake was independently negatively correlated with chronic constipation, despite the age range and the age at onset of constipation^[10]. Although there have been several randomized controlled trials (RCTs) studying the relationship between dietary fiber and constipation, no definitive quantitative summary is available, therefore we conducted a meta-analysis of RCTs, and report it below.

MATERIALS AND METHODS

Data selection

We searched Ovid MEDLINE (from 1946 to October 2011), Cochrane Library (2011) and PubMed to identify RCTs studying dietary fiber and constipation. We used the following terms: constipation as medical subject headings and free text terms, which were combined with fiber, cellulose, plant extracts, cereals, bran, psyllium or plantago. References of important articles were searched manually for relevant studies.

Study selection

Studies were included if they met the following criteria: (1) studies investigating the association between the intake

of dietary fiber and constipation; (2) RCTs with a trial quality greater than or equal to 3 points judged by Jadad score; (3) constipation was defined by symptoms according to the Roma criteria or clinical diagnosis; (4) studies reporting at least one of the following data: stool frequency, stool consistency, treatment success, laxative use, gastrointestinal symptom; and (5) dietary fiber was used as the only active intervention in treatment group.

Data extraction

The data were extracted independently by two researchers (Yang J and Wang HP) according to the described selection criteria. Disagreement was resolved by discussion with the third person. The following data were extracted: the first author's name, year of publication, study design, interventional method, study period, sample size, outcomes, method used to generate the randomization, level of blinding, withdrawn or drop-outs explanations.

Statistical analysis

Review manager version 5 software was used for all meta-analyses and tests for heterogeneity. Weighted mean difference with 95%CI was used for quantitative data, odds ratio with 95%CI was used for dichotomous data. Both I^2 statistic with a cut-off of $\geq 50\%$ and the χ^2 test with a P value < 0.10 were used to define a significant degree of heterogeneity. Random-effects model was applied. A two-tailed $P < 0.05$ was considered statistically significant.

RESULTS

We searched 1322 potential relevant articles, 19 of which were retrieved for further assessment, and 14 studies were excluded for the reasons as shown in Figure 1. As a result, five studies were included, and the characteristics of the included studies are listed in Table 1.

Stool frequency was reported by five RCTs^[11-15]. Results showed either a trend or a significant difference in favor of the treatment group, and an increased number of stools per week in treatment group compared with the placebo group [odds ratio (OR) = 1.19; 95%CI: 0.58-1.80, $P < 0.05$], with no significant heterogeneity among the studies ($I^2 = 0$, $P = 0.77$). Of note, stool frequency was expressed as median (interquartile range) in Chmielewska's study^[14], and we used the formula to transform it into mean \pm SD (Figure 2A)^[16].

Four studies evaluated stool consistency^[12-15], one of them presented outcome in terms of percentage of hard stool^[13], which was different from others, so we included the other three studies for analysis. Results showed no statistical difference between two groups (OR = 0.43; 95%CI: -0.24-1.11, $P > 0.05$), however substantial heterogeneity existed ($P < 0.1$, $I^2 > 75\%$) (Figure 2B).

Two studies reported treatment success^[12,14]. The pooled analysis (Figure 2C) for overall results found significant difference between groups (OR = 2.21; 95%CI: 0.35-12.69, $P > 0.05$). There was significant heterogeneity between stud-

Table 1 Basic characteristics of included studies

Study	Trial design	No. of patients	Interventional method	Duration (wk)	Randomized allocation/double-blind/description of withdrawn and dropouts	Jadad score
Badiali <i>et al</i> ^[11]	Double-blind crossover	24 (adults)	Bran 20 g (fiber 12.5 g) vs placebo	4	Y/Y/Y	4
Loening-Baucke <i>et al</i> ^[12]	Double-blind crossover	31 (children)	Glucmannan 100 mg/kg vs placebo	4	Y/Y/Y	5
Castillejo <i>et al</i> ^[13]	Double-blind	48 (children)	Fiber supplement 5.2 g (53.2% fiber) 2 or 4 sachets vs placebo	4	Y/Y/Y	5
Chmielewska <i>et al</i> ^[14]	Double-blind	72 (children)	Glucmannan 2.52 g vs placebo	4	Y/Y/Y	5
Staiano <i>et al</i> ^[15]	Double-blind	20 (children)	Glucmannan 200 mg/kg vs placebo	12	Y/Y/Y	4

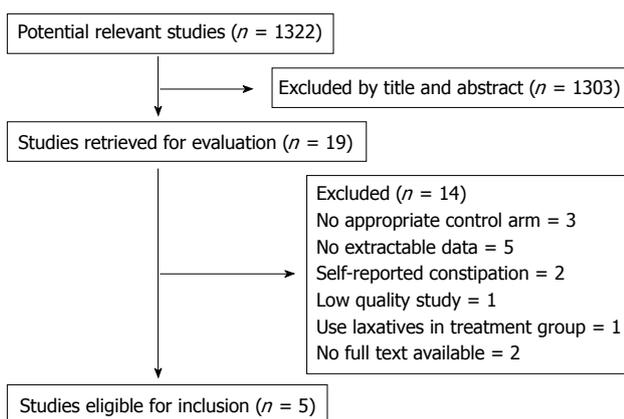


Figure 1 Study selection step.

ies ($P < 0.1$, $I^2 > 50\%$).

Three studies reported laxative use^[12,14,15], quantitative data was shown in one study^[15], and the pooled analysis (Figure 2D) of the other two studies showed no significant difference between treatment and placebo groups in laxative use (OR = 1.07; 95%CI: 0.51-2.25, $P = 0.85$), and no heterogeneity was present ($P = 0.84$, $I^2 = 0\%$).

Three studies evaluated painful defecation^[11,14,15]: one study presented both quantitative and dichotomous data^[14], the other two studies separately reported quantitative and dichotomous data^[11,15]. Evaluation method for quantitative data was different, one used actual frequency, the other two used frequency of occurrence (often/occasional/none). We used dichotomous data for analysis. The pooled estimate (Figure 2E) showed a nonsignificant trend in favor of treatment group (OR = 0.54; 95%CI: 0.15-1.91, $P = 0.34$). No statistically significant heterogeneity was present, but I^2 was moderate ($P > 0.1$, $I^2 = 43\%$).

DISCUSSION

This meta-analysis shows that the number of stools was increased significantly in dietary fiber group. Results demonstrated either a trend or a significant difference in favor of dietary fiber group. As for stool consistency, the overall results showed a trend in favor of fiber group, but no statistical difference was found. The substantial heterogeneity may influence the results, and the heterogeneity may be caused by different rating scale, ranging

from 0 to 4, from 0 to 5 and from 0 to 7 in three included studies, respectively. Although the rating sequence is unanimous, with a higher score indicating looser stools, different scale range may still influence the final results. Stool consistency was present as hard stool percentage in another included study^[13], 41.7% and 75% of the patients who received dietary fiber or placebo, respectively, reported hard stools, the percentage being obviously lower in the dietary fiber group.

Two high-quality RCTs compared dietary fiber with lactulose for treatment of constipation^[17,18], and found that dietary fiber and lactulose achieved comparable results in the treatment of childhood constipation. Dietary fiber is as effective as lactulose in improving stool frequency, stool consistency and treatment success, however, no difference was observed in treatment success between dietary fiber and placebo group in our meta-analysis. The possible reason was discussed. Constipation condition was more severe in the patients in Chmielewska's study, who had a lower baseline stool frequency per week and a higher percentage of hard stool than the studies mentioned above^[17,18] and the other study^[12] used for analysis. It suggests that dietary fiber may not be so effective in severe constipation and can only be used in mild to moderate constipation, or perhaps the dosage of glucmannan (2.52 g/d) used in Chmielewska's study is not high enough to exert effect. Nurko *et al*^[19] suggested that behavior modification, such as parental positive reinforcement and good patient-doctor relationship, may have impact on the treatment outcome. So the effect of dietary fiber on different grades of constipation should be explored in further studies and it is also important to balance the behavior factors in comparison.

Dichotomous data was used to analyze the laxative use in our study, which can only reflect how many patients used the laxatives, but not indicate how often it was used. Quantitative data about laxative use can better reflect the degree of dependence. If the time when laxative was used is described, the outcome will be more useful for overall analysis.

Gastrointestinal symptoms were reported by several studies. Because data was presented by different methods, only painful defecation was analyzed, and results showed that there was no significant difference between dietary fiber and placebo groups.

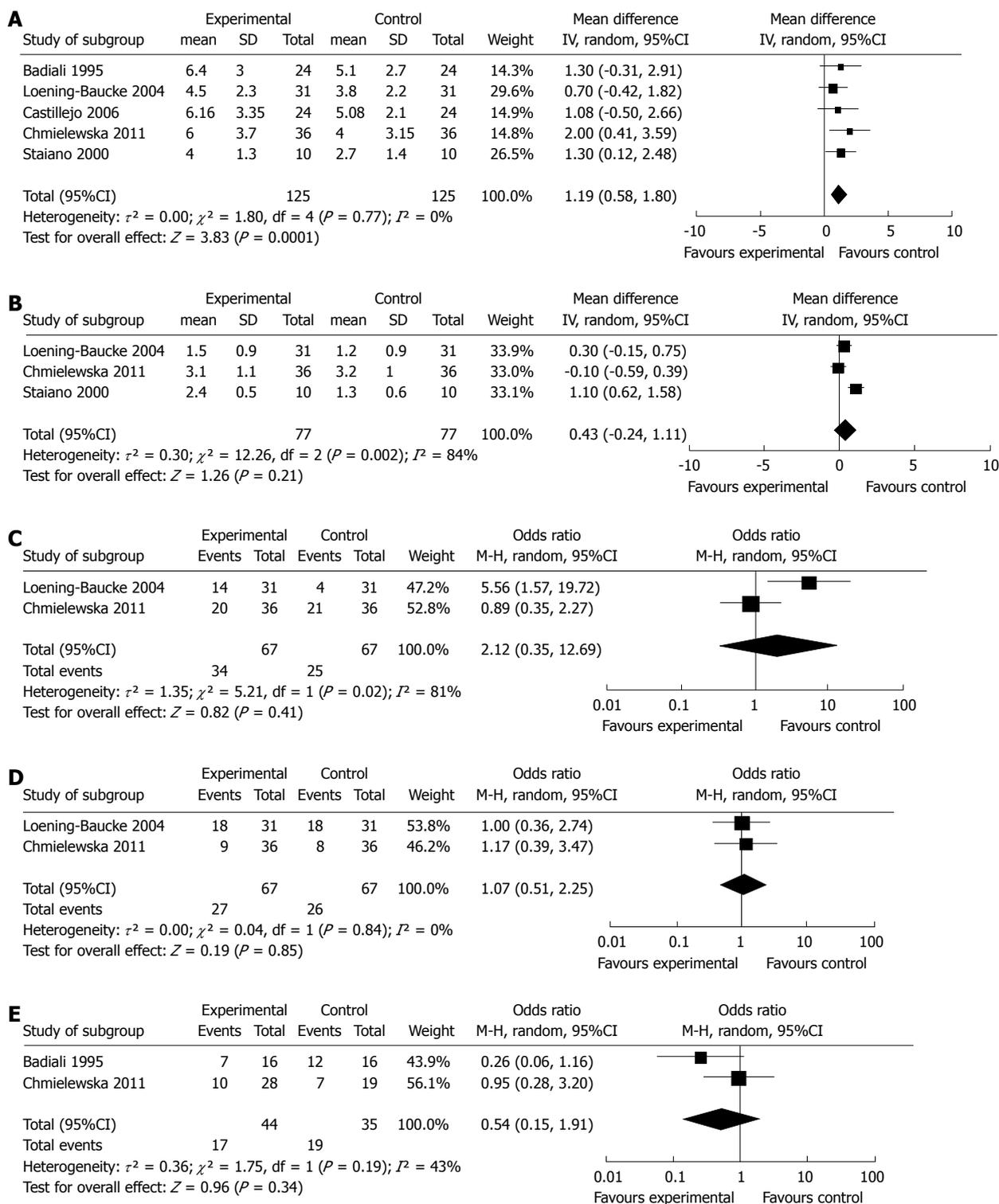


Figure 2 Pooled estimate of odds ratio and 95%CI. A: Stool frequency; B: Stool consistency; C: Treatment success; D: Laxative use; E: Painful defecation. IV: Inverse variance; M-H: Mantel-Haenszel.

We used rigid research methods and described search strategy, eligibility criteria and data extraction method in detail. We included high-quality studies (Jadad score ≥ 3) into the analysis. Because of the strict selection criteria, only five small sample-sized studies were included. Definite heterogeneity existed in the analysis. Neurologically impaired children were included, although no difference

was present between dietary fiber and placebo groups during baseline period, disease itself can interfere with the outcome. Some of the studies are limited to pediatric patients, stool withholding and stool toileting refusal often occurred^[20], which is uncommon among constipated adults, although no heterogeneity was found in the analysis of stool frequency, potential limitation still existed. As

scarce data of gastrointestinal syndromes was reported, and different evaluation and presentation methods were used, less data can be used for the analysis.

There were meta-analyses examining the efficacy of fiber in constipation previously, in which data of fiber and laxative were pooled for analysis^[21,22]. Recently, a systematic review of the efficacy of fiber in the management of chronic idiopathic constipation was published^[23], six RCTs were included, four RCTs compared the effect of soluble fiber with placebo^[24,27], and one study^[27] used the combined intervention with celandine, aloe vera and psyllium. Celandine and aloe vera contain several alkaloids with known aperient effect, which may influence the outcome of the patients. Of the two trials examining the effect of insoluble fiber^[11,28], one trial^[28] recruited patients with self-reported constipation, subjective error may be more obvious when the outcome was assessed by non-medical staff.

In summary, our meta-analysis demonstrated that dietary fiber can obviously increase stool frequency in patients with constipation. The result also showed that dietary fiber did not obviously improve stool consistency, treatment success, laxative use and painful defecation. However, there were some possible influential factors such as small sample-sized studies, severity of constipation, assessment method for outcomes, *etc.* So further large trials examining the effect of dietary fiber in the treatment of constipation are needed, the possible influential factors should be taken into consideration, and more gastrointestinal symptoms and adverse events should be reported before dietary fiber was formally recommended.

COMMENTS

Background

Constipation is one of the widespread health problems. There is a wide-range of treatment methods. Life-style modification is usually recommended as first-line treatment, but data on the effectiveness of these measures are limited. Laxatives are most commonly used for treatment of constipation, but frequent use of these drugs may lead to some adverse effects, and alternative treatment measure is therefore needed. Increase of dietary fiber intake has been recommended to treat constipation of children and adults. There have been several randomized controlled trials (RCTs) studying the relationship between dietary fiber and constipation, however no definitive quantitative summary is available.

Research frontiers

Some studies reported that dietary fiber can increase stool frequency, improve stool consistency and have no obviously adverse effects. Two studies concluded that dietary fiber is as effective as lactulose treatment and seems to have less side effects. However, in another study, dietary fiber was not found more effective than placebo in therapeutic success and it might increase the frequency of abdominal pain.

Innovations and breakthroughs

This meta-analysis demonstrated that dietary fiber can obviously increase stool frequency in patients with constipation. The result also showed that dietary fiber did not obviously improve stool consistency, treatment success, laxative use and painful defecation, however, there were some possible influential factors such as small sample-sized studies, severity of constipation, and assessment method for outcomes. Further large trials examining the effect of dietary fiber in the treatment of constipation are needed, the possible influential factors should be taken into consideration and more gastrointestinal symptoms and adverse events should be reported before dietary fiber was formally recommended.

Applications

The study results suggest that dietary fiber intake is a potential therapeutic method that could be used in the treatment of constipation.

Terminology

Constipation: Present with any two of the six symptoms of less than 3 defecations per week, straining, lumpy or hard stools, sensation of incomplete evacuation, sensation of anorectal obstruction or blockage, digital maneuvers; Dietary fiber: Dietary fiber is a broad category of non-digestible food ingredients that includes non-starch polysaccharides, oligosaccharides, lignin, and analogous polysaccharides with an associated healthful benefit.

Peer review

The effectiveness of dietary fiber on constipation is inconsistent. So far no definitive quantitative summary is available. This is a well performed meta-analysis, in which the authors analyzed the effect of dietary fiber in constipation. The results are interesting and suggest that dietary fiber intake is a potential therapeutic method that could be used in preventing and treating constipation. This analysis provides valuable information for further trials and clinical application.

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Effectiveness of 5-fluorouracil-based neoadjuvant chemotherapy in locally-advanced gastric/gastroesophageal cancer: A meta-analysis

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Abstract

AIM: To investigate the effectiveness of 5-fluorouracil-based neoadjuvant chemotherapy (NAC) for gastroesophageal and gastric cancer by meta-analysis.

METHODS: MEDLINE and manual searches were performed to identify all published randomized controlled trials (RCTs) investigating the efficacy of the fluorouracil-based NAC for gastroesophageal and gastric cancer, and RCTs of NAC for advanced gastroesophageal and gastric cancer *vs* no therapy before surgery. Studies that included patients with metastases at enrollment were excluded. Primary endpoint was the odds ratio (OR) for improving overall survival rate of patients with gastroesophageal and gastric cancer. Secondary endpoints were the OR of efficiency for down-staging tumor and increasing R0 resection in patients with gas-

troesophageal and gastric cancer. Safety analyses were also performed. The OR was the principal measurement of effect, which was calculated as the treatment group (NAC plus surgery) *vs* control group (surgery alone) and was presented as a point estimate with 95% confidence intervals (CI). All calculations and statistical tests were performed using RevMan 5.1 software.

RESULTS: Seven RCTs were included for the analysis. A total of 1249 patients with advanced gastroesophageal and gastric cancer enrolled in the seven trials were divided into treatment group ($n = 620$) and control group ($n = 629$). The quality scores of the RCTs were assessed according to the method of Jadad. The RCT quality scores ranged from 2 to 7 (5-point scale), with a mean of 3.75. The median follow-up time in these studies was over 3 years. The meta-analysis showed that NAC improved the overall survival rate (OR 1.40, 95%CI 1.11-1.76; $P = 0.005$), which was statistically significant. The 3-year progression-free survival rate was significantly higher in treatment group than in control group (37.7% *vs* 27.3%) (OR 1.62, 95%CI 1.21-2.15; $P = 0.001$). The tumor down-stage rate was higher in treatment group than in control group (55.76% *vs* 41.38%) (OR 1.77, 95%CI 1.27-2.49; $P = 0.0009$) and the R0 resection rate of the gastroesophageal and gastric cancer was higher in treatment group than in control group (75.11% *vs* 68.56%) (OR 1.38, 95%CI 1.03-1.85; $P = 0.03$), with significant differences. No obvious safety concerns about mortality and complications were raised in these trials. There were no statistically significant differences in perioperative mortality (5.08% *vs* 4.86%) (OR 1.05, 95%CI 0.57-1.94; $P = 0.87$ fixed-effect model) and in the complication rate between the two groups (13.25% *vs* 9.66%) (OR 1.40, 95%CI 0.91-2.14; $P = 0.12$ fixed-effect model). Trials showed that patients from Western countries favored NAC compared with those from Asian countries (OR 1.40, 95%CI 1.07-1.83). Monotherapy was inferior to

multiple chemotherapy (OR 1.40, 95%CI 1.07-1.83). Intravenous administration of NAC was more advantageous than oral route (OR 1.41, 95%CI 1.09-1.81).

CONCLUSION: Fluorouracil-based NAC can safely improve overall survival rate of patients with gastroesophageal/gastric cancer. Additionally, NAC can down the tumor stage and improve R0 resection.

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Key words: Gastroesophageal cancer; Gastric cancer; Neoadjuvant chemotherapy; Meta-analysis

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Ge L, Wang HJ, Yin D, Lei C, Zhu JF, Cai XH, Zhang GQ. Effectiveness of 5-fluorouracil-based neoadjuvant chemotherapy in locally-advanced gastric/gastroesophageal cancer: A meta-analysis. *World J Gastroenterol* 2012; 18(48): 7384-7393 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i48/7384.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i48.7384>

INTRODUCTION

Gastric and esophageal cancers are among the leading causes of cancer-related death worldwide^[1,2]. In spite of a declining incidence of the distal stomach cancer in the Western countries over the past decades, the incidence of adenocarcinoma of the lower esophagus and the gastroesophageal junction has dramatically increased in the world^[3]. Early-stage gastric and gastroesophageal cancers are curable with surgical treatment alone, with a 5-year overall survival rate of 90%. However, the majority of gastric and gastroesophageal cancer patients are diagnosed with advanced diseases (stages III or IV)^[4]. The advanced gastric and gastroesophageal cancer without distant metastasis is still a potentially curable disease, but the prognosis is poorer than the early-stage diseases. Treatment of advanced gastroesophageal cancer is still a challenge for gastrointestinal surgeons. Localized tumors, limited to the submucosa, can be best treated surgically, with a long-term survival of 70%-95%, but the prognosis of locally advanced tumors is poor due to a high unresectability rate at presentation, and a much higher relapse rate after radical surgery^[5,6], thus demanding further studies regarding neoadjuvant treatment.

Theoretically, the administration of the neoadjuvant chemotherapy (NAC) appears to have several potential benefits for gastroesophageal and gastric cancers: to reduce the tumor volume, to improve the R0 resection rate, to act on micrometastases and to evaluate tumor chemosensitivity to cytotoxic medications. Randomized trials and meta-analyses have demonstrated a benefit with neoadjuvant or perioperative chemotherapy in gastric and gas-

troesophageal cancers. However, the optimal approach in individual patients is not clear and remains controversial^[7,8]. The aim of the current meta-analysis under such circumstances was to evaluate the effectiveness of NAC in treatment of gastric and gastroesophageal cancers and explore the optimal strategy for chemotherapy delivery.

MATERIALS AND METHODS

Data collection and selection

MEDLINE and manual searches were carried out to identify all published RCTs that compared the fluorouracil-based NAC plus surgery with surgery alone for advanced gastric and gastroesophageal cancers. The search was done on PubMed using three sets of terms: “esophagogastric junction/gastroesophageal/gastric”; “carcinoma/cancer”; and “neoadjuvant chemotherapy/preoperative chemotherapy”. A limit was set on the randomized controlled trials (RCTs) and the terms were set to title/abstract.

Inclusion criteria

The following inclusion criteria were used: (1) RCTs that compared the fluorouracil-based NAC plus surgery with no treatment before surgery for gastric and gastroesophageal cancers; (2) blindness of the trial was not required; (3) patients with pathologically diagnosed esophagogastric junction or gastric adenocarcinoma, without prior treatment before entering the trial, but with a history of potentially curative surgery; and (4) studies which were considered updated.

Exclusion criteria

(1) Studies on preoperative radiotherapy or immunotherapy; and (2) studies with the control group receiving chemotherapy were excluded.

The data of each RCT were collected by two reviewers (Zhang GQ and Wang HJ) independently. The results were consistent.

Data checking and assessment

Methodological quality of trials was evaluated using the modified Jadad quality scores^[9], which include secure method of randomization, allocation concealment, double-blinding, and information on withdrawals, and losses to follow-up. Based on these criteria, the studies were divided into high-quality group (score ≥ 3) and low-quality group (score ≤ 2). Two reviewers independently assessed the eligibility of each trial.

Data extraction

The following data were extracted from each study and recorded using a predesigned form: authors, year of publication, patient population, country of investigators, sample size (total, eligible, and per arm), chemotherapy regimen, cycles of chemotherapy, follow-up period, curative effect (survival rate, rate of macroscopic radical resection and cancer stage at pathological examination), and adverse events. Two reviewers did the extraction independently.

Table 1 Characteristics of the included studies in the meta-analysis

No.	Authors and year of publication	Country	Patients (n)		Treatment group		Control group	
			Treatment	Control	Pre-op	Post-op	Pre-op	Post-op
1	Schuhmacher <i>et al</i> ^[15] , 2010	Germany	72	72	5-FU + DDP	None	None	None
2	Boige <i>et al</i> ^[11] , 2011	France	113	111	FP	FP	None	None
3	Cunningham <i>et al</i> ^[12] , 2006	United Kingdom	250	253	ECF	ECF	None	None
4	Hartgrink <i>et al</i> ^[13] , 2004	Holland	27	29	FAMTX		None	None
5	Zhang <i>et al</i> ^[17] , 2004	China	37	54	IV (no details)		None	None
6	Kobayashi <i>et al</i> ^[14] , 2000	Japan	91	80	5-FU (oral)	CT	None	None
7	Wang <i>et al</i> ^[16] , 2000	China	30	30	5-FU (oral)		None	None

5-FU: 5-fluorouracil; DDP: Cisplatin; FP: 5-FU/cisplatin; ECF: Epirubicin/cyclophosphamide/5-FU; FAMTX: 5-FU/adriamycin/methotrexate; CT: Chemotherapy; IV: Intravenous; Pre-op: Preoperative; Post-op: Postoperative.

Table 2 Origin of the included studies and Jadad score

No.	Authors	Titles	Jadad score
1	Schuhmacher <i>et al</i> ^[15]	Neoadjuvant chemotherapy compared with surgery alone for locally advanced cancer of the stomach and cardia: European Organization for Research and Treatment of Cancer randomized trial 40954	4
2	Boige <i>et al</i> ^[11]	Perioperative chemotherapy compared with surgery alone for resectable gastroesophageal adenocarcinoma: an FNCLCC and FFCD multicenter phase III trial	4
3	Cunningham <i>et al</i> ^[12]	Perioperative chemotherapy versus surgery alone for resectable gastroesophageal cancer	7
4	Hartgrink <i>et al</i> ^[13]	Neoadjuvant chemotherapy for operable gastric cancer: long-term results of the Dutch randomized FAMTX trial	5
5	Zhang <i>et al</i> ^[17]	Clinical significance of preoperative regional intra-arterial infusion chemotherapy for advanced gastric cancer	2
6	Kobayashi <i>et al</i> ^[14]	Long-term outcome of preoperative chemotherapy with 5'-deoxy-5-fluorouridine (5'-DFUR) for gastric cancer	3
7	Wang <i>et al</i> ^[16]	A favorable impact of preoperative FPLC chemotherapy on patients with gastric cardia cancer	3

FAMTX: 5-FU/adriamycin/methotrexate; FPLC: Fluorouracili polyphase liposome composita pro orale; FNCLCC: Fe'de' ration Nationale des Centres de Lutte Contrele Cancer; FFCD: Fe'de' ration Francophonede Cance' rologie Digestive Collaborative Groups.

Meta-analysis protocol

Data were obtained directly from included articles or calculated by percentage in each article. The meta-analysis was performed using Review Manager 5.1 software (provided by Cochrane Collaboration). Outcomes assessed by this meta-analysis included the overall survival, three-year progression-free survival rate, tumor down-staging rate, R0 resection rate, safety analysis and subgroup analysis. Overall survival was defined as the time between the treatment randomization and the date of the last follow-up or of the patient's death. Patients who were lost to follow-up were considered as dead. Locoregional recurrence was measured either from the date of treatment randomization to the occurrence of the event or to the date of last follow-up. Heterogeneity between the trials was assessed to determine which model would be used in the meta-analysis. A sensitivity analysis was performed by changing the meta-analysis model. An odds ratio (OR) was the principal measurement of effect. It was calculated as the treatment group *vs* the control group.

Statistical analysis

All statistical analysis were performed spontaneously using Review Manager 5.1 software^[10]. Heterogeneity between the trials was assessed using Chi-square test. The OR was presented with a 95%CI. I^2 statistics was used for the degree of heterogeneity evaluation, and $P < 0.05$ was considered statistically significant.

RESULTS

Eligible trials

Seven RCTs were identified, and the quality scores of the RCTs were assessed according to the method of Jadad. The details are shown in Table 1. The RCT quality scores ranged from 2 to 7 (5-point scale), with a mean of 3.75 (Tables 2 and 3).

Overall survival rates

There was no significant heterogeneity between the trials ($P = 0.52$), and the fixed effects model was used. The data in the seven studies^[11-17] were available for the analysis of overall survival. There were 620 patients in the treatment group and 629 patients in the control group in this meta-analysis. The median follow-up time was over three years. The OR, expressed as treatment group *vs* control group, was 1.40 (95%CI 1.11-1.76; $P = 0.005$). The difference of the overall survival between the treatment group and the control group was statistically significant. The overall survival was increased by 7.96% in the treatment group compared with the control group (Figure 1A). The number needed to treat (NNT) was 12. A sensitivity analysis was performed by changing the effect model into the random effect model. The results showed that the confidence interval of the odds ratio did not lie across the non-effect line, and the difference of the overall survival was statistically significant between the treatment group

Authors and year of publication	Randomization	Allocation concealment	Blinding	Withdrawal and dropout	Jadad score
Schuhmacher <i>et al</i> ^[15] , 2010	Without details	Without details	Without details	Well reported	4
Boige <i>et al</i> ^[11] , 2011	Without details	Without details	Without details	Well reported	4
Cunningham <i>et al</i> ^[12] , 2006	Well reported	Envelope	Double-blind	Well reported	7
Hartgrink <i>et al</i> ^[13] , 2004	Well reported	Envelope	No	Well reported	5
Zhang <i>et al</i> ^[17] , 2004	Without details	None	No	Well reported	2
Kobayashi <i>et al</i> ^[14] , 2000	Well reported	Envelope	No	Well reported	3
Wang <i>et al</i> ^[16] , 2000	Without details	Without details	No	Well reported	3

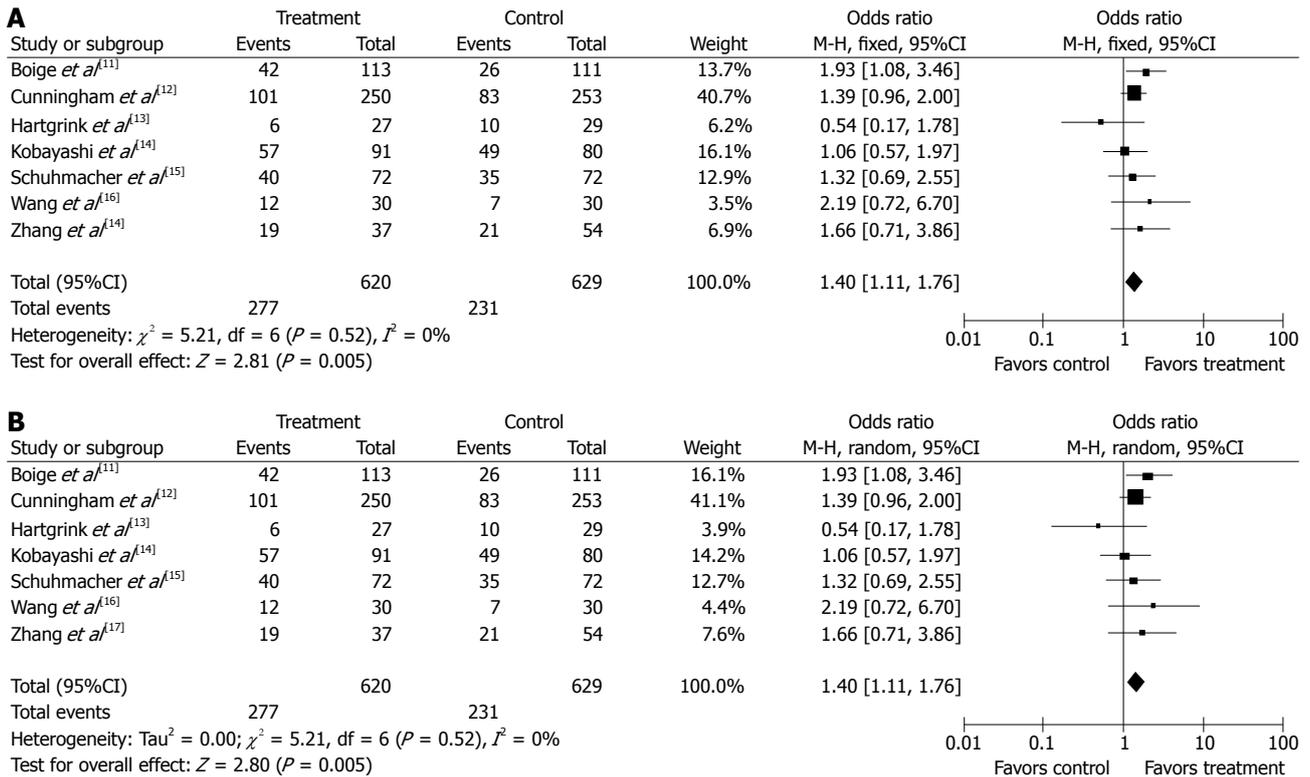


Figure 1 Effect of neoadjuvant chemotherapy on overall survival rate. A: Overall survival rate (fixed effects model); B Overall survival rate (random effects model).

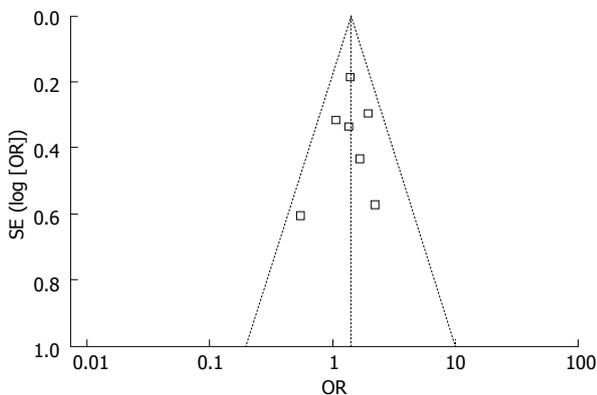


Figure 2 Publication bias in the included studies. Funnel plot analysis of potential publication bias. OR: Odds ratio.

and the control group when the random effect model was used. The fixed effect model and the random effect model were used respectively to evaluate the sensitivity of the results of the analysis (Figures 1B and 2).

Three-year progression-free survival rate

Three studies^[11,12,15] compared the 3-year progression-free survival (PFS) rates between the two groups. The 3-year PFS rate was higher in treatment group than in control group (37.7% vs 27.3%), (OR 1.62, 95%CI 1.21, 2.15; $P = 0.001$, fixed-effect model) and NNT was 10 (Figure 3A and B).

Tumor down-staging rate

Three studies^[12,13,15] describing the pathological staging of gastroesophageal and gastric cancers after resection (269 in treatment group and 290 in control group) were included in the analysis. The rate of pT1-2 was higher in treatment group than in control group (55.76% vs 41.37%) (OR 1.77, 95%CI 1.27, 2.49; $P = 0.0009$, fixed-effect model) and the NNT was 7 (Figure 4A and B).

R-0 resection rate

The resection rate of gastroesophageal and gastric cancers was reported in four trials^[11,12,13,15]. Since no obvious heterogeneity was observed in these studies ($P = 0.31$, I^2

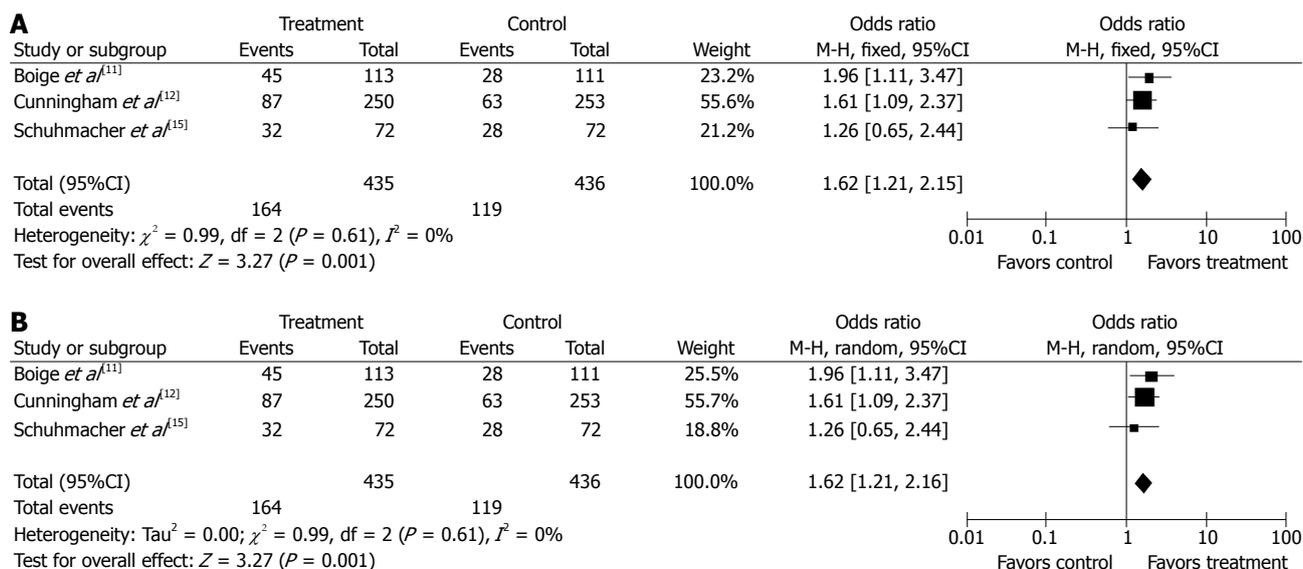


Figure 3 Effect of neoadjuvant chemotherapy in progression free survival rate. A: Progression free survival rate (fixed effects model); B Progression free survival rate (random effects model).

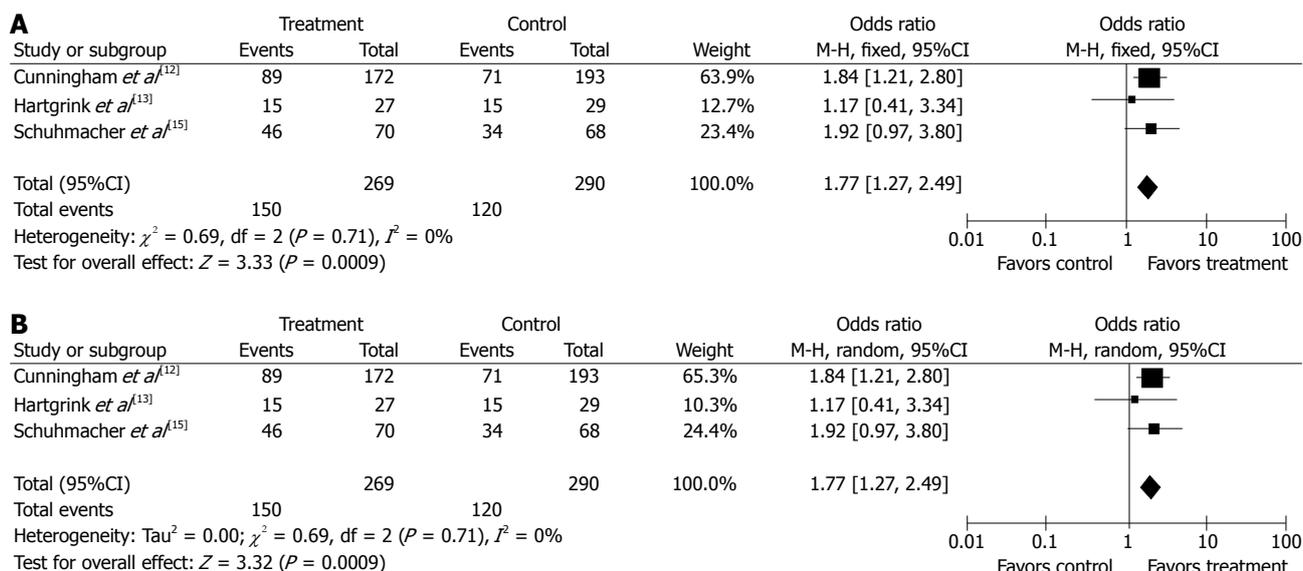


Figure 4 Effect of neoadjuvant chemotherapy in tumor down-staging. A: Tumor down-staging rate (fixed effects model); B Tumor down-staging rate (random effects model).

= 17%), the fixed-effect model was used. The R0 resection rate of the gastroesophageal and gastric cancers was higher in treatment group than in control group (OR 1.38, 95%CI 1.03-1.85, $P = 0.03$, fixed-effect model) and the NNT was 15 (Figure 5A and B).

Safety analysis

Safety analysis included both chemotherapy-induced adverse effects (grade 3/4, defined according to the Common Toxicity Criteria of the National Cancer Institute, version 2.0) and postoperative complication and mortality. Two studies reported^[11,12] grade 3/4 adverse effects of NAC, including gastrointestinal side effect in 18.1% (60/332) and leukopenia in 9.9% (33/332). Three stud-

ies^[11,12,15] reported perioperative mortality with no statistically significant difference ($P = 0.87$) between the two groups (5.08% *vs* 4.86%), (OR 1.05, 95%CI 0.57-1.94, fixed-effect model) (Figures 6 and 7).

Subgroup analysis

Factors that might influence the results in the two groups were studied (Figure 8). When the overall survival rate was set as the end point, gastroesophageal and gastric cancer patients were benefited more from perioperative chemotherapy than from NAC alone (OR 1.40, 95%CI 1.11-1.76, NNT = 12). Trials showed that patients from Western countries favored NAC compared with those from Asian countries (OR 1.40, 95%CI 1.07-1.83).

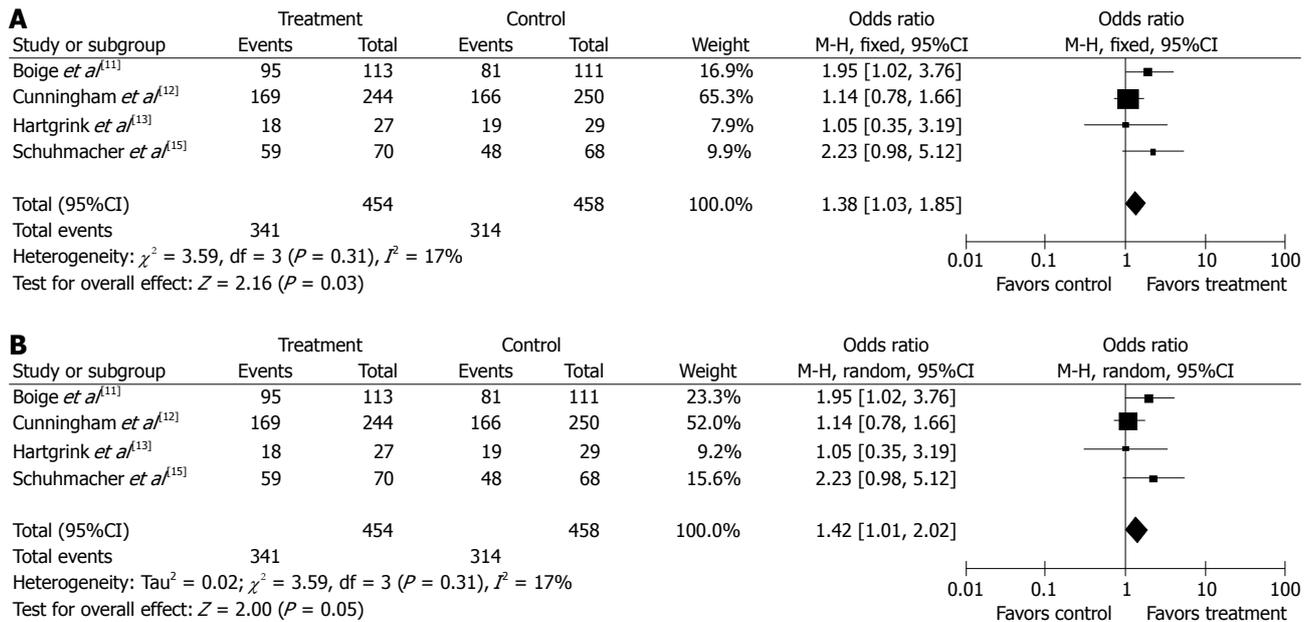


Figure 5 Effect of neoadjuvant chemotherapy in tumor R-0 resection. A: R-0 resection rate (fixed effects model); B: R-0 resection rate (random effects model).

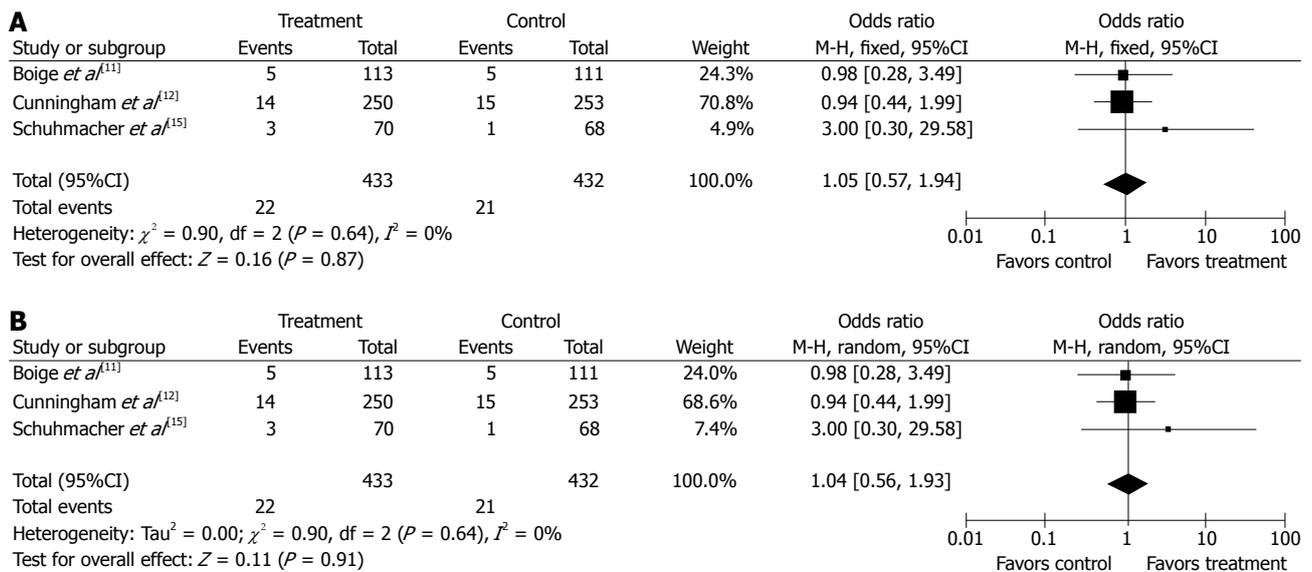


Figure 6 Effect of neoadjuvant chemotherapy in perioperative mortality. A: Perioperative mortality rate (fixed effects model); B: Perioperative mortality rate (random effects model).

Monotherapy was inferior to multiple chemotherapy (OR 1.40, 95%CI 1.07-1.83). Intravenous administration of NAC was more advantageous than oral route (OR 1.41, 95%CI 1.09-1.81) (Figure 7).

DISCUSSION

Gastric cancer remains to be an important health issue worldwide. Over the past two decades, the incidence of distal gastric cancer has been decreased, but the incidence of proximal and esophagogastric junction cancers has been increased significantly^[18]. The treatment of the gastroesophageal and gastric cancer is dependent on the TMN staging of the tumor. The advanced gastroesopha-

geal and gastric cancers (stage III-IVB) without evidence of distant metastasis are potentially curable, but these tumors usually present with a more advanced stage and are associated with a worse prognosis^[19], and a combination therapy, including surgery, chemotherapy and radiotherapy, is often needed. Chemotherapy is an adjuvant treatment modality in the form of adjuvant chemotherapy, NAC and concomitant chemoradiotherapy^[20]. NAC has several advantages: (1) it is well tolerated; (2) it can better control the micrometastasis^[21]; and (3) it might downstage the tumor to the greatest extent and increase the probability of R0 resection so as to facilitate the surgery^[22,23], thus improving the survival rate of the patients with gastroesophageal and gastric cancer. However, it might delay

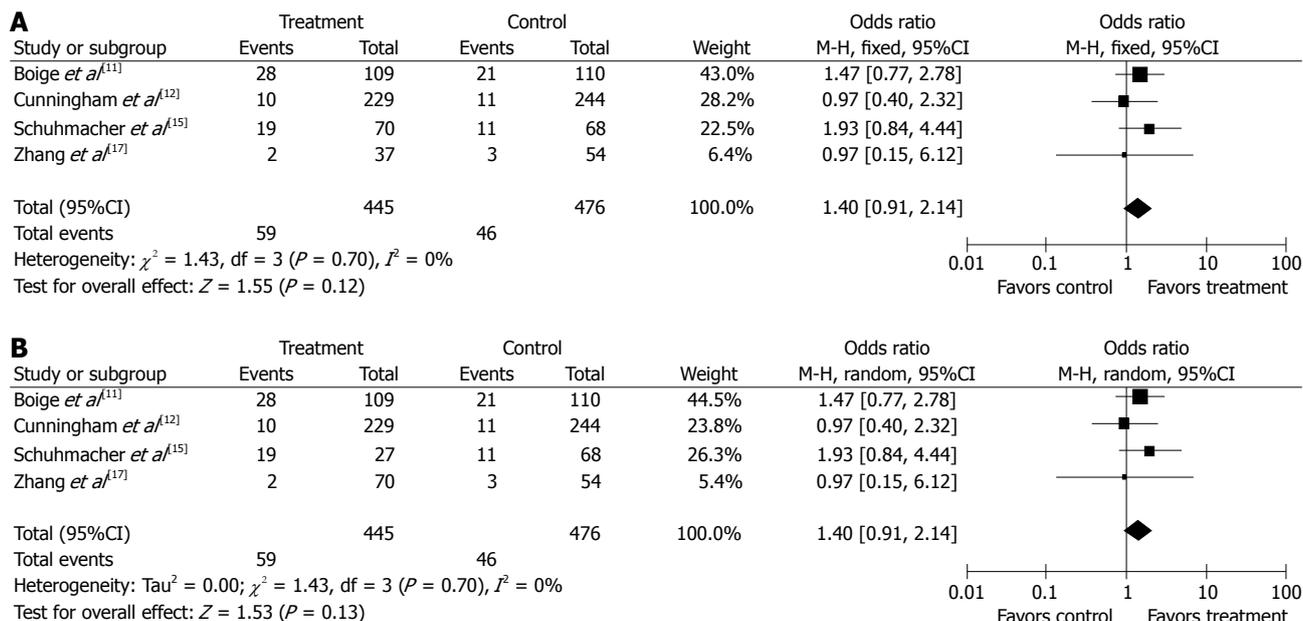


Figure 7 Effect of neoadjuvant chemotherapy in postoperative complications. A: Postoperative complications rate (fixed effects model); B: Postoperative complications rate (random effects model).

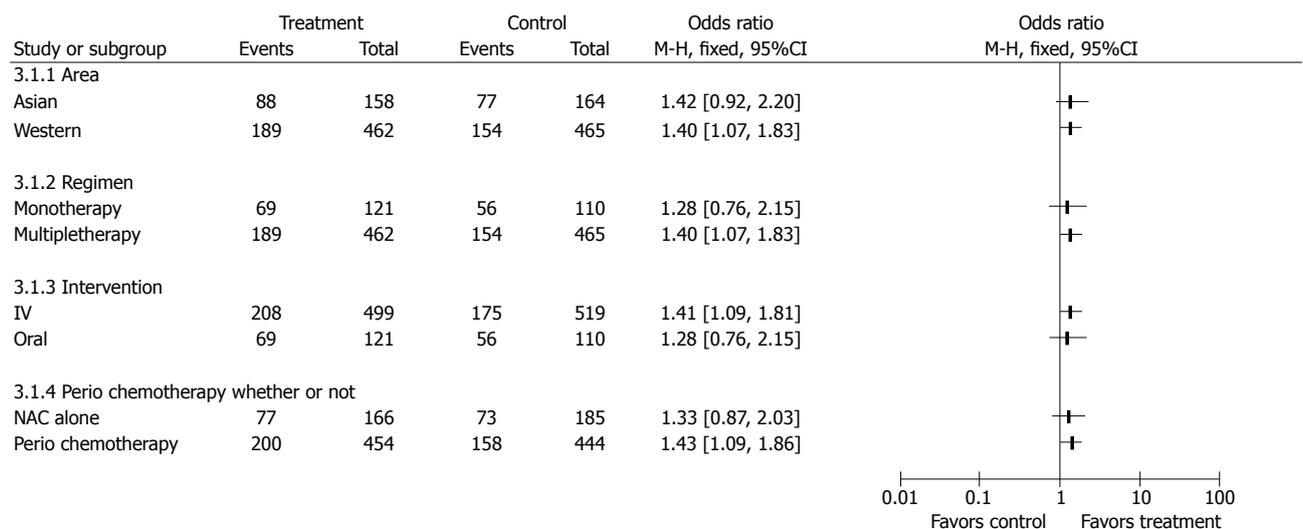


Figure 8 Subgroup analysis showing different overall survival rates in patients with advanced gastric cancer.

the curative treatment if the tumor does not respond to the NAC, which is also costly and may be detrimental to the patients. Theoretically, NAC can increase the survival rate and improve the quality of life of the patients. However, the meta-analysis by Him^[8] failed to show the benefit of NAC in patient survival. Li's study^[7] demonstrated a minor but significant benefit in patient survival. The result coincided with our studies, which shows that 5-fluorouracil-based NAC has a benefit on the overall survival of gastroesophageal and gastric cancer patients. Our meta-analysis demonstrated the feasibility of NAC for locally gastroesophageal and gastric cancer patients. NAC could downstage the tumor (NNT = 7) and increase the R0 rate of gastric cancer (NNT = 15). To examine the role of NAC alone in improving the overall survival rate

of gastroesophageal and gastric cancer patients who did not receive postadjuvant chemotherapy, data from four trials^[13,15-17] were further analyzed, showing that NAC had no effect on the overall survival rate of gastroesophageal and gastric cancer patients (OR 1.34, 95%CI 0.87-2.06). These may contribute to the factors that the included studies had a very small sample size with different follow-up time, different cycles of treatment as well as different tumor locations, which could be too underpowered to demonstrate any positive results. Alternatively, this might be attributed to the fact that currently used preoperative chemotherapeutic agents may not be effective enough to eradicate micrometastases. No clear conclusion could be reached as yet about the effect of NAC alone on the overall survival rate of gastroesophageal and gastric

cancer patients. Fortunately, the perioperative chemotherapy shows some effect in the overall survival rate of gastroesophageal and gastric cancer patients (OR 1.43, 95%CI 1.09-1.86). It has been found that 5-fluorouracil-based NAC benefits the overall survival of gastroesophageal and gastric cancer patients.

The regimen of the NAC might affect the outcome of the treatment. Therefore, the regimen factors concerning NAC for gastroesophageal and gastric cancer should be taken into account. Several regimens have been used in the NAC of gastroesophageal and gastric cancer. Our current meta-analysis showed that fluorouracil-based combination regimen and intravenous route of NAC had a high efficiency for gastroesophageal and gastric cancer patients. The effective response rate will help down-stage tumors to the greatest extent and increase the probability of R0 resection, thus improving the survival rate of the patients.

Subgroup analysis also showed that the outcome of NAC for gastroesophageal and gastric cancer was better in trials from Western countries than in those from Asian countries and in multi-agent regimens as well. The increasingly higher incidence of the gastroesophageal cancer in Western countries compared with Asian countries^[12] and the different constituent of tumor stage may influence the final results.

Another major concern in our meta-analysis is the efficiency and safety of NAC in the studies included. Mortality directly links with treatment failure, which mainly comprises locoregional recurrence, secondary primary malignancy, and distant metastasis. In this meta-analysis, the outcomes of 3-year PFS were analyzed. The difference of 3-year PFS was statistically significant between the treatment group and the control group, which suggests that NAC with a 5-fluorouracil-based regimen is effective in the locoregional control of gastroesophageal and gastric cancer when a locoregional treatment is administered. Our meta-analysis showed that gastric cancer patients could well tolerate NAC. Grade 3/4 gastrointestinal and leukopenia adverse events of NAC occurred in 32% (112/343) of gastroesophageal and gastric cancer patients. The rate of complications in the treatment group was not obviously higher than in the control group, indicating that NAC is a safe modality for gastric cancer (OR 1.05, 95%CI 0.75-1.48). The efficiency and the safety were assessed in treatment group, but disease progression during NAC is another potential concern in patients with a loss of opportunity for surgery. In this meta-analysis, three trials^[11,12,15] showed a disease progression rate of 10.6% (46/435). The R-0 resection rate was higher in treatment group than in control group (OR 1.57, 95%CI 1.21-2.02, NNT = 11), indicating that disease progression after NAC is not a major concern for its resection. The results were consistent with the study showing that lack of response to NAC may delay curative surgery, and chemotherapy-induced toxicity may lead to increased surgical complications^[24,25].

In addition, funnel plot observation did not indicate obvious publication bias in the two subgroups (Figure 2).

The sensitivity analysis showed similar results by excluding the trials with Jadad score less than 2. In this meta-analysis, the primary endpoint about the overall survival rate with a fixed model OR of 1.40 (95%CI 1.11-1.76) vs a random model OR of 1.40 (95%CI 1.11-1.76) (Figure 1A, B), indicated that the trial quality was good, which was not influenced by the factors, such as selection bias and other trials.

NAC has been proven effective against some cancers, such as breast cancer^[26], head and neck squamous cell carcinoma^[27]. However, it is not recommended as a standard regimen for gastroesophageal and gastric cancer, primarily because of the significantly different strategies used around the world. However, adjuvant chemoradiotherapy is a standard treatment in the USA, perioperative chemotherapy is the first choice in Europe, and surgery and adjuvant chemotherapy are recommended in Japan, where D2 surgery is effective and safe^[28-30]. Some studies have demonstrated that NAC and D2 surgery can effectively improve the overall survival^[31], whereas a recent study showed no benefits^[32]. Whether NAC benefits D2 dissection lacks strong evidence. Fortunately, our meta-analysis provided the up-to-date evidence for the positive effect of NAC in gastroesophageal and gastric cancers, but further studies are required to determine its best regimen and response-based neoadjuvant concept.

In conclusion, our study provides information on the efficacy of NAC with 5-fluorouracil-based regimen in gastroesophageal and gastric cancer patients. This regimen was regarded as the most effective one before the emergence of Taxanes. The incorporation of Taxanes into the 5-FU/cisplatin (FP) regimen makes up the Taxol/5-FU/cisplatin (TPF) regimen, which is a promising treatment strategy for gastroesophageal and gastric cancers^[33]. A number of trials have been registered to examine the role of NAC in treatment of advanced gastric cancer, such as S-1 plus cisplatin or S-1 and cisplatin plus Taxanes^[34,35]. However, its effectiveness is yet to be confirmed by the meta-analysis. With all these clinical and scientific efforts, these treatment strategies will definitely continue to further improve the outcome of gastroesophageal and gastric cancer patients.

COMMENTS

Background

Although the prevalence of distal gastric cancer has declined, the world-wide incidence of gastroesophageal junctional adenocarcinoma is increasing. For patients who present with gastric and gastroesophageal cancer, surgery remains the cornerstone of treatment, which can potentially improve the long-term survival. However, the prognosis of the patients remains poor. Chemotherapy has been proven to be effective for advanced gastric cancer. Neoadjuvant chemotherapy (NAC) plays a role in improving the prognosis of the patients with advanced gastric cancer and gastroesophageal junctional adenocarcinoma, but its value remains controversial because of lack of well-powered trials.

Research frontiers

Meta-analysis was used to evaluate the effectiveness of NAC for advanced gastric/gastroesophageal cancer in this study.

Innovations and breakthroughs

The meta-analysis provided the up-to-date evidence for the positive effect of NAC in locally advanced gastric and gastroesophageal cancer. NAC im-

proved the R0 resection rate (95%CI: 1.03-1.85), tumor down-staging (95%CI: 1.27-2.49) and survival rate (95%CI: 1.11-1.76) for the 1249 patients enrolled in seven trials. Surgery was safe after preoperative 5-fluorouracil-based chemotherapy while no obvious morality and complication concerns were raised in these trials. These findings suggest that NAC can improve the survival rate of patients with advanced gastric and gastroesophageal cancer.

Applications

Based on the studies on the efficacy and feasibility of the preoperative chemotherapy with 5-fluorouracil-based regimen and with the increasing acceptance of the concept of NAC, additional studies on new regimens and well-designed powerful trials are highly encouraged in patients with locally advanced gastric and gastroesophageal cancer. More experiments are expected to focus on individualized treatment under the guidance of molecular marker for the NAC.

Terminology

D₂ surgery: the extent of lymphadenectomy (D₁₋₃), in which complete dissection of up to the second group nodes was defined as D₂ according to the rules of the Japanese Research Society for Gastric Cancer; TNM classification of malignant tumors: "pT" indicates the pathological stage after surgery and "cTNM" indicates the clinical pretreatment tumor stage; Taxanes: they represent one of the more effective targets in current cancer therapy, which block cell cycle progression through centrosomal impairment, induction of abnormal spindles and suppression of spindle microtubule dynamics. Paclitaxel (Taxol) is the prototype of the taxane family of anti-tumor drugs, which has been approved for treatment of metastatic gastric cancer; Number needed to treat: It is often used to describe how many patients would need to be treated to prevent one event. It is determined from the absolute difference between one treatment and another.

Peer review

This is an excellent analysis. The authors submit a meta-analysis of the available randomized controlled trials investigating the utility of neoadjuvant therapy for gastric and gastroesophageal cancer. Overall, the paper is very strong, the data appropriately analyzed and the conclusions reasonable. The supposition the neoadjuvant therapy for gastroesophageal cancers remaining controversial is not entirely the case. Most centers across the world have embraced this approach based on the strong data supporting efficacy.

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Entering the duodenal diverticulum: A method for cannulation of the intradiverticular papilla

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Abstract

Successful cannulation of the common bile duct may be difficult in patients in whom the papilla is located entirely within a diverticulum. In this study, we report successful biliary cannulation in three patients following intubation of the distal tip of the duodenoscope into the duodenal diverticulum and locating the major papilla. No complications occurred during the operation or during the postoperative period. This method didn't need second intubation an endoscope and might lower the burden of patients. So this skill is useful to deal with the papilla hidden inside the large diverticulum because of its safety and convenience.

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Key words: Endoscopic retrograde cholangiopancrea-

tography; Intradiverticular papilla; Duodenoscope

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INTRODUCTION

Periampullary diverticula (PAD) are found in 9% to 32.8% of patients who undergo endoscopic retrograde cholangiopancreatography (ERCP)^[1]. The papilla often exists at the superior or inferior border of the PAD. Occasionally, identification of the papillary orifice and successful cannulation of the common bile duct (CBD) may be more difficult in patients in whom the papilla is located entirely within the diverticulum, even for endoscopic experts. In this report, we present three cases where the papilla was hidden in a large duodenal diverticulum. Successful biliary cannulation was achieved following intubation of the distal tip of the duodenoscope into the duodenal diverticulum.

CASE REPORT

Three male patients (aged 70, 78 and 82 years) were admitted to our hospital as a result of right upper quadrant pain and fever and jaundice. Magnetic resonance cholangiopancreatography revealed intra- and extra-hepatic bile duct dilation (CBD stones were presented in patient one and patient two). On ERCP, the papilla could not be found at the rim of large duodenal diverticula (one with septa). We therefore suspected that the papilla was hidden inside the diverticula. With careful searching, the papil-

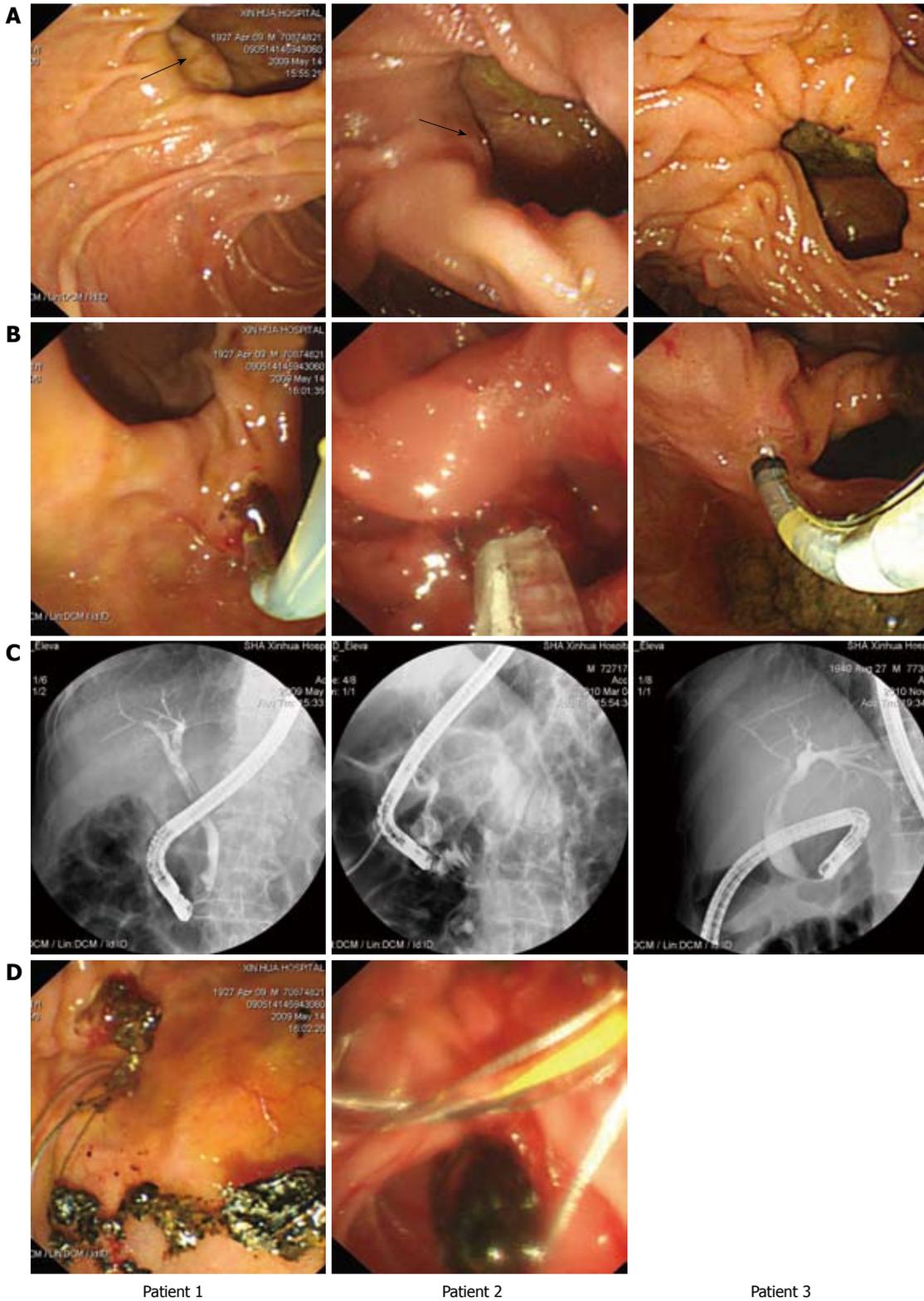


Figure 1 The duodenal diverticulum to be located intradiverticular papilla for biliary cannulation. A: The papillary orifices can be indistinctly seen at the left side of the inner diverticular borders in patient 1 and patient 2 (arrow), but not seen at all in patient 3; B: Successful biliary cannulation was achieved after facing the intradiverticular papilla; C: Endoscopic retrograde cholangiography showing stones in the common bile duct in patient 1 and patient 2 and dilation of the common bile duct in patient 3; D: Extracted bile stones in the duodenal diverticulum in patient 1 and patient 2.

lary orifices were indistinctly seen at the left side of the inner diverticular borders in patient one and patient two, but were not visualized in patient three (Figure 1). Several attempts were made to evert the papilla out of the diver-

ticulum, including dilating the diverticular rim, using two devices in one channel (a catheter and a sphincterotome), but without success. As the diverticulum was judged to be large enough to accommodate the distal tip of a duo-

denoscope, we decided to intubate the duodenoscope (JF-260V, Olympus Medical System, Tokyo, Japan) into the duodenal diverticulum by gently adjusting the knob, and by push-pull the duodenoscope. Eventually, we discovered that the orifices in all three patients were on the left side of the inner diverticular borders, opening towards the medial and posterior wall of the diverticulum. Subsequently, the orifices were faced by finely adjusting the direction of the endoscope, and by carefully exploring the papillary alignment (Figure 1). Biliary cannulation and stone extraction was then conducted using a small biliary sphincterotomy and balloon dilatation (Figure 1). No complications occurred during the procedure or during the postoperative period. All patients were well on discharge shortly after surgery.

DISCUSSION

PAD is outpunching from the duodenum that represents herniation of the mucosal or submucosal layers through a defect in the muscular wall within a radius of 2 to 3 cm of the ampulla of Vater. The prevalence of PAD increases with age and has been reported in 65% of elderly patients^[2]. There is now clear evidence of an association between duodenal diverticula and bile duct stones, particularly in the absence of gallbladder stones^[3]. The location of the papilla with respect to the diverticulum is divided into two categories. In type I the papilla is at the rim or within 2 cm of the edge of the diverticulum, and this is called a peri-diverticular papilla (PDP). In type II cases, the papilla lies inside or at the middle of the bottom edge of the diverticulum or between two adjacent diverticula, and this is termed an intradiverticular papilla (IDP)^[4]. According to a report by Tham *et al*^[5], type I and type II cases accounted for 74.7% and 25.3%, respectively, in a cohort of 83 patients with diverticular papilla. Previously, successful cannulation in patients with PAD has varied from 61% to 95.4%, which is significantly lower than that observed in those without PAD^[1,5]. Two recent studies reported that PAD did not cause any technical difficulties at ERCP or increase the risk of complications, however, this was true only when patients with the undetectable papilla were excluded^[3,6]. It is reasonable to speculate an even lower successful cannulation rate in IDP patients in whom papilla are often more difficult to locate than in PDP patients. Therefore great efforts are needed to find new methods to achieve successful cannulation in patients with IDP.

In recent years, new devices and new manipulations for successful biliary cannulation in patients with IDP have been reported. These include using a two-devices-in-one-channel method^[7], balloon dilation of a narrow-necked diverticulum^[8], using an ultrathin gastroscope to locate the papilla^[9], an endoscopic ultrasound-guided rendezvous technique^[10], endoclip-assisted biliary cannulation^[11], and the double-endoscope method^[12].

Our study suggests an alternative skill for biliary cannulation in patients with IDP, which didn't need second incubation an endoscope. In contrast to colon diverticu-

la, the PAD is relatively fixed as they are located behind the retroperitoneum. Also the adjacent kidney and pancreas could provide further support to diverticula wall. Thus, we can relatively easily control the position of the duodenoscope in the duodenal diverticulum. To avoid sudden movements in the PAD, the duodenoscope can also be pulled back to the duodenal lumen for stone extraction. There were no complications, such as bleeding or perforation, during ERCP treatment of these three patients. So this skill is a safe and convenient method which may lower the burden of patients.

We conclude that intubating the distal tip of the duodenoscope into the large duodenal diverticulum, thereby locating the hidden papilla, is a safe and convenient skill that facilitates both biliary cannulation and endoscopic treatment in the hands of experienced practitioners, which didn't need second incubation an endoscope and may lower the burden of patients.

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Endoscopic ultrasound features of gastric schwannomas with radiological correlation: A case series report

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Abstract

Gastric schwannomas are rare mesenchymal tumors of the gastrointestinal tract. They are usually misdiagnosed as other submucosal tumors preoperatively. Experience of the imaging features of gastric schwannomas is extremely limited. In this report, we summarize the features of a series of endoscopic ultrasound (EUS) images of gastric schwannomas in an effort to improve the diagnosis and differential diagnosis rate. We retrospectively reviewed the endosonographic features of four patients with gastric schwannomas and their computed tomography imaging results. Gastric schwannomas had heterogeneous hypoechogenicity or isoechogenicity, and a well-demarcated margin. The tumors originated from the fourth layer. Cystic changes and calcification were uncommon. Marginal hypoechoic haloes were observed in two patients. The results described here were different from those of previous studies. In the EUS evaluation, the internal echogenicity of gastric schwannomas was heterogeneous and low, but slightly higher than that of muscularis pro-

pria. These features might help us differentiate gastric schwannomas from other submucosal tumors. Further investigation is needed to differentiate these mesenchymal tumors.

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Key words: Schwannomas; Endosonography; Stomach; Radiological examination; Imaging

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INTRODUCTION

Schwannomas of the gastrointestinal (GI) tract are relatively rare, representing about 3% of all mesenchymal tumors of the GI tract^[1]. They are usually encapsulated benign tumors of the peripheral nerve sheath composed of differentiated schwann cells, with an excellent prognosis after surgical resection. Malignant transformation of gastric schwannomas is extremely rare^[2,3]. It is difficult to differentiate a gastric schwannoma from gastrointestinal stromal tumors (GISTs). Schwannomas are often misdiagnosed as GISTs on radiological examination^[4]. However, GISTs have greater malignant potential and require surgical resection and imatinib-based adjuvant therapy. Therefore, it is important to differentiate be-

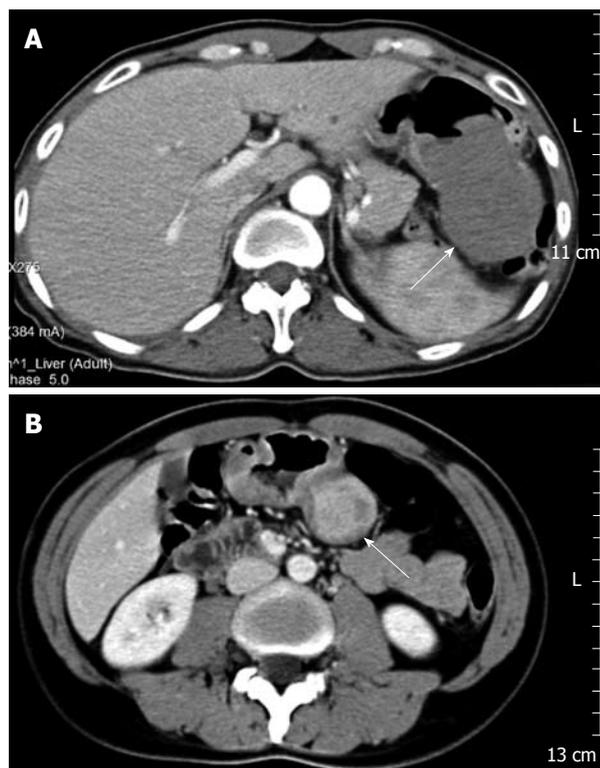


Figure 1 Computed tomography images showed rounded masses in the stomachs, with homogeneous ($n = 3$) or heterogeneous ($n = 1$) internal contrast enhancement. A: Contrast-enhanced computed tomography (CT) showed a solitary, exophytic, soft, internal homogeneous tissue mass (arrow) in the greater curvature of the stomach, the mass exhibited central ulceration (Case 3); B: CT during the portal venous phase of contrast enhancement showed a heterogeneous contrast enhanced mass (arrow) in the body of the stomach (Case 1).

tween gastric schwannomas and other potentially malignant gastrointestinal tumors, especially GISTs. To date, there is only one report describing the endoscopic ultrasonography (EUS) features of gastric schwannomas^[5]. In this study, we analyzed 4 gastric schwannomas using EUS imaging. The results we observed were unlike those of previous studies.

CASE REPORT

From October 21, 2008 to December 27, 2011, four tumors were histologically identified as gastric schwannomas after surgery at the Second Affiliated Hospital of Zhejiang University School of Medicine. Clinical data and tumor size were recorded. This retrospective review was approved by the Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine. All patients provided written informed consent before performing EUS and surgery.

Endosonography was applied using ultrasound endoscopes [the Olympus GF-UM200, GF-UCT240-AL5 and GF-UE260-AL5 (7.5 MHz; Olympus Optical Co. Japan)]. EUS scanning showed many features that were evaluated, including the following: presence or absence of mucosal ulceration; the regularity of the marginal

border (regular or irregular); the presence of lobulation of the surface; the shape of the tumor (oval to round or non-oval distorted); the presence of a marginal halo, echogenicity (hypoechoic or hyperechoic); homogeneity (homogeneous or heterogeneous); presence of internal echogenic or cystic foci; and presence of exophytic development (tumor development outside the gastric wall). Computed tomography (CT) images and radiological reports were retrospectively reviewed. The CT features were analyzed with the pattern of contrast enhancement.

Two patients underwent laparotomy and resection of tumor and two other patients underwent subtotal gastrectomy. Three of 4 patients underwent regional lymphadenectomy. Immunohistochemical studies were performed on the resected tissue to evaluate the expression of S100 protein, α -smooth muscle actin (α -SMA), desmin, CD117, CD34 and Ki67. All four patients underwent EUS and CT examinations before surgery.

Follow-up data were obtained from clinical records and from the treating doctors.

The clinicopathological characteristics of the patients and the surgical data and outcomes are summarized in Table 1. All 4 cases were female (age ranged from 32 to 50 years). One patient was asymptomatic, and the schwannoma was accidentally discovered in this patient by ultrasonic examination in the follow-up of a hysteromyoma. Three patients had symptoms including ructus, bloating and epigastric pain, weight loss, melena and hematemesis.

The patients underwent multiple examinations before surgery, but none had a correct preoperative diagnosis of schwannoma. They were misdiagnosed as having GISTs. Upper endoscopy revealed that one patient had a submucosal tumor with a central ulcer and that the other patients did not have ulceration. All the tumors were located in the gastric body (2 in the greater curvature and 2 in the lesser curvature).

The CT images showed rounded masses in the stomachs, with homogeneous ($n = 3$) (Figure 1A) or heterogeneous ($n = 1$) (Figure 1B) internal contrast enhancement. One of the masses was accompanied by enlargement of the lymph nodes.

The EUS evaluation revealed that all the tumors were hypoechoic, with a connection between the tumor and the muscularis propria. The echogenicities of the tumors were heterogeneous with an internal high-echo region (Figure 2A). Two of the patients had tumors with marginal hypoechoic regions (Figure 2B). The shapes of all tumors were oval, with well-demarcated margins, and the growth patterns of the tumors were exogastric. The tumors had smooth surfaces, except one which had an ulcer. None had internal cystic lesions, lobulations, or calcification (Table 2). Endoscopic ultrasound-guided fine needle aspiration was performed in one patient, but failed to confirm the diagnosis because of insufficient tissue biopsy for immunohistochemistry.

Histological examination showed that the tumors were composed of spindle cells. There were lymphoid cuffs

Case	Age (yr)	Sex	Size (cm)	Clinical Presentation	Site (gastric body)	Management	Outcome (mo)
1	32	Female	3.3 × 4.0	Ructus, bloating, epigastric pain	Greater curvature	Laparoscopic wedge resection	14
2	39	Female	4.9 × 4.0	Weight loss	Lesser curvature	Laparoscopic wedge resection	4
3	50	Female	8.1 × 5.3	Melena, hematemesis (GI bleeding)	Great curvature	Subtotal gastrectomy	5
4	49	Female	5.0 × 3.5	Incidental on ultrasonic examination for hysteromyoma follow-up	Lesser curvature	Subtotal gastrectomy	39

Case	Echogenicity	Ulcer	Shape	Margin	Lob	Halo	Cyst	Spots	Cal	Growth
1	Low	-	Oval	Regular	-	+	-	+	-	In < out
2	Low	-	Oval	Regular	-	+	-	+	-	In < out
3	Low	2	Oval	Regular	-	-	-	+	-	In < out
4	Low	-	Oval	Regular	-	-	-	+	-	In < out

Lob: Lobulation; Halo: Marginal halo; Cyst: Internal cystic lesion; Spots: High spots; Cal: Calcified lesion.

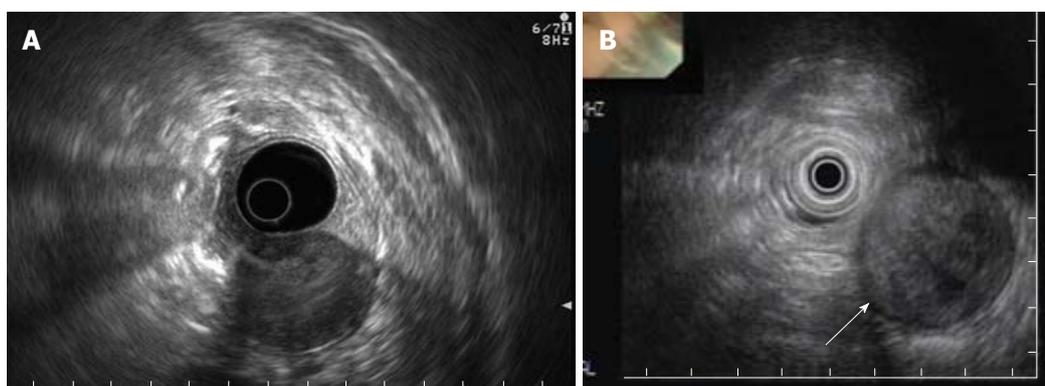


Figure 2 Endoscopic ultrasonography of tumors. A: Endoscopic ultrasonography (EUS) showed a well-circumscribed heterogeneous hypoechoic mass with internal flake high-echo arising from the proper muscle layer of the stomach (case 3); B: EUS showed a round heterogeneous hypoechoic mass with marginal halo (arrow) (case 1).

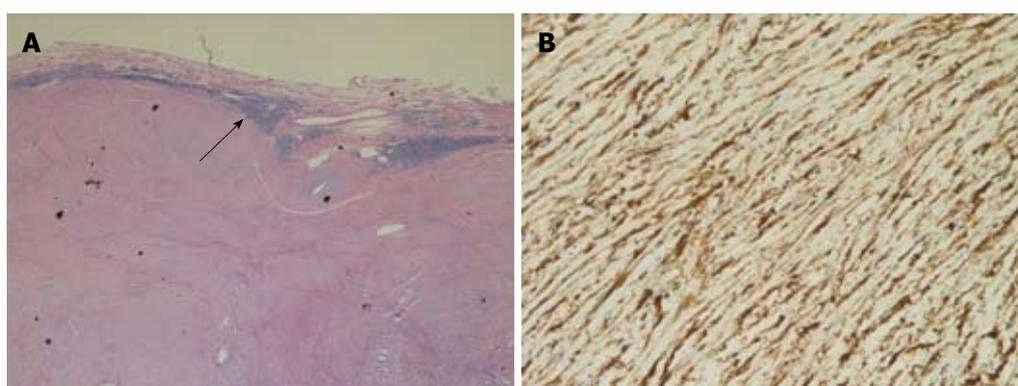


Figure 3 Histological examinations showed that the lymphoid cuff (arrow) (A) and immunohistochemical evaluation revealed the spindle tumor cells stained positive for S-100 protein(B).

surrounding the tumors (Figure 3A). Immunohistochemical evaluation revealed strong expression of S-100 protein in all tumors (Figure 3B). None of the tumors showed expression of CD117, CD34 or desmin. All tumors revealed low proliferation, as estimated from the low proportion of Ki67-positive cells (Ki67 < 5%).

DISCUSSION

GI schwannomas are benign, slow growing tumors regarded as tumors distinct from conventional schwannomas. These tumors arise from the nerve sheath of the gut wall, rather than from the central nervous system and

Table 3 Endoscopic ultrasound for differential diagnosis of gastric schwannomas versus gastric gastrointestinal stromal tumors

	Gastric schwannomas	Low-risk GIST	High-risk GIST
Echogenicity	Heterogeneous and hypoechoic, but slightly higher than that of muscularis propria	Homogeneous and hypoechoic	Heterogeneous and hypoechoic
Halo	Frequent	Uncertain	Uncertain
Growth	In < out (mostly)	In ≥ out (mostly)	Variety
Margin	Regular	Regular	Irregular
Lobulation	Rare	Uncommon	Common
High echo spot	Common	Occasional	Common
Cyst	Very rare	Frequent	Very frequent
Calcification	Scarce	Occasional	Occasional

GIST: Gastrointestinal stromal tumors.

from soft tissues^[6]. The stomach is the most common site of origin of GI schwannomas^[2]. The tumors are most commonly located in the body of the stomach^[3]. The tumors predominantly occur in older adults (mean age is 58 years) with a marked female predominance^[6,7]. Gastric schwannomas are usually asymptomatic or associated with non-specific abdominal discomfort. They are accidentally discovered or when complications, such as GI bleeding, arise. Endoscopically, gastric schwannomas appear as elevated submucosal masses, with or without central ulcers. Endoscopic biopsies always yield false-negative results. The definitive diagnosis of gastric schwannomas is determined by pathological and immunohistochemical examination of surgical specimens. The tumors are typically negative for CD117, desmin, α-SMA and positive for S100. They stain variably with CD34^[2].

It may be helpful to gain limited information through EUS, CT, magnetic resonance imaging (MRI), and positron emission tomography (PET) to differentiate gastric schwannomas from other gastric submucosal tumors. In previous studies, gastric schwannomas displayed well-circumscribed masses with heterogeneous or homogeneous contrast enhancement on CT^[7,8]. On MRI examination, gastric schwannomas are sharply demarcated, strongly enhanced tumors, having low to medium signal intensity on T1 weighted images, and high signal intensity on T2 weighted images^[9]. However, the radiological imaging features of gastric schwannomas are not specific. They are quite similar to those of gastric stromal tumors. Recent reports described several cases of gastric schwannoma with increased fluorodeoxyglucose (FDG) uptake on PET. It is therefore necessary to distinguish schwannomas from low risk GISTs when assessing submucosal tumors of the stomach that show a high FDG accumulation^[4,10].

Presently, reports on the EUS features of gastric schwannomas are extremely limited, due to the rarity of these tumors. The EUS features of gastric schwannomas have been described as round submucosal masses with marginal haloes, and homogeneous internal echogenicity

without internal echogenic foci^[5,11]. One study indicated that the echogenicities of gastric schwannomas were much lower than the normal surrounding muscle layers. Therefore, the authors considered that these findings may be useful for differentiating schwannomas from GISTs^[5]. Nevertheless, EUS findings of gastric schwannomas revealed a hypoechoic mass, with some hyperechoic foci, in some other case reports^[12,13]. According to our results, the internal echogenicity of gastric schwannomas was heterogeneous and low, but slightly higher than that of muscularis propria, with internal patch high echo. They were not homogeneous and extremely low. CT imaging showed that the tumors showed homogeneous or heterogeneous contrast enhancement. This meant that these tumors were hypervascular. The blood within the schwannomas was very slow-flowing. It is usually not possible to demonstrate flow with color or power Doppler sonography. The slow flow of blood, in spite of the hypervascular nature, can be explained by the observation that the internal echoes of the four tumors appeared similar to or slightly higher than those of muscularis propria, with some patchy hyperechoic areas. Although a lymphoid cuff was observed in each patient, it was not always continuous, which may explain why the marginal hypoechoic halo was only found in two cases. All 4 gastric schwannomas in our study lacked a cystic change, indicating that cystic changes are uncommon in this type of tumor. Only 2 cases with cystic changes on CT examinations have been reported in the literature^[8]. Another clinicopathological study found no cases of cystic change or gross necrosis in 51 cases^[3]. No calcification within gastric schwannomas was reported in previous reports. However, cysts, hemorrhagia, and necrosis were common in GISTs, and calcification was seen in 6% of GISTs^[14]. The differential diagnosis of gastric schwannomas and GISTs in EUS images are summarized in Table 3.

In conclusion, gastric schwannomas are rare benign mesenchymal tumors of the stomach. However, it is necessary to differentiate these tumors from other submucosal tumors of the stomach, particularly GISTs. The EUS features of gastric schwannomas are varied. On EUS evaluation, heterogeneous hypoechoic or isoechogenicity, a well-demarcated margin, fourth-layer origination, and lack of cystic change and calcification may be considered to be helpful findings for the diagnosis of gastric schwannoma. A marginal halo was perhaps the characteristic feature, but not an essential one. Ulceration may be seen in large tumors. More EUS studies should be conducted to delineate the characteristic features that can help differentiate these mesenchymal tumors.

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Treatment of radiation-induced hemorrhagic gastritis with prednisolone: A case report

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Abstract

Radiation-induced gastritis is an infrequent cause of gastrointestinal bleeding. It is a serious complication arising from radiation therapy, and the standard treatment method has not been established. The initial injury is characteristically acute inflammation of gastric mucosa. We presented a 46-year-old male patient with hemorrhagic gastritis induced by external radiotherapy for metastatic retroperitoneal lymph node of hepatocellular carcinoma. The endoscopic examination showed diffuse edematous hyperemic mucosa with telangiectasias in the whole mucosa of the stomach and duodenal bulb. Multiple hemorrhagic patches with active oozing were found over the antrum. Anti-secretory therapy was initiated for hemostasis, but melena still occurred off and on. Finally, he was successfully treated by prednisolone therapy. We therefore strongly argue in favor of prednisolone therapy to effectively treat patients with radiation-induced hemorrhagic gastritis.

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INTRODUCTION

Radiation-induced gastritis is a serious complication of radiation therapy and can cause upper gastrointestinal bleeding. There have been no effective options to manage this complication. We encountered a 46-year-old male patient with hemorrhagic gastritis induced by external radiotherapy for the treatment of metastatic retroperitoneal lymph node of hepatocellular carcinoma (HCC). Successful hemostasis was achieved with treatment of prednisolone.

CASE REPORT

A 46-year-old man diagnosed with HCC received left lobectomy in December 2010. Two courses of transcatheter arterial chemoembolization with 5-fluorouracil, oxaliplatin, mitomycin and lipiodol (5-10 mL) were administered from January 2011 to July 2011 for intrahepatic recurrence. He received liver transplantation in September 2011. He was treated with sorafenib 400 mg *bid* from January 2011. In November 2011, he received

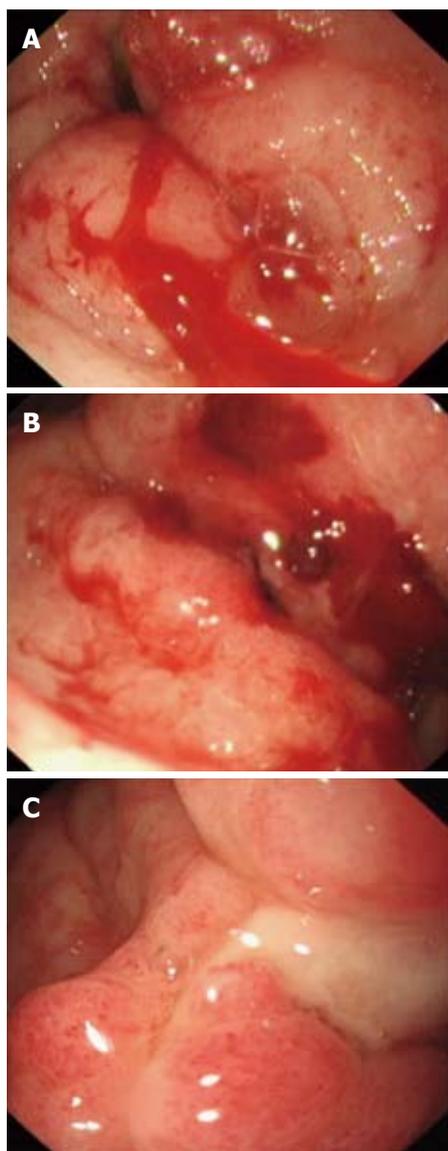


Figure 1 Endoscopic appearance of antral mucosa. A: Multiple telangiectasias spontaneously bleeding at diffuse antral mucosa after radiation therapy; B: After prednisone was discontinued, multiple hemorrhagic spots were still visible at antral mucosa; C: After 1 mo of prednisone maintenance therapy, there were residual telangiectasias with no bleeding tendency.

helical radiotherapy using tomotherapy (total 63.7 Gy) for the treatment of metastatic retroperitoneal lymph node. The dosages delivered to the stomach, intestine and liver were 18.70 Gy, 18.10 Gy and 10.78 Gy, respectively. During the course of radiotherapy, sirolimus was continued against transplantation rejection and sorafenib was discontinued. Three months after radiotherapy, the patient had hematemesis and melena. He was admitted to the emergency room. His vital signs were quite unstable: blood pressure was 84/56 mmHg; heart rate was 92 beats/min and respiratory rate was 20 breaths/min. Laboratory evaluation revealed severe anemia with a hemoglobin level of 37 g/L, prothrombin time was 11.4 s, activated partial thromboplastin time was 27.5 s, international normalization ratio was 0.98 and platelets was $68 \times 10^9/L$. Blood transfusion was administered.

The endoscopic examination showed diffuse edematous hyperemic mucosa with telangiectasia in the whole mucosa of the entire examined stomach and duodenal bulb. Multiple hemorrhagic patches with active oozing were visible over the antrum (Figure 1A). He was diagnosed as having radiation-induced gastritis. Omeprazole (40 mg *bid iv*) and sucralfate (1 g *tid oral*) were given for a week. However, upper gastrointestinal bleeding still occurred off and on. Due to the inaccessibility of argon plasma coagulation, he was treated with prednisolone. The starting dose of prednisolone was 40 mg daily. Hematemesis and melena stopped 3 d later, and fecal occult blood test was negative. Then, he received oral prednisone, 25 mg daily. The dosage was gradually reduced by 5 mg every 3 d and down to zero within 15 d after hospital discharge. His hemoglobin levels improved to 60 g/L. Unfortunately, 2 wk after prednisone was discontinued, his melena recurred. The endoscopic examination still showed multiple hemorrhagic spot at antral mucosa (Figure 1B). The hemoglobin levels decreased to 34 g/L. We treated him with prednisolone again. Within 3 d, his hematocrit stabilized and the melena resolved. He then received maintenance prednisone (10 mg daily). One month later, he underwent follow-up endoscopy, which revealed some residual telangiectasias with no bleeding friability (Figure 1C). No gastrointestinal bleeding recurred. His hemoglobin levels improved to 88 g/L without blood transfusions or iron supplements.

DISCUSSION

Radiation-induced gastritis is a serious complication of radiation therapy and difficult to manage. It usually happens 2-9 mo after initial radiotherapy^[1-3]. A high total dose and, above all, high daily fraction appear to be the main risk factors in gastric injuries. Radiologic durability dosage of the stomach and intestine is defined as 45 Gy, and that of rectum is defined as 55 Gy. The initial injury is characteristically acute inflammation of gastric mucosa. If injury progresses, vasculopathy may evolve to progressive obliterative endarteritis and endothelial proliferation, leading to mucosal ischemia, ulceration, and telangiectasias^[4]. The characteristic endoscopic finding is the presence of telangiectasia. Other endoscopic findings include diffuse erythema of mucosa, shallow or deep ulcers and scar formation.

Radiation-induced hemorrhagic gastritis is a diffuse process with multiple bleeding sites^[4]. The standard method has not been established. Repeated endoscopies and the anti-secretory agents all yielded unsatisfactory control of bleeding. Argon plasma coagulation had been reported for successful hemostasis of radiation-induced hemorrhagic gastritis, colitis and proctitis^[1,2,5-7]. Surgery may be necessary if other treatment fails, but it is associated with a high morbidity.

Rectal steroids have often been recommended for the treatment of radiation-induced proctitis. Kochhar *et al*^[8] reported that steroids successfully treated radiation-induced proctosigmoiditis. But only few instances of

steroids therapy for radiation induced gastritis have been reported. In our case, we chose to use prednisolone because prednisolone can reduce inflammation. Although the pathogenesis of radiation-induced gastritis is not entirely clear, it was presumed to be an inflammatory process. A large number of cell types, interacting molecular signals including cytokines and growth factors, and various molecules on the endothelial cell surface are involved. It was reported that the mucosal levels of interleukin (IL)-2, IL-6, and IL-8 were significantly higher in patients with radiation proctitis^[9]. Prednisolone can inhibit inflammation by a diverse array of mechanisms, including decreasing chemotaxis of monocytes and neutrophils, inhibiting adhesive molecule synthesis and decreasing eicosanoid production. Prednisolone's anti-inflammatory functions may reduce gastric mucosa damage and degeneration as well.

In this case, the upper gastrointestinal bleeding recurred when prednisone was discontinued. It indicated that sufficient time of prednisone administration is needed for the effective treatment. In fact, when the patient received prednisone for a prolonged duration, no gastrointestinal bleeding recurred. Therefore, the patients with radiation-induced hemorrhagic gastritis should continue with maintenance prednisone because the radiation-induced injury is a chronic course and may last a long time.

During radiotherapy, the patient continued taking sirolimus against transplantation rejection, which also has an anti-angiogenic effect. Many studies have shown that anti-angiogenic agent in combination with radiotherapy produces a synergistic anti-tumor efficacy without increasing toxicity^[10,11].

This report described a further potential treatment modality for radiation-induced hemorrhagic gastritis. More patients and longer follow-up are necessary to confirm the effectiveness of prednisolone in this form of gastritis.

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Delayed liver laceration following transjugular intrahepatic portosystemic shunt for portal hypertension

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Abstract

The transjugular intrahepatic portosystemic shunt (TIPS) is an acceptable procedure that has proven benefits in the treatment of patients who have complications from portal hypertension due to liver cirrhosis. Delayed liver laceration is a rare complication of the TIPS procedure. We describe a patient with portal hypertension due to liver cirrhosis, who suddenly presented with abdominal hemorrhage and liver laceration 8 d after TIPS. Few reports have described complications after TIPS placement. To the best of our knowledge, this is the first report describing delayed liver laceration. This potential and serious complication appears to be specific and fatal for TIPS in portal hypertension. We advocate careful attention to the technique to avoid this complication, and timely treatment is extremely important.

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Key words: Transjugular intrahepatic portosystemic shunt; Portal hypertension; Liver cirrhosis; Postoperative complications; Hemorrhage

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INTRODUCTION

Since its first introduction in the 1990s, transjugular intrahepatic portosystemic shunt (TIPS) has played an increasingly important role in the management and treatment of the complications of portal hypertension (PH). Now, established indications for TIPS include active variceal hemorrhage not controlled by endoscopic and pharmacologic treatment (mainly from gastric and ectopic varices), secondary prevention of bleeding, refractory hydrothorax and ascites, Budd-Chiari and veno-occlusive syndrome, hepatopulmonary syndrome, hepatorenal syndrome, and prophylaxis of complications in patients with cirrhosis who need major abdominal surgery^[1-3].

TIPS as a treatment for PH has been performed for > 20 years in our hospital. Five hundred cases of PH have been treated^[4,5]. With improvement in the technical skills of interventional radiologists over time, technical complications such as abdominal bleeding, stent migration, hemobilia, and hepatic artery injury have become extremely rare^[6,7]. This paper reports a case of delayed liver laceration 8 d after TIPS in a patient with PH.

CASE REPORT

A 45-year-old female patient was admitted to our institution



Figure 1 Gastroscopy showing severe esophageal and gastric varices.

because of hematemesis. She had a few years history of PH due to liver cirrhosis of unclear etiology, and had been hospitalized twice for gastrointestinal bleeding. On examination, she was emaciated and had moderate ascites requiring diuretic treatment. Endoscopy showed severe hypertensive gastropathy with severe esophageal and gastric varices (Figure 1). Abdominal computed tomography demonstrated a classical PH appearance with liver cirrhosis and splenomegaly (Figure 2A). Plain chest film showed left hydrothorax. Abnormal laboratory findings on admission were as follows: hemoglobin concentration, 71 g/L; platelet count, $3 \times 10^{10}/L$. Child-Pugh class A and normal renal function are not laboratory findings and were not abnormal.

The TIPS procedure was performed according to the standard method under local anesthesia (1% lidocaine hydrochloride). After the right internal jugular vein was percutaneously punctured, a 10-Fr, 41-cm long sheath (Rösch-Uchida TIPS Puncture Set; Cook, Bloomington, IN, United States) was placed in the suprahepatic portion of the inferior vena. After catheterization of the right hepatic vein, measurement of hepatic venous pressure and portal puncture were performed. At no stage during attempted puncture of the portal vein was contrast agent seen to enter the hepatic arterial system, bile ducts, or peritoneal cavity. Portal venography was performed to evaluate the anatomy and determine the direction of portal flow (Figure 2B). With the use of a hydrophilic guide wire (Radiofocus guide wire; Terumo Europe, Leuven, Belgium), the portal vein was catheterized. The dilation of the intrahepatic parenchymal tract was performed using a low-profile balloon with 8 mm diameter. In this particular case with large varices of the gastric coronary vein, embolization using coils and gelatin sponge was performed to reduce the risk of rebleeding. Polytetrafluoroethylene (PTFE)-covered Fluency stent grafts (8 mm \times 6 cm, manufactured by Angiomed GmbH Co., subsidiary of C.R. Bard, Murray Hill, NJ, United States) were used. After placement of the stents, portography was performed to ensure no contrast extravasation within the shunt, and the portal pressure was measured (Figure 3). The portosystemic gradient reduced from 50 to 34 cm H₂O. The 5-Fr catheter should be routinely retained inside the portal vein through the right internal jugular vein after the procedure

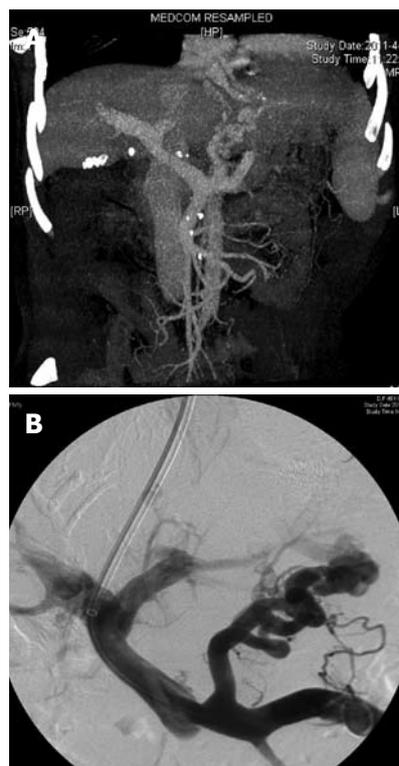


Figure 2 Portal venous-phase computed tomography and portal venography. A: Portal venous-phase computed tomography showing classical appearance of portal hypertension with liver cirrhosis and splenomegaly; B: Portal venography showing the anatomy and direction of portal flow.

for the purpose of postoperative regional anticoagulation (heparin 4000 U/d) and re-examination.

Doppler ultrasound of the liver on d 1 and 7 following TIPS confirmed shunt patency and showed no evidence of liver parenchymal hemorrhage. The patient was transferred onto low molecular weight heparin (4100 U/d) and hepatoprotective drugs (such as magnesium isoglycyrrhizinate injection 200 mg/d, polyene phosphatidylcholine injection 930 mg/d, and ornithine aspartate injection 20 g/d), and made good clinical progress.

On d 8 following TIPS she developed sudden severe right upper abdominal pain, which did not significantly improve after analgesic treatment. Laboratory findings showed a drop in hemoglobin to 49 g/L, with deterioration of liver and coagulation function: serum alanine aminotransferase (ALT), 202 U/L; serum aspartate aminotransferase (AST), 300 U/L; total bilirubin, 38 μ mol/L; prothrombin time, 22 s; and international normalized ratio, 1.8. At the same time, she developed clinical evidence of hypovolemic shock, with confusion, cool periphery, and systemic hypotension. Emergency ultrasound showed massive hemoperitoneum. The patient's anticoagulation therapy was stopped, and vasoactive drugs were given to maintain blood pressure. The same day the patient was transferred to the intensive care unit due to hemorrhagic shock with an Acute Physiology and Chronic Health Evaluation II score of 17. A large volume of blood (about 1 L) was drained from the abdominal cavity by paracent-



Figure 3 Completion venography of transjugular intrahepatic portosystemic shunt showing good flow and no contrast extravasation.

sis. Exploratory laparotomy revealed massive hemoperitoneum (3 L). The active bleeding source was located in the liver parenchyma behind the gallbladder bed of the right hepatic lobe and was controlled with direct suturing and perihepatic packing. Erythrocyte suspension, 25 U, fresh frozen plasma, 2 L, platelet concentrate, 10 U, and cryoprecipitate, 20 U, were infused during and after surgery. The bleeding had ceased and the packs were removed after 6 d. However, postoperative liver dysfunction gradually appeared. Total bilirubin continued to increase, up to 505 $\mu\text{mol/L}$, but ALT and AST were normal (cholenzyme separation). Finally, she died of liver failure 23 d after TIPS.

DISCUSSION

Since the first clinical application was presented by Rössle *et al*^[8] in 1989, TIPS has gained widespread popularity and has become the treatment of choice in many clinical scenarios for complications related to PH^[9]. TIPS is an interventional radiology procedure that results in decompression of the splanchnic venous system in patients with PH by creating a low-resistance channel between an intrahepatic branch of the portal vein and a main hepatic vein^[9]. When compared with endoscopic ligation and sclerotherapy, TIPS shows a significant advantage with respect to hemostasis (87% *vs* 45%, $P = 0.03$), rebleeding (31% *vs* 54%, $P = 0.0005$) and mortality (29% *vs* 48%, $P = 0.05$)^[10]. TIPS is as effective as surgical shunting in preventing variceal bleeding and is also more cost-effective^[11,12]. Evidence has now been provided that the use of TIPS at an early stage may represent a new perspective and improve the outcome and survival of high-risk bleeding patients selected by simple clinical prognostic indicators^[13]. It has been reported that, in patients with Child-Pugh class C disease or class B disease with active bleeding who were admitted for acute variceal bleeding, the early use of TIPS with an e-PTFE covered stent was associated with significant reductions in failure to control bleeding, rebleeding, and mortality, with no increase in the risk of hepatic encephalopathy^[14].

TIPS can markedly reduce portal pressure, but associated complications are also common. Direct procedure-

related complication rates during TIPS have been reported in up to 20% of procedures^[6]. Gaba *et al*^[15] have conducted an in-depth discussion of complications and how to avoid TIPS-related operations in 2010. Freedman *et al*^[6] have described a large series of TIPS complications, after analyzing TIPS procedures and complications at four American centers and reviewing the literature. They have reported that the most dangerous complications were related to transhepatic needle puncture, such as biliary duct injury, hemobilia, transcapsular tearing, arterial puncture, or portal venous rupture. In the event of such complications, direct surgical suture as well as local and perihepatic gauze packing can often effectively stop bleeding. However, if there has been uncontrollable bleeding despite repeated surgical interventions, postoperative evolution toward hepatic insufficiency (acute or progressive), and portal vein injuries that cannot be reconstructed, liver transplantation may be the only option to salvage the patient^[16].

The current patient with decompensated liver cirrhosis was admitted to hospital with upper gastrointestinal bleeding. After adjustment of the liver and blood coagulation function, TIPS was performed to prevent variceal rebleeding. Routine anticoagulation with low molecular weight heparin was given in order to prevent early stent thrombosis and improve the stent patency rate. However, delayed liver laceration suddenly occurred 8 d after surgery. We consider that it may have been related to the following factors: (1) patients with liver cirrhosis suffer from coagulation disorders; (2) small blood vessels of liver parenchyma were damaged during the TIPS procedure; and (3) stent anticoagulation was given with low molecular weight heparin when thrombosis had not formed in a timely manner.

To the best of our knowledge, delayed liver laceration after TIPS has not been reported previously. There have been only three previous reports about late intrahepatic hematoma following TIPS, occurring in patients with Budd-Chiari syndrome (BCS). In each case the hematoma resolved with conservative management. It was considered that the cause of hematoma may have been related to the use of postoperative heparin^[17].

With the introduction of the e-PTFE covered stent, the long-term patency of shunts has significantly improved^[2,4,11,18]. Regular anticoagulation is given to prevent early shunt thrombosis through systemic or local application. A randomized study of anticoagulation following TIPS has reported reduced incidence of early thrombosis but no instances of intrahepatic hemorrhage, although this study comprised only 49 patients, 25 of whom stopped anticoagulation 3 d after TIPS^[19].

Advanced cirrhosis is often accompanied by severe liver atrophy, rigidity of the liver, and enlargement of liver fissure. Anatomical variations of intrahepatic branches of the portal and hepatic veins affect the success rate of the puncture. This often requires repeated puncture or appropriate adjustments of the angle^[20]. Many candidates for TIPS have cirrhosis and are more prone to arterial

injuries because of enlargement and increased flow in the hepatic arteries. Therefore, multiple puncture and early postoperative anticoagulation may be an important risk factor for bleeding. So, early and timely examination has become an effective means of diagnosis and treatment of such complications. Some studies have reported that dynamic gadolinium-enhanced serial magnetic resonance examination may be useful to demonstrate and localize active bleeding, and if performed serially, may have the potential to quantify the rate of bleeding, which may potentially decrease the time needed to identify a site of hemorrhage during surgery^[21].

In conclusion, delayed liver laceration is a rare but fatal complication of TIPS, which to date has not been reported in patients with PH. TIPS is increasingly accepted as an important management option for PH and other diseases such as BCS, therefore, this complication must be borne in mind. We advocate that careful attention to technique is vital to avoid complications. However, when complications do arise, the first step to stop bleeding is immediate imaging and secure local hemostasis with direct suturing and perihepatic gauze packing. If the bleeding does not stop, based on the clinical status and concomitant liver failure, liver transplantation may be necessary.

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¹⁸F-fluorodeoxyglucose PET/CT findings of a solitary primary hepatic lymphoma: A case report

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pathological findings were consistent with lymphoma. The patient was discharged two weeks after surgery and did not receive any further treatment. After 25 mo follow-up, she is in good health. ¹⁸F-fluorodeoxyglucose PET/CT is useful in confirming the diagnosis of primary hepatic lymphoma by demonstrating no other foci with high uptake in other parts of the body.

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Key words: Fluorodeoxyglucose; Positron emission tomography/computerized tomography; Primary hepatic lymphoma; Diffuse large B cell lymphoma; Solitary lesion

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Abstract

Primary hepatic lymphoma is extremely rare, and only a few cases have been described on positron emission tomography (PET) or PET/computed tomography (PET/CT) imaging in the English literature. We report a case of a 55-year-old woman who presented with low-grade fever and weight loss of three months. On CT scanning, a mass was identified which appeared to be a hypodense lesion, on ultrasonographic imaging, the mass was hypoechoic, therefore, liver abscess or hepatic metastasis from a gastrointestinal primary was initially suspected. Tumor markers such as alpha-fetoprotein, carcinoembryonic antigen and carbohydrate antigen 19-9 were within normal limits. PET/CT demonstrated a large abnormal ring-like hypermetabolic focus in the right liver lobe. The lesion was resected and the histo-

INTRODUCTION

Primary hepatic lymphoma (PHL) is an uncommon malignancy of the liver, and has been described in only a few reports in the English literature. The prevalence of PHL is 0.4% in extranodal non-Hodgkin's lymphomas, and 0.016% in all non-Hodgkin's lymphomas^[1]. The incidence of PHL has increased in recent years, and may be related to the human immunodeficiency virus (HIV) epidemic. Due to its rarity, clinical manifestations and laboratory tests are non-specific, the disease is usually misdiagnosed preoperatively, and the final diagnosis depends on histological examination from liver biopsy. In

the present report, we present a patient with pathologically confirmed primary hepatic diffuse large B cell lymphoma originating from the germinal center.

CASE REPORT

A 55-year-old woman presented with intermittent low-grade fever and weight loss of 10 kg in the previous 3 mo. She had no remarkable past medical history except for an operation for a benign gastric stromal tumor. She was admitted to our hospital due to right upper quadrant abdominal pain in June 2009. Gastroscopy of the esophagus suggested stromal tumor, and the patient underwent endoscopic resection of the esophageal stromal tumor. Immunohistochemical examination showed that the markers CD34, alpha-smooth muscle actin and Des were positive, and CD117 as well as S-100 were negative. On examination, the patient was in good spirits, the skin and mucosa were anicteric, there was no splenomegaly or enlarged superficial lymph nodes, and the liver was not palpable. Laboratory findings on admission were as follows: total white cell count $7.9 \times 10^9/L$, hematocrit 0.29 L/L, hemoglobin 8.7 g/dL; total bilirubin 11.6 $\mu\text{mol/L}$ (normal range 3.4–20.5 $\mu\text{mol/L}$), aspartate aminotransferase 17 IU/L (normal range 0–60 IU/L), alanine aminotransferase 7 IU/L (normal range 0–60 IU/L), alkaline phosphatase 123 IU/L (normal range 40–150 IU/L); lactate dehydrogenase 209.0 IU/L (normal range 135.0–215.0 IU/L), C-reactive protein 52.50 mg/L (normal range 0.00–80.00 mg/L), calcium 2.09 mmol/L (normal range 2.00–2.60 mmol/L); and positive antibody for hepatitis B virus, and negative antibody for hepatitis C virus. Serology for HIV was negative. The levels of tumor markers, such as alpha-fetoprotein, carcinoembryonic antigen, and carbohydrate antigen 19-9 were within normal limits. Chest X-ray did not reveal any abnormalities. On ultrasound, the lesion showed heterogeneous hypo-echoicity relative to normal hepatic parenchyma in the right liver and its border was clear, dotted blood flow could be seen around the edge of the lesion on Color Doppler imaging. Computed tomography showed a solid, cystic hypodense lesion in the right hepatic lobe measuring 10.0 cm \times 6.1 cm on plain image. The enhancement image after injection of iodinated contrast revealed mild rim enhancement in the arterial phase. Obvious enhancement in the portal venous phase was observed (Figure 1). Primary hepatocarcinoma, liver abscess, and metastasis from the gastrointestinal tract were initially suspected. In order to make an accurate diagnosis and stage the lesion, the patient was referred for a whole body positron emission tomography/computed tomography (PET/CT) scan to identify other sites of involvement. The patient was injected with 6.1 millicuries of ^{18}F -fluorodeoxyglucose (FDG) and after 60 min of uptake time, she underwent a whole body scan in a dedicated PET/CT scanner. An abnormal ring-like metabolic focus in the right liver lobe was observed, with a maximum FDG uptake of 17.7 (Figure 2), the center exhibited less FDG uptake, and other body sites

were negative. Hepatocarcinoma and liver abscess were suspected. In order to establish a diagnosis, ultrasound-guided percutaneous liver biopsy was performed, and five strips of liver tissue were extracted from the lesion. However, examination of the tissue was inconclusive as it showed fibroplasia and hyaline degeneration interspersed with lymphocytes. The patient underwent open laparotomy and partial hepatectomy for diagnosis and treatment.

There was no significant mesenteric or retroperitoneal lymphadenopathy, no ascites were observed in the abdominal cavity, and the gastrointestinal tract was normal. The tumor was located in segments IV and I of the right liver lobe adhered to the hepatic flexure of the colon and renal capsule.

The intraoperative frozen section revealed a small round cell tumor. Microscopic histopathological examination showed a multinucleated giant cell tumor and hepatic sinusoidal infiltration, interstitial lymphocyte reactive hyperplasia with spotting hemorrhagic areas and necrosis. The surrounding liver tissues revealed intrahepatic cholestasis and lymphocytic infiltration around the bile ducts. Immunohistochemical staining was positive for CD20, Bcl-6, Mum-1 and Ki-67 (> 80%), and negative for CD30, Bcl-2, and cytokeratin (Figure 3). The diagnosis of diffuse B-cell lymphoma originating from the germinal center was made.

The patient was discharged 2 wk after surgery, and did not receive chemotherapy or radiotherapy. After 25 mo follow-up, she was in good health.

DISCUSSION

PHL, which is defined as a lesion or lesions confined to the liver only without the involvement of any other organ or lymph nodes, is extremely rare, accounting for less than 0.01% of all non-Hodgkin's lymphomas, and can occur in any age group but is usually found in middle-aged men. The pathogenesis of PHL is unclear, and it is usually seen in organ transplant recipients, in patients receiving immunosuppressive therapy and in individuals with AIDS. In recent years, studies have indicated that hepatitis C infection is strongly related to PHL, a possible cause is hepatitis C virus stimulating B lymphocytes and chronic polyclonal proliferation, leading to liver lymphoma^[2,3].

The clinical manifestations are atypical including fever, weight loss, night sweat and right upper abdominal pain. Lactate dehydrogenase and blood calcium are sometimes elevated^[4,5]. Primary hepatic lymphoma presenting as fulminant hepatic failure with hyperferritinemia was described by Haider *et al*^[6]. The most frequent pathology in PHL is diffuse large B cell followed by small lymphocytic, T cell, follicular and marginal B cell lymphoma. PHL responds to therapy and may have a better prognosis than hepatocellular carcinoma, as it is chemosensitive, and early aggressive combination chemotherapy may result in sustained remission^[7].

Primary hepatic lymphoma has its own characteristic imaging features. It can be classified into 3 morphologic



Figure 1 Obvious enhancement in the portal venous phase was observed. A: A hypodense area in the right liver lobe was seen on plain computed tomography; B: The hypodense lesion showed mild rim enhancement in the arterial phase; C: Obvious enhancement was seen in the portal phase.

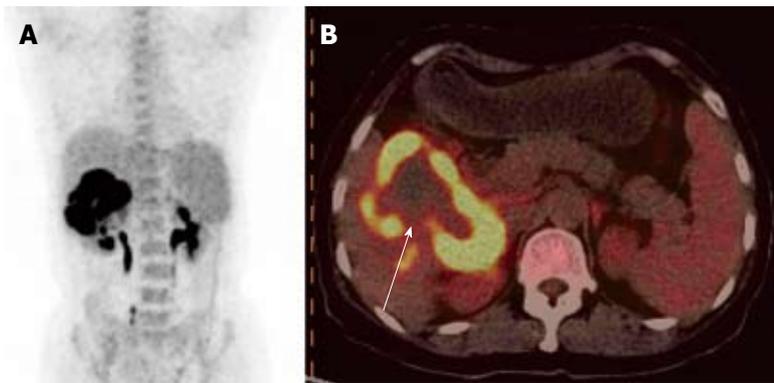


Figure 2 An abnormal ring-like metabolic focus in the right liver lobe was observed, with a maximum fluorodeoxyglucose uptake of 17.7. A: Positron emission tomography/ computed tomography imaging showed a high metabolic focus in the right liver lobe; B: Fusion imaging revealed a ring-like high uptake focus with lower uptake in the center of the lesion (as shown by the white arrow).

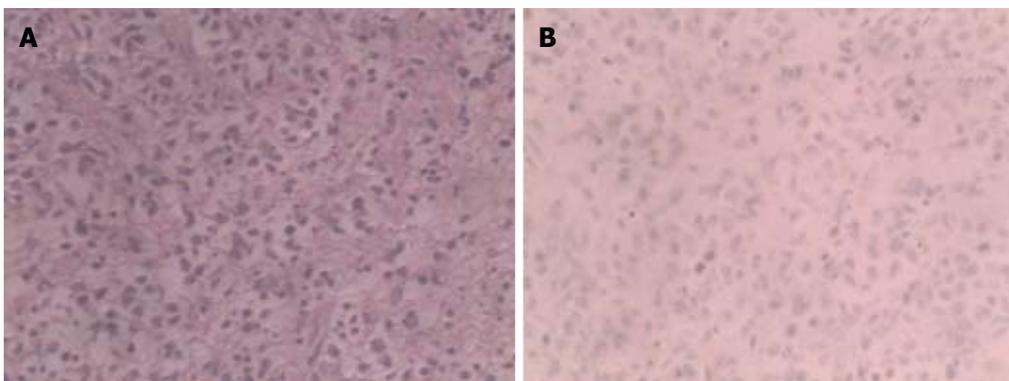


Figure 3 Immunohistochemical staining was positive for CD20, Bc1-6, Mum-1, Ki-67 and negative for CD30, Bcl-2, cytokeratin. A: The normal structure of liver tissue was damaged, and showed dysplastic, almost naked nuclear lymphocytes, diffuse infiltration of liver tissue, tumor cells were mainly composed of round and oval cells, and spindle, polygonal, and multinucleated giant tumor cells were also seen (hematoxylin and eosin, $\times 400$); B: Bcl-6 was positive, and CD10 as well as Mum-1 were negative (immunohistochemistry, $\times 400$).

patterns: solitary liver mass, multiple focal nodules and diffuse infiltrative disease, the first 2 patterns are the most common^[8], and an imaging study showed a solitary space-occupying lesion or multiple heterogeneous lesions which were well-defined suggesting hepatocarcinoma or metastasis from the gastrointestinal tract^[9]. Elsayes *et al*^[10] described 12 cases of PHL, three of which presented with a single focal lesion (25%), eight (67%) patients presented with multiple well-defined lesions, and one patient (8%) presented with diffuse hepatic involvement on CT imaging. The lesions in three patients demonstrated rim enhancement following intravenous iodinated contrast administration. The features of rim enhancement were

similar to those in our case. PHLs are usually hypoechoic relative to normal liver on ultrasound imaging, and in a minority of cases, the lesions can be anechoic, hypoechoic areas with high perinodular and low intranodular vascularization on Doppler sonography, which are easily misdiagnosed as angiomas^[11,12]. On contrast-enhanced ultrasonography, the lesion has mild inhomogeneous hyperenhancement in the arterial phase and wash-out in the portal and late phases^[13]. On magnetic resonance imaging (MRI), PHLs usually have moderate to high signal intensity on T2-weighted images and are mild to moderate hypointense relative to liver on T1-weighted images^[14,15]. The enhanced image is variable, mainly manifestations

of early intense ring or predominantly ring enhancement. The imaging features of PHL on ultrasound, MRI and CT have been reported, while the imaging features of PHL on PET and single photon emission computed tomography are relatively rare (the number of PET or PET/CT studies is small compared to the large number of ultrasound CT and MRI studies on PHL). PET/CT scanning which can produce whole body imaging data and distinguish primary liver lesions from metastatic disease is superior to CT, MRI and ultrasound which only visualize a limited portion of the body. Thus, PET/CT is advantageous in the diagnosis and treatment of liver lymphoma. To the best of our knowledge, three cases of multiple PHL and one case of diffuse infiltrative disease have been described using PET/CT imaging. While diffuse infiltrative disease is extremely rare, Kang *et al*^[16] reported one case using PET imaging in the English literature, this type of PHL is easily misdiagnosed as hepatitis or diffuse cholangiocellular carcinoma. However, a solitary PHL on PET/CT imaging has not been reported. Whether primary or secondary lymphoma of the liver is present, imaging with FDG or Ga67 PET showed high uptake. In our case report, the lesion demonstrated a ring-like hypermetabolic area, which suggested tumor viability. The level of isotope uptake may correlate with disease activity and tumor proliferation^[17,18].

In conclusion, PHL is a rare disease, its clinical symptoms, laboratory results, and imaging results are non-specific, therefore, it is difficult to establish a diagnosis immediately. Thus, when a solitary hypermetabolic lesion on PET/CT scanning is found in the liver and no other organ or tissue is involved, PHL should be considered, hepatoma and liver abscess should be excluded and a liver biopsy performed for further management.

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May 19-22, 2012
 2012-Digestive Disease Week
 San Diego, CA 92121, United States

June 2-6, 2012
 American Society of Colon and
 Rectal Surgeons Annual Meeting
 San Antonio, TX 78249,
 United States

June 18-21, 2012
 Pancreatic Cancer: Progress and
 Challenges
 Lake Tahoe, NV 89101, United States

July 25-26, 2012
 PancreasFest 2012
 Pittsburgh, PA 15260, United States

September 1-4, 2012
 OESO 11th World Conference
 Como, Italy

September 6-8, 2012
 2012 Joint International

Neurogastroenterology and Motility
 Meeting
 Bologna, Italy

September 7-9, 2012
 The Viral Hepatitis Congress
 Frankfurt, Germany

September 8-9, 2012
 New Advances in Inflammatory
 Bowel Disease
 La Jolla, CA 92093, United States

September 8-9, 2012
 Florida Gastroenterologic Society
 2012 Annual Meeting
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September 15-16, 2012
 Current Problems of
 Gastroenterology and Abdominal
 Surgery
 Kiev, Ukraine

September 20-22, 2012
 1st World Congress on Controversies
 in the Management of Viral Hepatitis
 Prague, Czech

October 19-24, 2012
 American College of
 Gastroenterology 77th Annual
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 Course
 Las Vegas, NV 89085, United States

November 3-4, 2012
 Modern Technologies in
 Diagnosis and Treatment of
 Gastroenterological Patients
 Dnepropetrovsk, Ukraine

November 4-8, 2012
 The Liver Meeting
 San Francisco, CA 94101,
 United States

November 9-13, 2012
 American Association for the Study
 of Liver Diseases
 Boston, MA 02298, United States

December 1-4, 2012
 Advances in Inflammatory Bowel
 Diseases
 Hollywood, FL 33028, United States

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1352 experts in gastroenterology and hepatology from 64 countries.

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Acknowledgments

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Chinese journal article (list all authors and include the PMID where applicable)

- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

In press

- 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

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

- 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]

No author given

- 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]

Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

- 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]

No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS A Careaction* 2002; 1-6 [PMID: 12154804]

Books*Personal author(s)*

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 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

Author(s) and editor(s)

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiecezorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

- 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

Electronic journal (list all authors)

- 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>

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean \pm SD or mean \pm SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as ν (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 $24.5 \mu\text{g/L}$; CO₂ volume fraction, 50 mL/L CO₂, not 5% CO₂; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

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