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EDITORIAL

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.

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 *et al*[14]	Steroid-naïve 8 (steroid-refractory 31)	Adacolumn 11/11	Naïve 88 (refractory 81)
Suzuki *et al*[15]	20	Adacolumn 5-10/2.5-5	85
Tanaka *et al*[25]	Steroid-naïve 26 (steroid-dependent 19)	Adacolumn 11 /12	Naïve 85 (dependent 58)
Nishioka *et al*[26]	9	Cellsorba 10 /10	33 (89 improved)
Umehara *et al*[27]	18	Cellsorba 5 /5	61

[1]Remission 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*[24], 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 difference 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 suggest 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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TOPIC HIGHLIGHT

Giovanni Tarantino, Professor, *Series Editor*

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, PPARγ, 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.

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 physiopathology 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 ($O_2^{\bullet}$) 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 (PPARγ) 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., PPARα, PPARγ, SREBP-1, ChREBP), can control key pathways involved in hepatic lipid metabolism[30,31]. In more detail, n-3 PUFAs are potent activators of PPARα, 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 PPARγ, 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 NALFD. 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 (PPARγ), 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 PPARα, 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], PPARα regulates fatty acid binding and their export in very low density lipoprotein[65,66]. In PPARα (-/-) knockout animals, hepatic steatosis, which was minimal under normal conditions, drastically increased after a fasting period. The absence of PPARα 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-β1, 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 demonstarted 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) × 3/daily, while Group B received the recommended diet and 2 g placebo × 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 *et al*[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 *et al*[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	Limits of the study are lack of placebo, and the non blinding of participants and investigators
Zhu *et al*[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	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)	
Tanaka *et al*[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 *et al*[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%)	

Nobili *et al*[78]	Randomized	DHA (250 and 500 mg/d) *vs* placebo for 6 mo	60 children with biopsy-proven NAFLD randomly assigned to receive DHA 250 mg/d (n = 20), DHA 500 mg/d (n = 20) or placebo (n = 20)	Primary: change in liver fat content as detected by US; secondary: changes in ISI, ALT, TG and BMI	DHA 250 mg *vs* placebo: odds of more severe *vs* less severe steatosis (OR = 0.01, robust 95% CI: 0.002 to 0.11, P < 0.001); ↑ of ISI (P < 0.01), ↓TG (P < 0.05); ALT and SDS of BMI; DHA 500 mg *vs* placebo: (OR = 0.04, 0.002 to 0.46; P = 0.01); ↑ of ISI (P < 0.01), ↓TG (P < 0.05); ALT and SDS of BMI; DHA 250 mg *vs* DHA 200 mg: NS	
Vega *et al*[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% (P < 0.03), VLDL + IDL by 21% (P < 0.03), ApoB by 15% (P < 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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GUIDELINES FOR BASIC SCIENCE

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.

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-1α, IL-1β, 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-1α and IL-1β. For both IL-1α 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-1β 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-1β, 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 if 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-1β, 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-4Rα and IL-13Rα2, 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-β1 administration or the inhibition of STAT6, a key adaptor molecule in IL-13 signaling, by small interfering RNA or a histone deacetylase inhibitor result in significant epithelial healing, supporting the aforementioned hypothesis[67-69].

Recently, it was also shown that IL-13 signaling through IL-13Rα2 led to the increase of transforming growth factor (TGF)-β1 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 $IL\text{-}17A^{-/-}$ $CD45RB^{hi}$ CD4+ T cells correlated with higher expression of genes encoding Th1 type cytokines in colon tissue. Furthermore, $IL\text{-}17RA^{-/-}$ 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 proprial 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-β1 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-βRII, phosphorylates sma-and mothers against decapentaplegic-related protein transcription factors that have primarily immunosuppressive function[101]. Genetic mutations in TGF-βRII 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-βDNRII 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-βRII 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+ ββ 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 form 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-1β, 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 proprial 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 EBI3 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 immunity. 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-1β, 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
Interleukin-6 family	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 ↑
	IL-35	Possible controlling of T-cell dependent inflammation	NI
Interleukin-13	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 invloved 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[149]. 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-

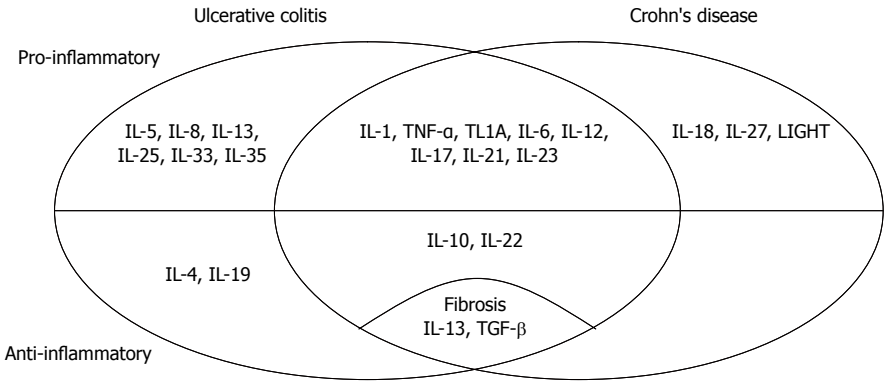

Figure 1 The disease-related pathogenic role of cytokines with pro-inflammatory, anti-inflammatory and pro-fibrogenic effects in ulcerative colitis and Crohn's disease. LIGHT: Lymphotoxins, exhibits inducible expression, and competes with herpes sipmlex virus *g*lycoprotein D for herpesvirus entry mediator, 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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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 investigations 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 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.

Key words: Children; Adolescents; Crohn's disease; Ulcerative colitis; Inflammatory bowel diseases

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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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ORIGINAL ARTICLE

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.

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 intratissue 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 times × 373 K / 273 K = 1700 times. Protein coagulation occurs at a temperature of approximately 60 ℃, while vaporization occurs above 100 ℃. 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 intratissue 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

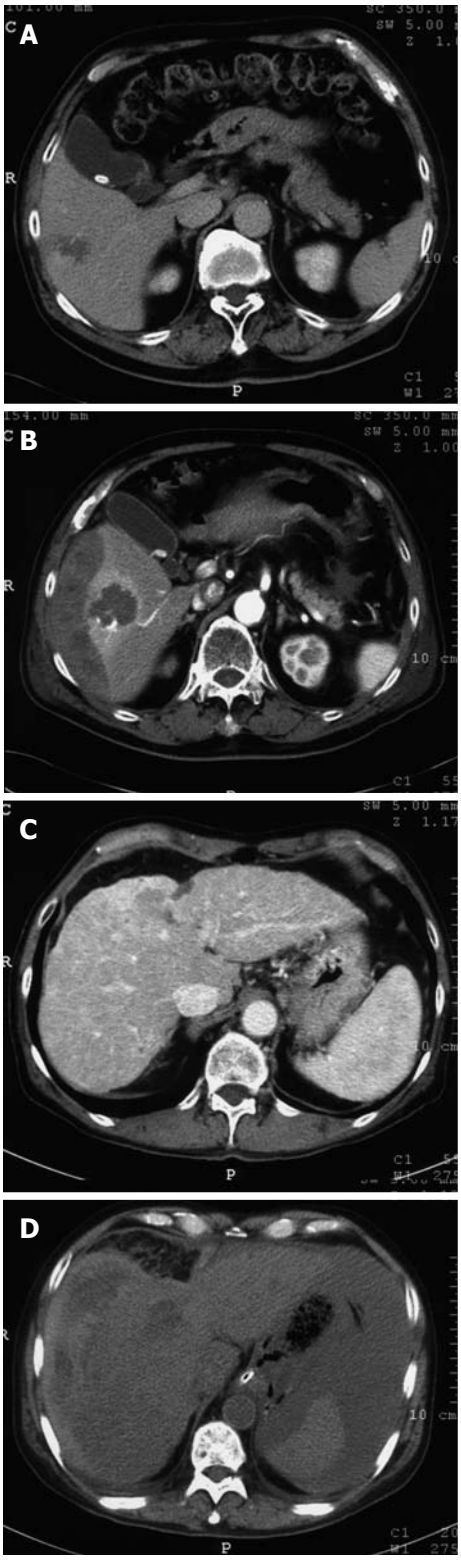

Figure 1 A case showing subcapsular (A, B) and intraperitoneal (C, D) hemorrhage after popping, which occurred during radiofrequency ablation for hepatocellular carcinoma. A, C: Before ablation; B, D: After ablation. RFA: Radiofrequency ablation; HCC: Hepatocellular carcinoma.

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 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.

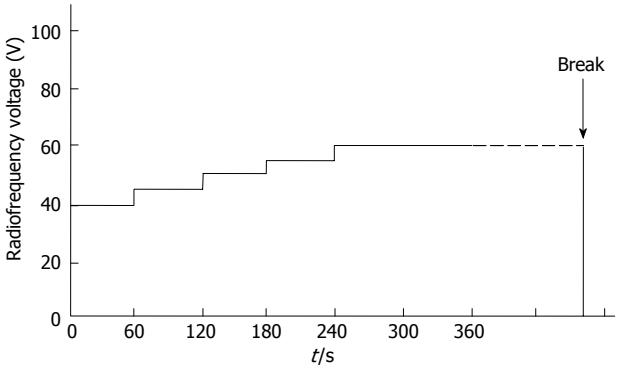

Figure 2 The ablation protocol to avoid popping involves the initiation of power delivery from 40 V with increases of 5 V every minute to a maximum of 60 V until a break is observed.

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 (℃), and impedance (Ω) simultaneously (Figure 3).

Statistical analysis

All statistical analyses were conducted using JMP 8.0.2

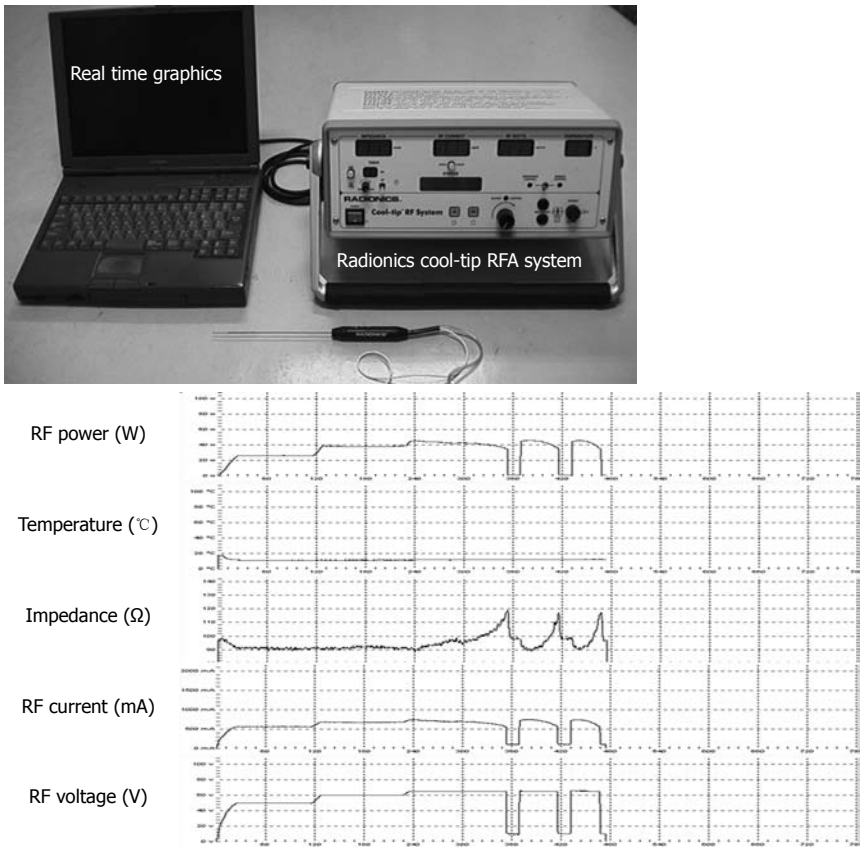

Figure 3 Radio frequency power, radio frequency current, radio frequency voltage, temperature and impedance were intraoperatively monitored using the equipment as shown in the diagram. RF: Radio frequency.

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

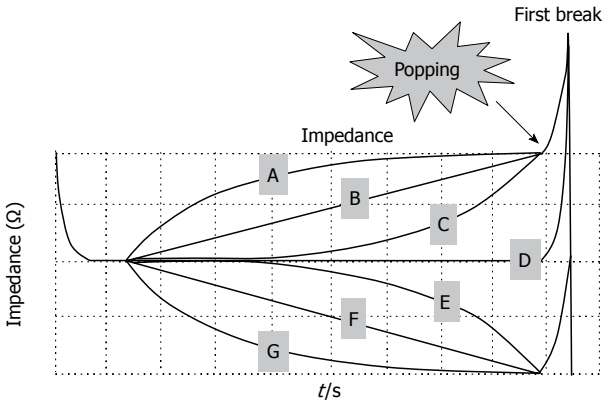

Figure 4 The graph showing the characteristic impedance curve shapes leading to the break point. The graph showing the seven characteristic impedance curves leading to the break point, which dictated the subgroups into which the previously classified nodules were further sorted. A-C: Increasing type; 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 (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.

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	(0,1,0,0,0,0,0,1)
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). [1]Data are means ± SD; [2]Data are numbers of nodules. ICGR 15: Indocyanine green retention rate at 15 min.

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

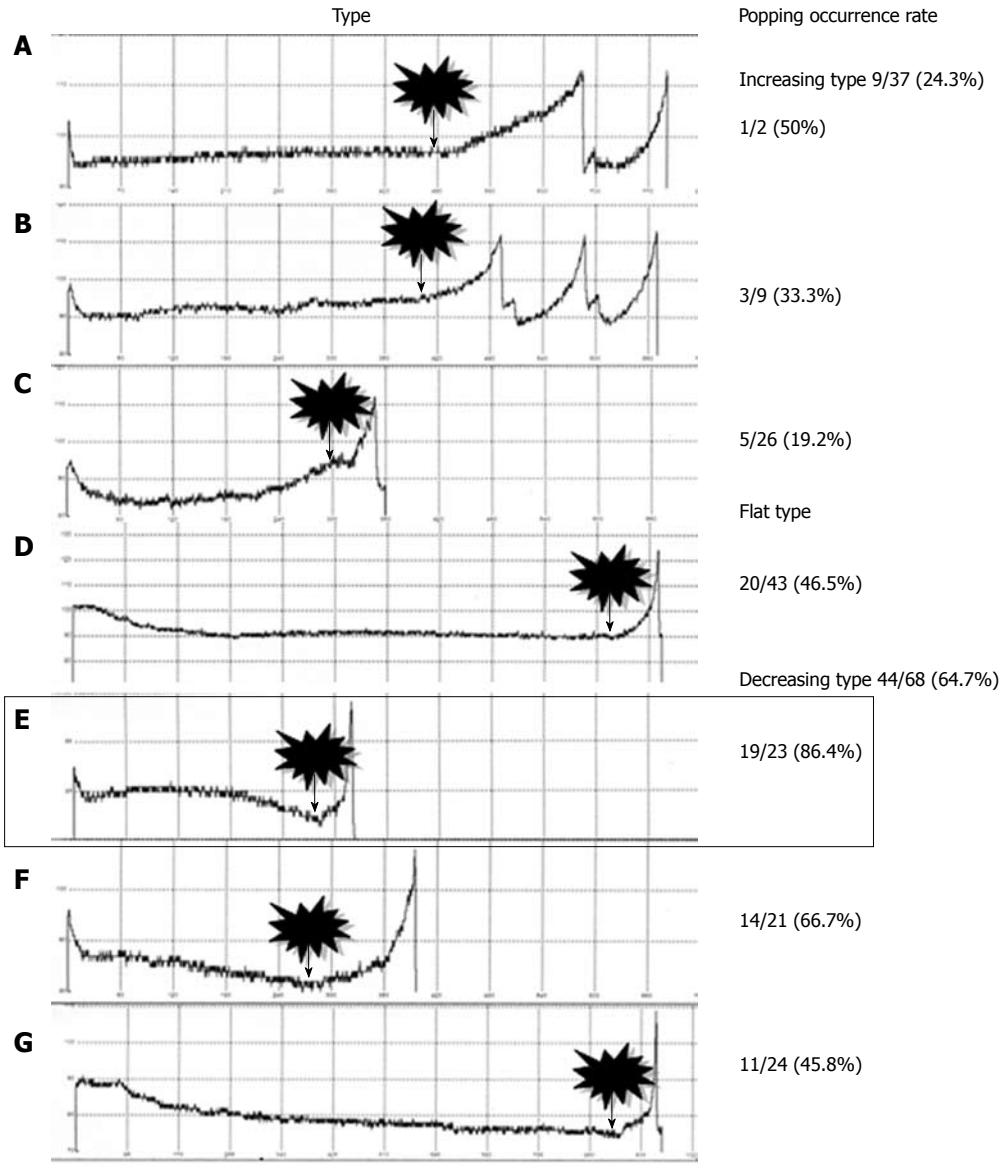

Figure 5 Subtype E (nodules that showed a rapid decrease in impedance during the latter half of the ablation) exhibited a significantly higher rate of popping compared to subtypes B, C, D and G (*P* < 0.05). Decreasing-type nodules exhibited a significantly higher rate of popping compared to increasing-type 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 (℃), and k_{25} is the electrical conductivity at 25 ℃. 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 ℃ 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 ℃. However, if the temperature rises above 100 ℃ 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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ORIGINAL ARTICLE

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 m^2)], 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.

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

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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 μg/kg or peg-IFN α-2a at a dose of 180 μg 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 ≤ 60 kg, 800 mg for > 60 kg to ≤ 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) × BW (kg)]/(72 × Scr) (Scr, serum creatinine; Ccr × 0.85 for female). Estimated glomerular filtration rate (GFR, mL/min/1.73 m^2) was

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

Variable	Overall cohort (*n* = 561)	Derivation group (*n* = 374)	Confirmatory group (*n* = 187)
Demographic feature			
Age (yr)	59.1 ± 10.9	59.1 ± 10.8	59.5 ± 11.3
Sex (female/male)	302/259	201/173	101/86
Weight (kg)	59.8 ± 11.4	59.9 ± 11.5	58.5 ± 10.9
BMI (kg/m^2)	23.2 ± 3.3	23.2 ± 3.4	23.2 ± 3.2
Height (cm)	160.3 ± 9.0	160.4 ± 9.1	158.5 ± 8.7
BSA (m^2)	1.62 ± 0.18	1.62 ± 0.18	1.59 ± 0.18
Laboratory data			
ALT (IU/L)	63 ± 54	63 ± 56	58 ± 47
GGT (IU/L)	58 ± 63	56 ± 60	58 ± 72
Albumin (g/dL)	4.1 ± 0.4	4.1 ± 0.4	4.1 ± 0.3
Creatinine (mg/dL)	0.70 ± 0.16	0.70 ± 0.17	0.70 ± 0.17
WBC count (× 10^3/mL)	5.0 ± 1.5	5.0 ± 1.5	5.1 ± 1.4
Hemoglobin (g/dL)	13.7 ± 1.5	13.7 ± 1.5	13.8 ± 1.3
Platelet count (× 10^4/mL)	16.7 ± 5.7	16.5 ± 5.7	17.0 ± 6.2
Estimated calculation value			
Ccr (mL/min)	91.8 ± 27.9	91.6 ± 28.0	87.9 ± 25.6
GFR (mL/min/1.73 m^2)	77.5 ± 16.8	79.6 ± 17.0	78.2 ± 15.9
CL/F (L/h)	11.3 ± 5.4	11.2 ± 5.3	10.9 ± 5.1
Liver histopathology			
Stage of fibrosis 0-1/2/3-4	221/131/161	146/89/107	75/42/54
Grade of inflammation 1/2/3	267/222/19	170/156/14	97/66/5
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 ± 1.5	11.3 ± 1.9	11.4 ± 1.9
Peg-IFN α-2a/-2b	82/479	52/322	30/157
Virology			
Viral load (log10 IU/mL)	6.2 ± 0.8	6.2 ± 0.8	6.2 ± 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 × Scr$^{-1.094}$ × Age$^{-0.287}$ (GFR × 0.739 for female). Apparent clearance of ribavirin (CL/F, L/h) was determined as follows[9]: CL/F =32.3 × BW × (1 - 0.0094 × age) × (1 - 0.42 × gender)/Scr (gender = 0 for male and 1 for female; Scr is in μmol/L). All liver biopsy specimens were reviewed by using the established ranking system for staging of fibrosis and grading of necroinflammation activity with some modification[22].

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, $\cdots$, x_p):

$S = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \cdots + \beta_p x_p$ (β_0: Intercept, β_1, β_2, $\cdots$, β_p: 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 the groups. Most of the patients complained of dyspnea on effort, easy fatigability or lightheadedness. None received erythropoiesis-stimulating agents throughout the treatment period.

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

Variable	*P* 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 (log10 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.

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 ($P = 4.71 \times 10^{-4}$), higher Hb ($P = 7.75 \times 10^{-8}$), lower GFR ($P = 5.69 \times 10^{-4}$), and SNP rs1127354 major

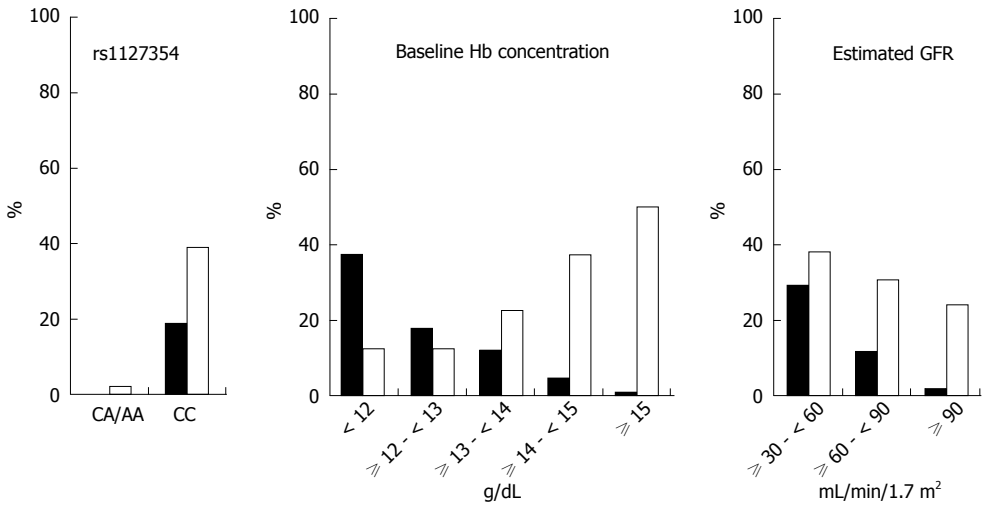

Figure 1 Anemic event rates in subset groups of each significantly independent baseline factor. The inosine triphosphatase single nucleotide polymorphism rs1127354 genotype, baseline hemoglobin (Hb) concentration and estimated glomerular filtration rate (GFR) were significantly associated with anemic 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 m^2), 95%CI: 0.942-0.977]. The model was expressed as: S = -9.369 + 0.635 × baseline Hb + 3.342 × SNP rs1127354 (where genotype CC was 1 and CA/AA was 0) -0.041 × 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 m^2), 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 ($P = 5.437 \times 10^{-9}$).

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

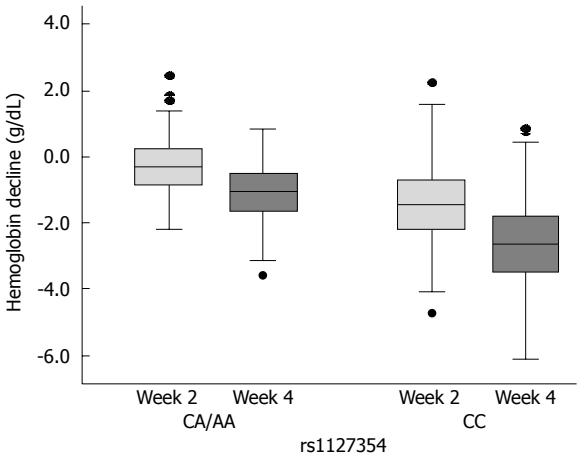

Figure 2 Hemoglobin decline from baseline at week 2 and 4 of treatment according to the inosine triphosphatase single nucleotide polymorphism rs1127354 genotypes. Bars within boxes denote the median value of hemoglobin (Hb) decline from baseline. The boxes and the lower and upper bars represent the 25th 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).

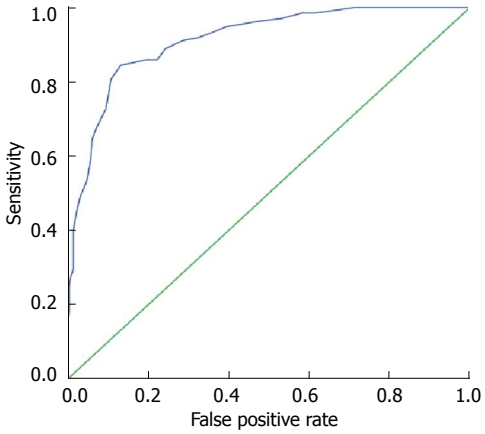

Figure 3 Receiver operating characteristic curves generated with every cut-off point of predicted probability of significant hemoglobin decline (> 3.0 g/dL at week 4 of treatment) corresponding to each hemoglobin decline from baseline at week 2 of treatment. The area under the curve was 0.913 (95% confidence 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 [$P = 2.16 \times 10^{-4}$, OR = 0.962 (mL/min/1.73 m^2), 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 × Hb decline at week 2 -0.039 × GFR + 2.393 × SNP rs1127354 (where genotype CC was 1 and CA/AA was 0) + 0.405 × 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.

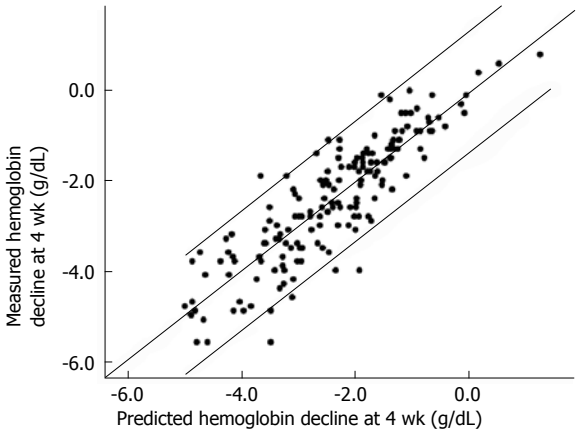

Figure 4 Correlation between predicted and measured values of hemoglobin decline at week 4 of treatment. Predicted values were yielded by the multiple linear regression model that was constructed in the derivation group. Measured values were derived from the confirmatory group. There was a significant correlation between predicted and measured values (Spearman's ρ = 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.

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 × Hb decline at week 2 - 0.878 × SNP rs1127354 (where genotype CC was 1 and CA/AA was 0) - 0.178 × baseline Hb + 0.012 × 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 ρ = 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 × SNP rs1127354 (where genotype CC was 1 and CA/AA was 0) - 0.276 × baseline Hb + 0.008 × 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 ⩾ 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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ORIGINAL ARTICLE

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% ± 4.0% *vs* 87.0% ± 7.8%, $P < 0.01$) by deslanoside in 8 low contractile states, and inhibitory effects were induced (180.6% ± 17.8% *vs* 109.9% ± 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% ± 4.1% *vs* 96.0% ± 8.1%, $P < 0.01$) and high contractile state (139.2% ± 8.5% *vs* 105.5 ± 7.34, $P < 0.01$) was also bidirectional. Bidirectional 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.

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 ℃-4 ℃) 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 ℃, 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 μmol/L)-ACh (5.0 μmol/L) Krebs buffer, quercetin (10.0 μmol/L)-capsaicin (10.0 μmol/L) and nitric oxide (NO) donor sodium nitroprusside (SNP) (5 μmol/L)-erythromycin (10 μmol/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 μmol/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 μmol/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 ℃ 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 Ca^{2+}-free assay condition, and 20 μmol/L deslanoside did not stimulate the contractility of JSMF pre-incubated with the Ca^{2+}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

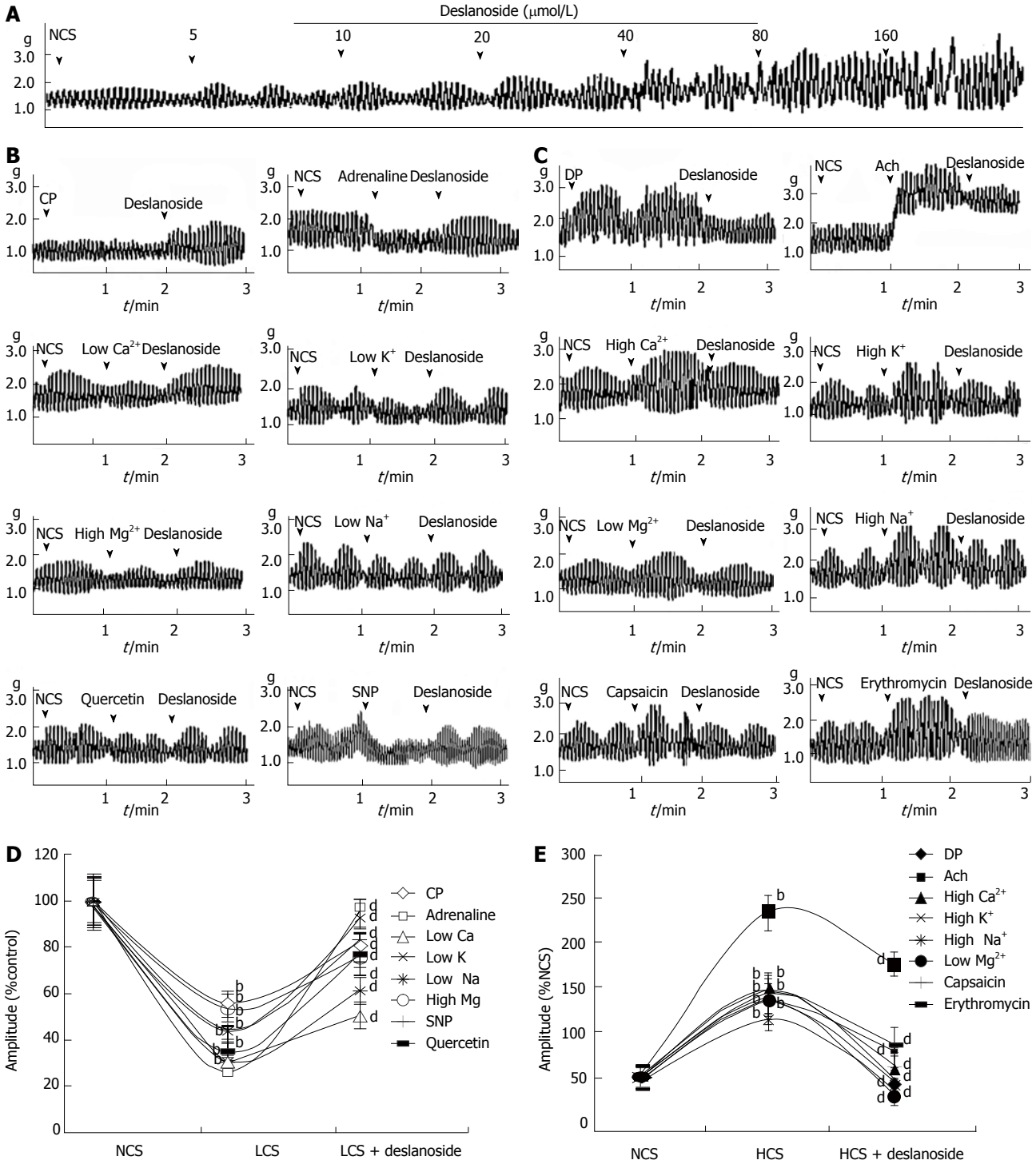

Figure 1 Deslanoside-induced bidirectional regulation on the contractility of jejunal smooth muscle fragment. A: Dose response relationship of caslano side; B, D: Representative traces and statistical analysis of total traces from six independent experiments of deslanoside-induced bidirectional regulation 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, $n = 6$); $^{b}P < 0.01$ *vs* the control; $^{d}P < 0.01$ *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

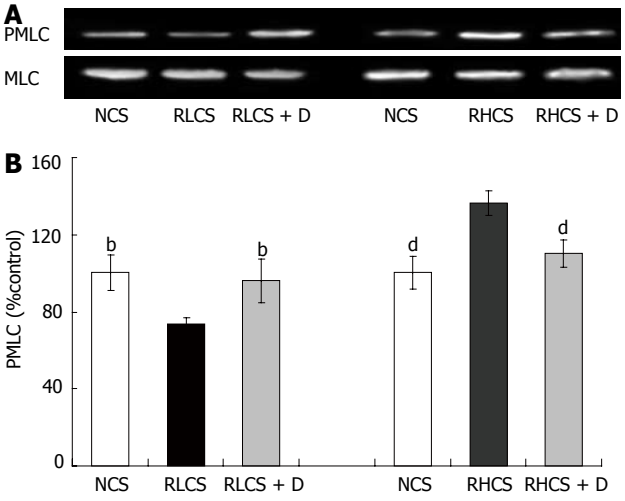

Figure 2 Western blotting analysis of the phosphorylation of myosin light chain. A: Representative images of Western blotting of the phosphorylation of myosin light chain (PMLC) of jejunum; B: Statistical analysis of band intensities of the PMLC in 4 independent experiments in normal contractile state (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 ± SE from 4 independent experiments; $^{b}P < 0.01$ *vs* RLCS; $^{d}P < 0.01$ *vs* RHCS. D: Deslanoside.

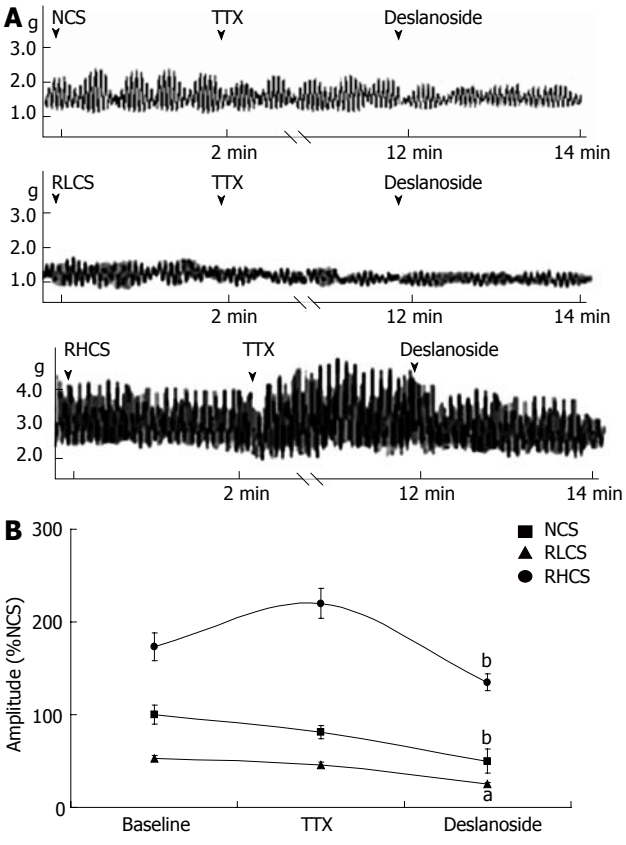

Figure 3 Effects of deslanoside on the contractility of jejunal smooth muscle fragment pretreated with tetrodotoxin. A: Representative traces of deslanoside on the contractility of jejunal smooth muscle fragment (JSMF) pre-treated with tetrodotoxin in normal contractile state (NCS), representative low 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 ± SE (%NCS, $n = 6$); $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* contractile amplitude of JSMF after treatment with tetrodotoxin (TTX), (0.1 μmol/L).

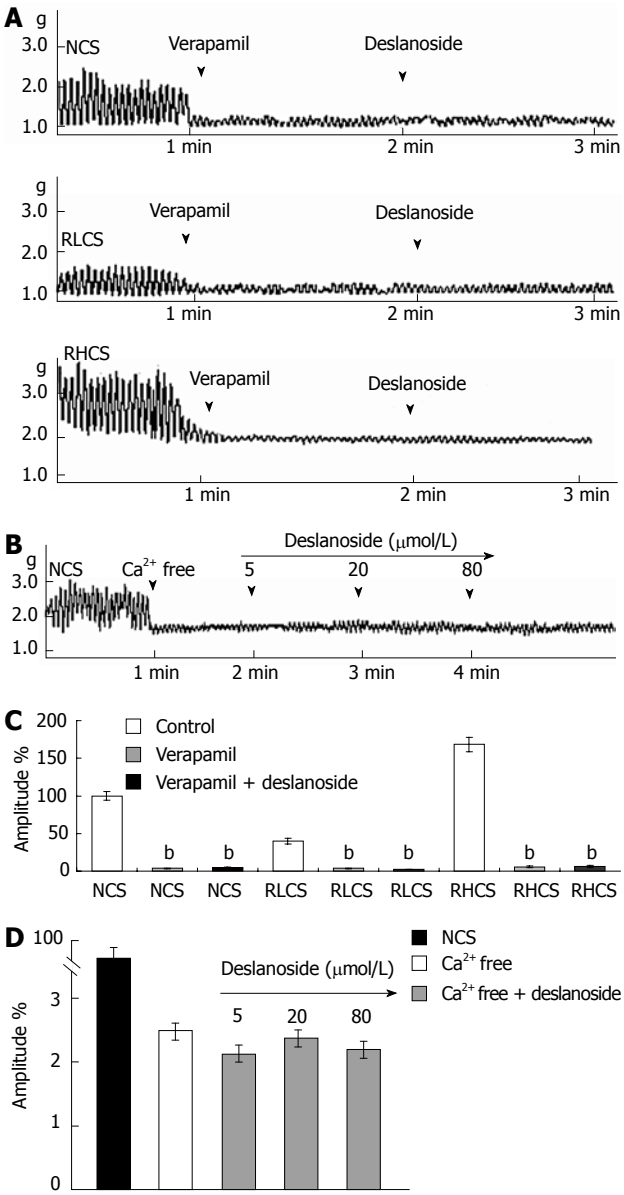

Figure 4 Effects of deslanoside on the contractility of jejunal smooth muscle fragment in Ca^{2+}-free conditions. A: Representative traces of deslanoside (20 μmol/L) on the contractility of jejunal smooth muscle fragment (JSMF) pre-treated with verapamil (0.1 μmol/L) in normal contractile state (NCS), representative low contractile state (RLCS) and representative high contractile state (RHCS); B: Representative traces of deslanoside (5-80 μmol/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 ± 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-

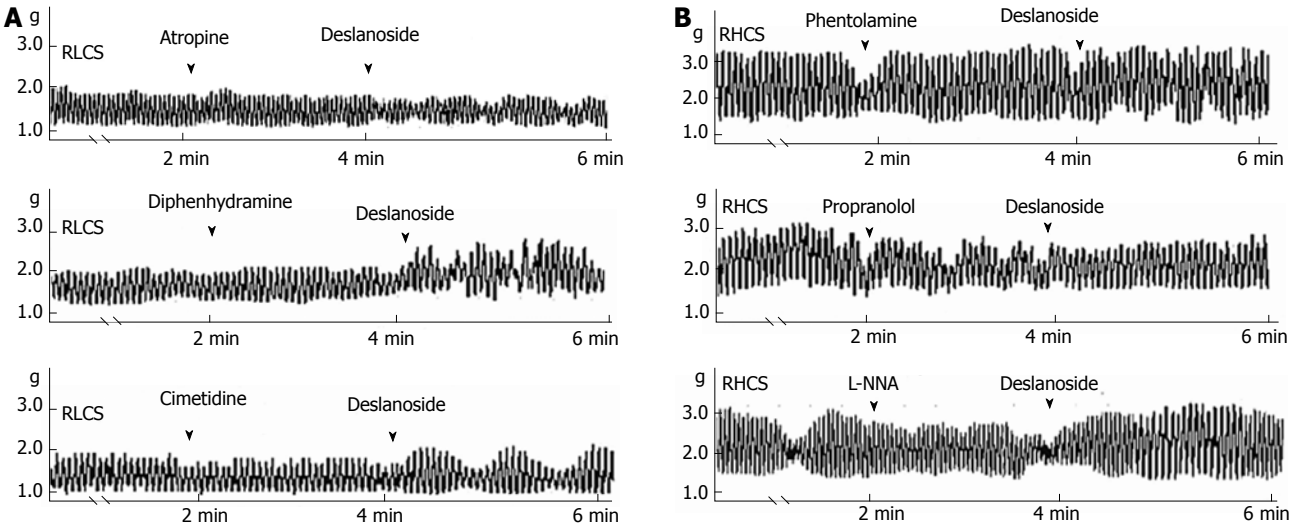

Figure 5 Effects of deslanoside on the contractility of jejunal smooth muscle fragment pretreated with receptor antagonist. A: Effects of deslanoside on the contractility of jejunal smooth muscle fragment (JSMF) pretreated with 10 μmol/L atropine, 10 μmol/L diphenhydramine and 10 μmol/L cimetidine in the representative low contractile state (RLCS), respectively; B: Effects of deslanoside on the contractility of JSMF pretreated with 10 μmol/L phentolamine, 5 μmol/L propranolol and 10 μmol/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 JMSF 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.

ACKNOWLEDGMENTS

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 Ca^{2+} 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 (hypo-motility), 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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ORIGINAL ARTICLE

Expression characteristics and diagnostic value of annexin A2 in hepatocellular carcinoma

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Author contributions: Zhang HJ and Yao M contributed equally to this work, designed the research, analyzed the data, and wrote the first draft; Yan XD and Chen J collected liver specimens; Yan MJ performed quantitative real time polymerase chain reaction, Western blotting, and enzyme-linked immunosorbent assay analysis; Wu W and Huang H performed immunohistochemistry and evaluation; Yao DF is the guarantor and contributed to the design and interpretation of the study.
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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 cancer, 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.

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

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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 (Uscn Life Science Inc., Wuhan, China) according to the manufacturer's instructions. To each well was added 100 μL of serum sample or standard separately, and then 100 μL of detection reagent A was

added and incubated for 1 h at 37 ℃. Subsequently, 100 μL of detection reagent B was added and incubated for 0.5 h at 37 ℃. Then, 90 μL of substrate solution was added and incubated for 25 min at 37 ℃. Finally, 50 μL 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 μg 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 μL 2 × SYBR Premix Ex Taq (TaKaRa, Japan), 2 μL primer mix, 1 μL 50 × ROX Reference Dye I, 4 μL cDNA, and 18 μL deionized water to make a total volume of 50 μL. ANXA2 primers were as follows: forward, 5'-TGAGCGGGATGCTTTGAAC-3', and; reverse, 5'-ATCCTGTCTC 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 (H_2O) was included in each reaction run. The optimized PCR conditions were as follows: 1 cycle at 95 ℃ for 2 min; 40 cycles at 95 ℃ for 10 s, 62 ℃ for 1 min and final extension at 60 ℃ 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 $MgCl_2$, 0.2 mmol/L dithiothreitol, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L Na_3VO_4, 20 mmol/L sodium pyrophosphate, 20 mmol/L β-phosphoglycerol, 1 mmol/L p-nitrophenyl phosphate, 1 mmol/L benzamidine, 1 mmol/L phenylmethylsulfonyl fluoride, and 5 μg/mL each of leupeptin, aprotinin, and pepstatin A. The homogenates were centrifuged at 800 *g* for 10 min at 4 ℃. 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. $RR = SI_{ANXA2}/SI_{\beta\text{-actin}}$.

Immunohistochemistry for ANXA2

The 3 μm 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 μm 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 ℃ 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 ± SD. Differences between different groups were evaluated by using a Student

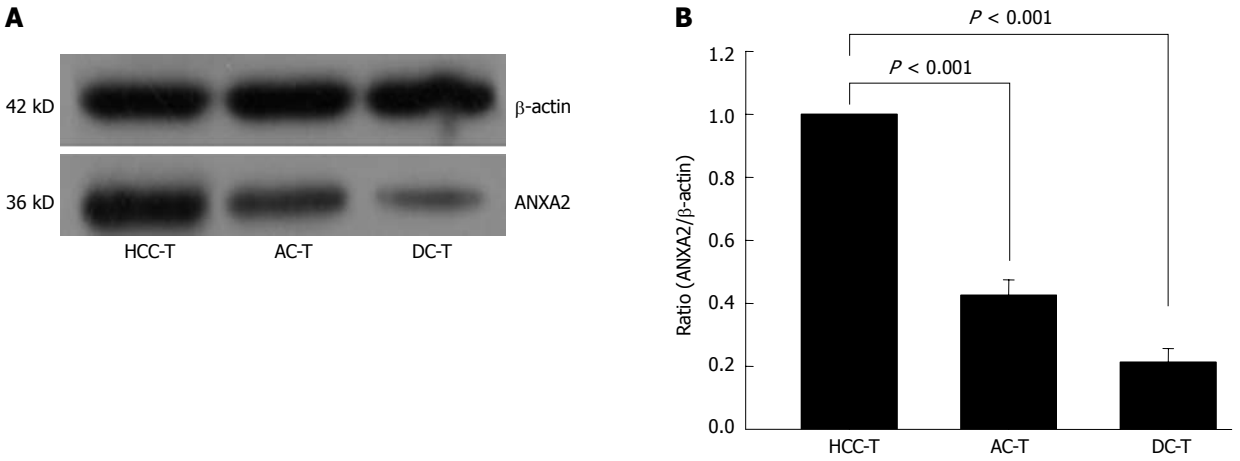

Figure 1 Annexin A2 protein level in liver tissue of hepatocellular carcinoma. A: Representative images of Western blotting. The protein levels of annexin A2 (ANXA2) and β-actin were detected and the latter served as the control; B: The statistical results indicated that the level of ANXA2 expression 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 ± 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 ± SD)

Group	Ct_{ANXA2}	$Ct_{β\text{-actin}}$	ΔCt	$2^{-\Delta\Delta Ct}$	Annexin A2 intensity				Z
					-	+	++	+++	
HCC tissues	21.63 ± 0.09	21.19 ± 0.07	0.44 ± 0.06	1	0	1	7	22	
Adjacent cancerous tissues	24.29 ± 0.09	22.60 ± 0.08	1.70 ± 0.12	0.43 ± 0.10[b]	3	16	11	0	6.113[b]
Distant cancerous tissues	24.71 ± 0.06	22.13 ± 0.06	2.57 ± 0.07	0.23 ± 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 ± 0.10) or the distant cancerous tissues ($2^{-\Delta\Delta Ct}$ = 0.23 ± 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 (χ^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

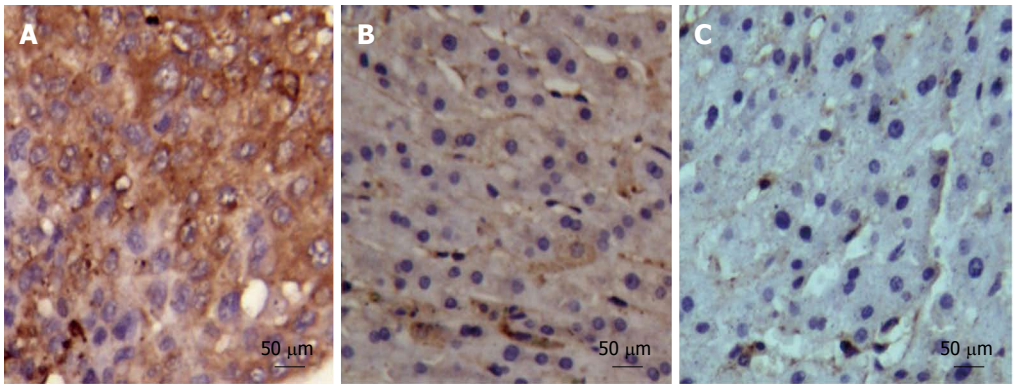

Figure 2 Immunohistochemistry of hepatic annexin A2 expression and distribution. Representative images of immunohistochemistry in the hepatocellular carcinoma (HCC), self-controlled adjacent- and distant- cancerous tissues (400 × magnification). A: Hepatocyte annexin A2 (ANXA2) expression in HCC tissue; 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 ± SD)

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

[1]Serum annexin A2 (ANXA2) level > 18 ng/mL or [2]Serum α-fetoprotein (AFP) level > 50 ng/mL was abnormal; [3]Compared 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 ± SD)

Group		*n*	(ng/mL)	*t*	*P* value	Pos. (%) > 18 ng/mL	χ^2	*P* value
Sex	Male	88	24.56 ± 5.84	0.150	0.881	78 (88.64)	0.933	0.334
	Female	27	24.79 ± 5.33			22 (81.48)		
Age (yr)	≥ 50	87	24.84 ± 6.07	0.798	0.427	76 (87.36)	0.050	0.822
	< 50	28	23.83 ± 4.56			24 (85.71)		
Tumor size (cm)	≥ 5.0	75	24.38 ± 5.68	0.850	0.397	66 (88.00)	0.207	0.649
	< 5.0	40	25.35 ± 5.95			34 (85.00)		
α-fetoprotein (ng/mL)	≥ 400	53	24.91 ± 5.52	0.531	0.596	45 (84.91)	0.365	0.546
	< 400	62	24.34 ± 5.96			55 (88.71)		
HBsAg	Positive	79	27.38 ± 5.67	6.820	< 0.001	73 (92.41)	6.605	0.010
	Negative	36	18.58 ± 7.83			27 (75.00)		
Differentiated grading	Well	37	20.43 ± 4.97			25 (67.57)		
	Moderate	43	26.19 ± 5.34	4.966[1]	< 0.001[1]	41 (95.35)	10.631[1]	0.001[1]
	Poor	35	27.05 ± 5.13	5.561[1]	< 0.001[1]	34 (97.14)	10.633[1]	0.001[1]
TNM staging	Stages Ⅰ-Ⅱ	52	21.16 ± 5.97			38 (71.43)		
	Stages Ⅲ-Ⅳ	63	27.44 ± 6.01	5.594[2]	< 0.001[2]	62 (97.06)	16.122[2]	< 0.001[2]
Extrahepatic metastasis	With	62	26.11 ± 5.43	3.191	0.002	58 (93.55)	5.514	0.023
	Without	53	22.79 ± 5.64			42 (79.25)		
Portal vein thrombus	With	60	26.03 ± 5.99	2.859	0.005	56 (93.33)	4.498	0.034
	Without	55	23.06 ± 5.03			44 (80.00)		

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

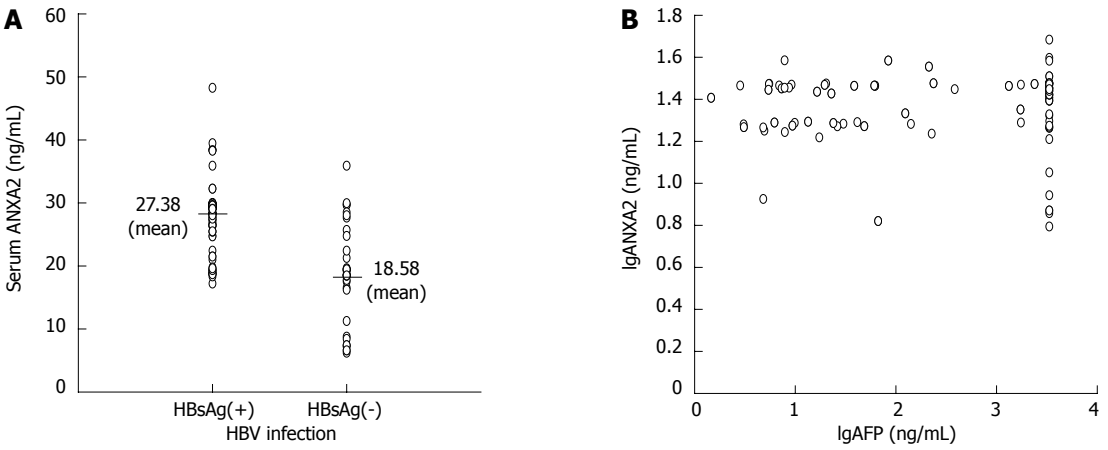

Figure 3 Relationship between serum annexin A2 level and hepatitis B virus infection or serum α-fetoprotein level in hepatocellular carcinoma patients. A: The scatter figure indicated that serum annexin A2 (ANXA2) level was obviously correlated with hepatitis B virus (HBV) infection. The serum ANXA2 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

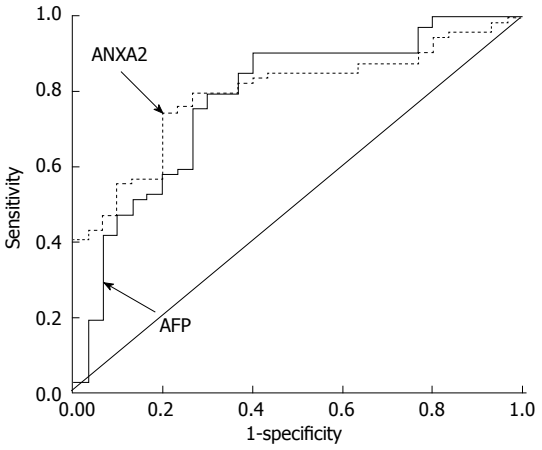

Figure 4 Receiver operating characteristic curves for circulating annexin A2 level in hepatocellular carcinoma diagnosis. The area under the receiver operating characteristic curves was 0.782 for α-fetoprotein (AFP) and 0.796 for annexin A2 (ANXA2).

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 cularis 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/and α-fetoprotein levels for hepatocellular carcinoma diagnosis[1]

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

[1]Serum 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/and AFP 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 μg/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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BRIEF ARTICLE

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.

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

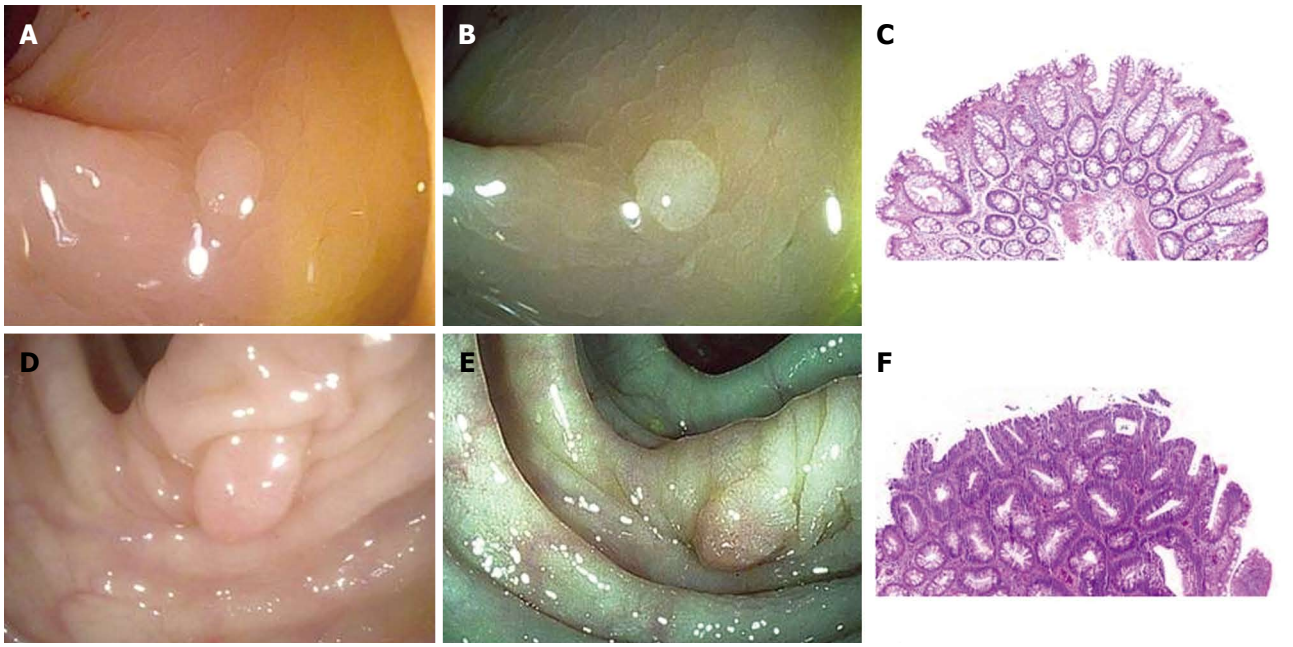

Figure 1 Correct prediction of small colonic polyps. A: Correctly predicted as hyperplastic, visualized under high-definition white light; B: Correctly predicted as hyperplastic, visualized under *i*-SCAN; C: Pathology demonstrating colonic mucosa with serrated architecture extending midway down the 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[11]. 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 (*n*)	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)

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

Table 2 Endoscopist quality measures

	Endoscopist A	Endoscopist B
Patients (*n*)	48 (64)	27 (36)
Polyps/patient (*n*/*n*), 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 *n* (%)	29 (60.4)	14 (51.9)
Cases in which adenomatous polyp identified *n* (%)	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 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".

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-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)

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 magnification, 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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BRIEF ARTICLE

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.

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 gastrorenal 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 patients 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 according 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, α1-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

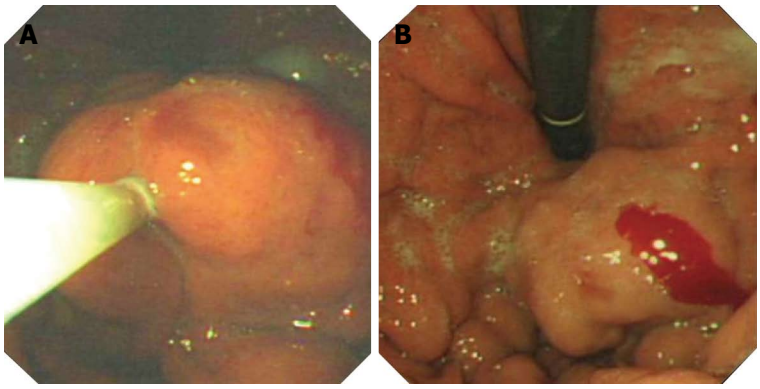

Figure 1 Injection of gastric varices with thrombin. A: During thrombin injection the gastric varix swells; B: Post thrombin injection.

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*[2]. 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 Ramond *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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BRIEF ARTICLE

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 Gastroenterology 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 ± 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 ± 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 ± 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©.

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.

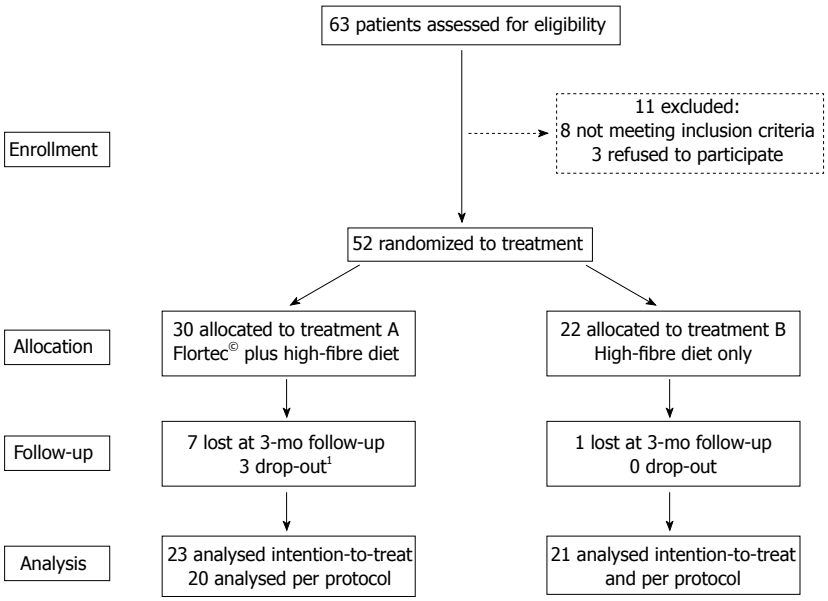

Figure 1 Flowchart of the patients throughout the study. [1]3 patients dropped out after 3 mo of treatment, 1 patient for new onset of constipation and 2 patients for worsening of abdominal symptoms.

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., occurrence 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 ℃) 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

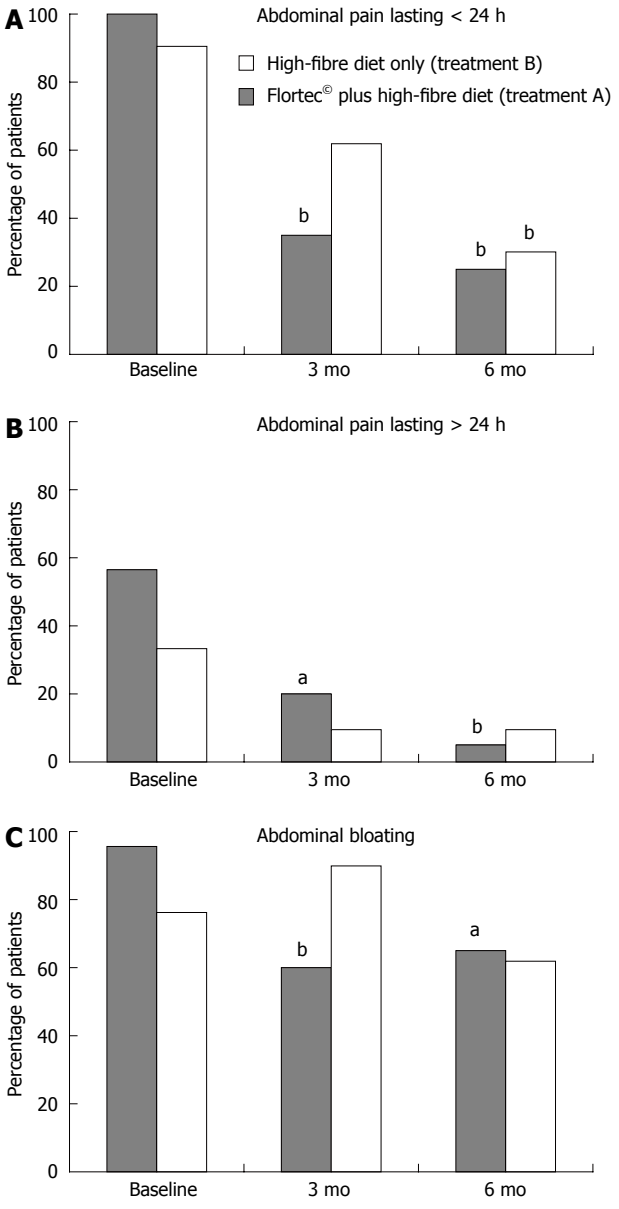

Figure 2 Change of abdominal symptoms after treatment according to intention-to-treat analysis. A: Abdominal pain lasting < 24 h; B: Abdominal pain lasting > 24 h; C: Abdominal bloating. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* baseline.

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 ± 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 (n = 30)	High-fibre diet alone (n = 22)	P value
Demographics			
Age, yr	68.1 ± 8.6	63.8 ± 10.3	0.10
Gender, female	22 (73.3)	13 (59.1)	0.43
Body mass index	26.4 ± 2.9	24.9 ± 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 colon	3 (10.0)	2 (9.1)	0.28
Symptoms			
Dyspeptic symptoms	3 (10.0)	3 (13.6)	0.97
Abdominal pain lasting < 24 h	28 (93.3)	20 (90.9)	0.48
VAS	4.6 ± 2.2	4.6 ± 2.8	0.97
Abdominal pain lasting > 24 h	14 (46.7)	8 (36.4)	0.49
VAS	6.7 ± 1.7	5.2 ± 2.8	0.12
Abdominal bloating	25 (83.3)	17 (77.2)	0.49
VAS	5.4 ± 2.2	5.2 ± 3.1	0.80

VAS: Visual analogic scale. Data are presented by mean ± 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 ± 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 ± 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 ± 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 antiinflamnatory 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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BRIEF ARTICLE

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 experiment. 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.

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, doctoral 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, consistency 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 ℃ 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 ℃. 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 ℃. 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 μg/mL (final concentration) of trypsin (Sigma, St Louis, United States) for 30 min at 37 ℃. 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 μg/mL of trypsin was added, and the cultivation was continued for 48 h at 37 ℃. 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 ℃ 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 × glutamine, penicillin 100 IU/mL, streptomycin 100 μg/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 ℃ 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 ℃. 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 μg/mL aprotinin (Sigma-Aldrich, St. Louis, MO, United States) 100 μg/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 ℃ for 10 min in a water bath. The lysate was centrifuged with QIAshredder (Qiagen) (2 min, 12 000 × *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 ℃, and RT was performed by incubating the reaction mixture for 10 min at 25 ℃, 120 min at 37 ℃, and 5 min at 85 ℃. 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 × SYBR Premix Ex Taq™ (Takara, Dalian, China), 0.5 μL of each primer (20 μmol/L final concentrations), 0.5 μL ROX Ref Dye II (50 ×), 4 μL of RV cDNA template, and 7 μL of sterile water (Sigma-Aldrich). The thermocycling profile included initial denaturation at 95 ℃ for 30 s, followed by 45 cycles of 95 ℃ for 5 s, 58 ℃ for 20 s, and 72 ℃ for 30 s. Finally, the melting curve analysis was performed at 95 ℃ for 1 min, 55 ℃ for 30 s, and 95 ℃ 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 ℃. 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 ± SE. In cases of significant global *P*-values,

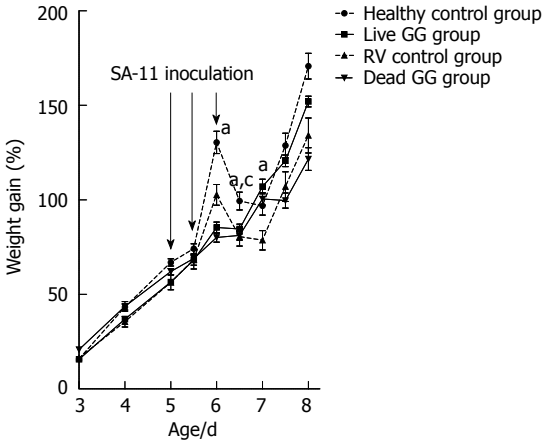

Figure 1 Percentage weight gain of the rats. The rat pups were weighed 1-2 times daily after day 2 during the experiment. On days 5 and 6, rotavirus (RV) was inoculated orally to the rats. Weight gain is expressed as percentual weight gain from the beginning of the experiment. [a]*P* < 0.05 *vs* RV control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

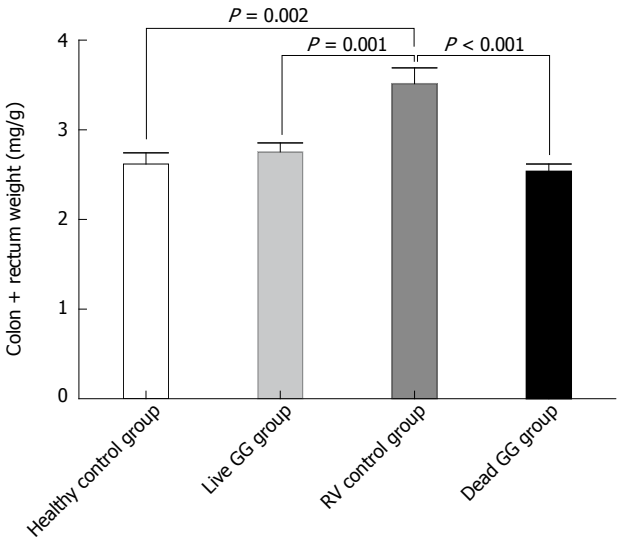

Figure 2 Weight of colon without feces. Results between groups are presented as the weight ratio of colon/animal. *P* = 0.002, *P* = 0.001, *P* < 0.001 *vs* rotavirus (RV) control.

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 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].

Table 1 Classification of diarrhea

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. [1]Animal deceased during the experiment due to technical error in dosing. RV: Rotavirus; NA: Not analyzed.

Table 2 Number of rotavirus polymerase chain reaction-positive samples in the indicated test groups

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.

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).

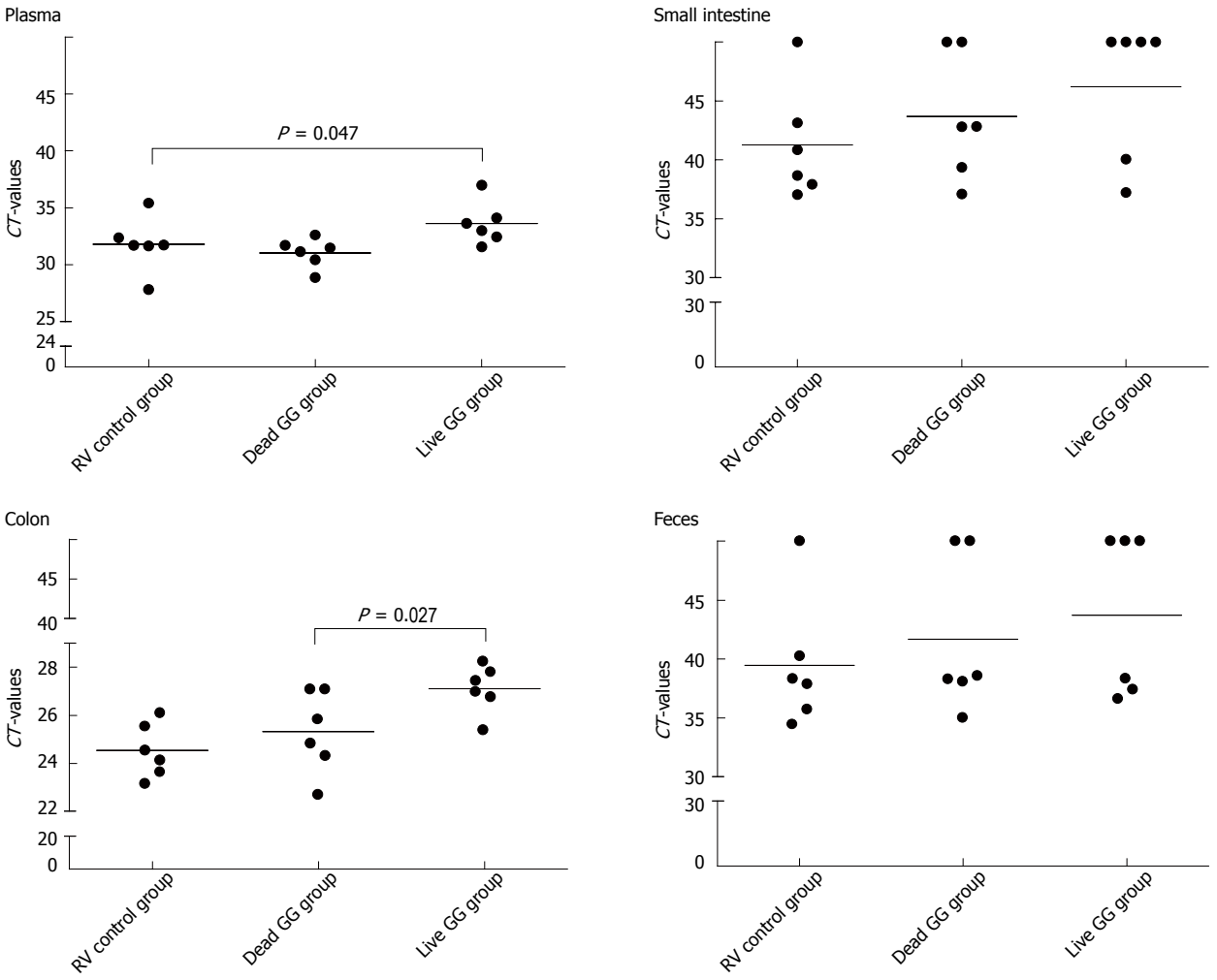

Figure 3 Rotavirus detection in plasma, small intestine, colon, and feces by real-time quantitative polymerase chain reaction. Limit of detection of the reaction was cycle threshold (*CT*) 45. The lines represent the mean of the *CT*-values. $P = 0.047$ *vs* RV control group, $P = 0.027$ *vs* dead GG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

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 (H_2) and methane (CH_4) breath test in order to assess small intestinal bacterial overgrowth (SIBO). SIBO was diagnosed when there was an increase in H_2 $\geqslant$ 20 ppm or CH_4 $\geqslant$ 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.

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 participants 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 ℃ 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 (H_2) and methane (CH_4) 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 $H_2 \geqslant 20$ ppm or $CH_4 \geqslant 10$ 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 hypothesis.

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]

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

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 (χ^2 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 ($H_2 \geqslant 20$ ppm), 23.1% (6/26) had increased methane concentrations with respect to the fasting value ($CH_4 \geqslant 10$ 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 (n = 26)	Without SIBO (n = 58)	P 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[4] (77.2)	0.594[2]
Total coliforms	13/26 (50.0)	30/57[4] (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]

[1]mean ± SD, Student's t-test; [2]χ^2 test; [3]Exact Fisher test; [4]Water 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).

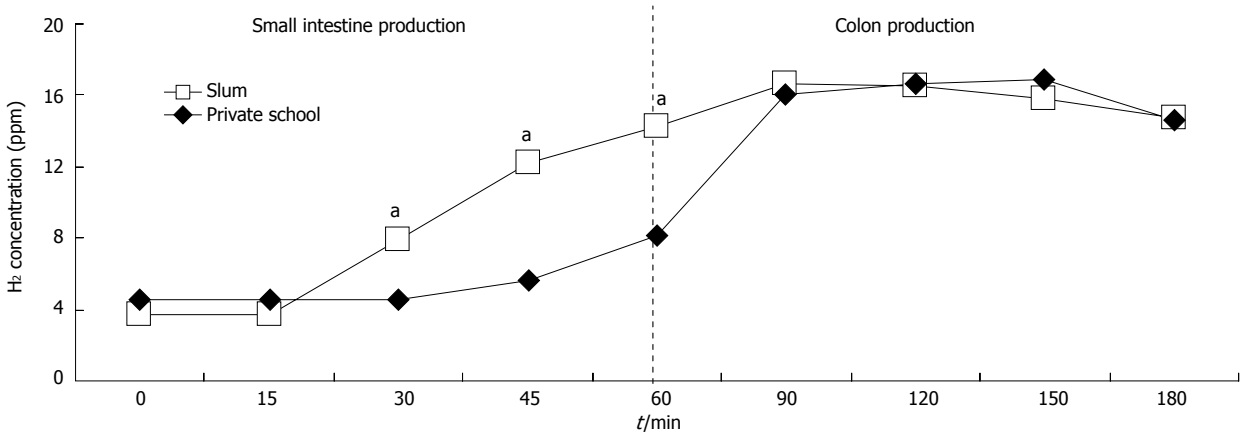
Figure 1D shows the concentration of hydrogen (ppm) in the breath for individual methane producers (n = 53) and non-producers (n = 31) in the slum group. An analysis of the area under the curve revealed that between 0 min and 60 min after lactulose ingestion, no difference existed in the levels of hydrogen 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

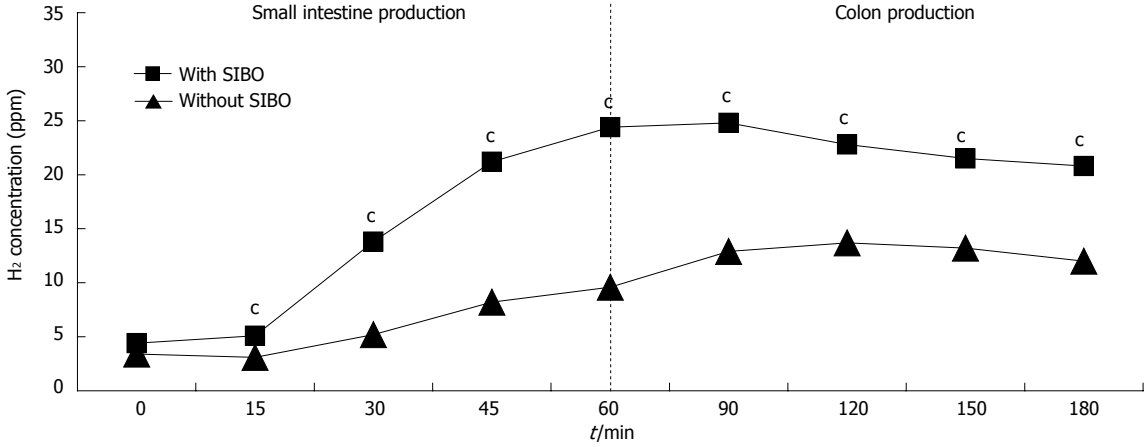
SIBO was found in 30.9% of the children in the slum group and in 2.4% of the children in the private school group. These values are similar to those previously reported in Brazil[5] for children living in a slum and those attending a private health clinic (37.5% and 2.1% SIBO in each group, respectively). 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

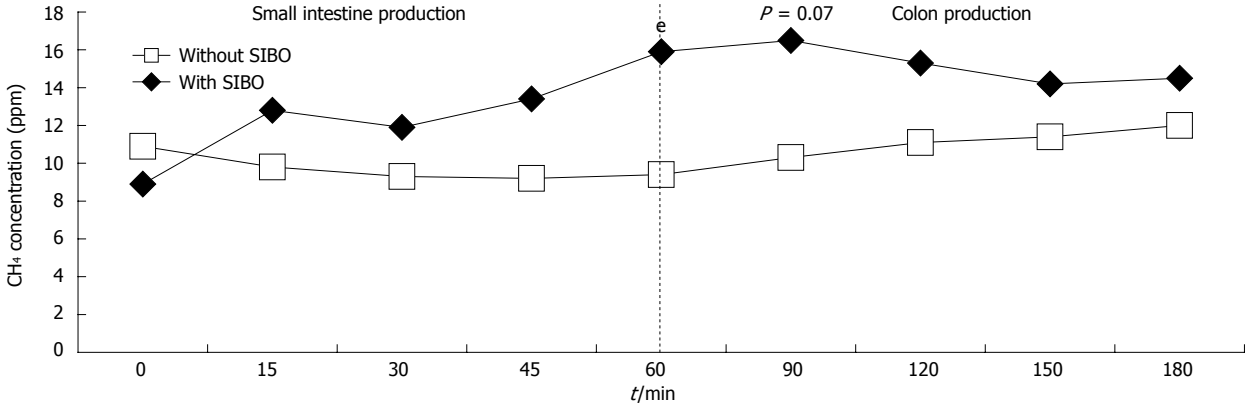
A


Small intestine production
Colon production
Slum
Private school
H2 concentration (ppm)
t/min
a

B


Small intestine production
Colon production
With SIBO
Without SIBO
H2 concentration (ppm)
t/min
c

C


Small intestine production
P = 0.07
Colon production
Without SIBO
With SIBO
CH4 concentration (ppm)
t/min
e

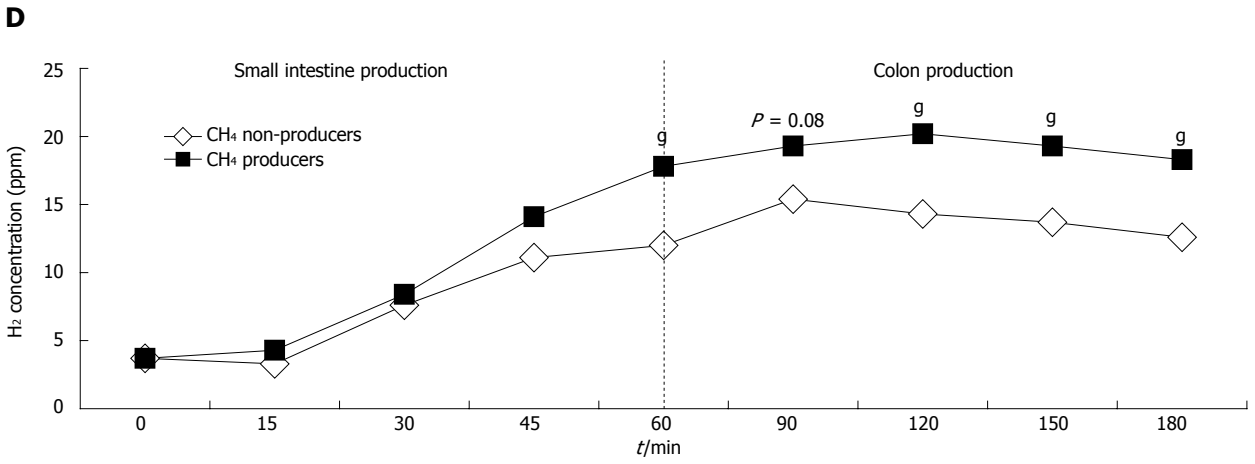


Figure 1 Small intestinal bacterial overgrowth in school-aged children and the relationship between hydrogen and methane production in breath test. A: Mean concentrations of hydrogen (ppm) in breath samples collected after fasting and at 15, 30, 45, 60, 90, 150 and 180 min after lactulose ingestion from 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 hydogen production in breath test; [e]$P < 0.05$ *vs* the slum group without SIBO in relation of the methane production in breath test; [g]$P < 0.05$ *vs* the slum group non-methene 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 CH_4 excretion, different to that which occurred in H_2 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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BRIEF ARTICLE

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

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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 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

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.

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

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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 pathophysiology 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 EndoCinch™ (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/m^2); 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 following 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].

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.

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 (n = 19)	3 mo (n = 19)	12 mo (n = 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

	n	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 (n = 10)	3 mo (n = 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.

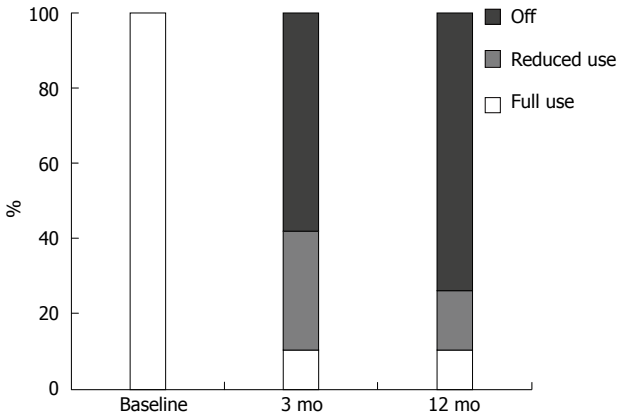

Figure 1 Rate of discontinuation or reduction by more than 50% of proton pump inhibitor use.

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,

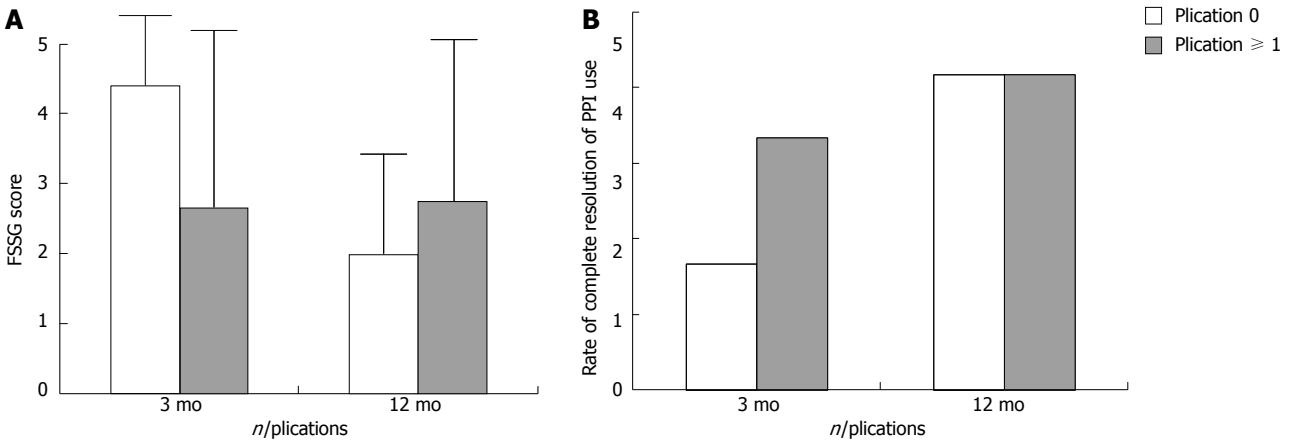

Figure 2 Relationship with the plication number and symptom or proton pump inhibitor use. A: Relationship between the number of plications and the score of the frequency scale for symptoms of gastro-esophageal reflux disease. There is no significant difference between the two groups (remaining pliccations 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.

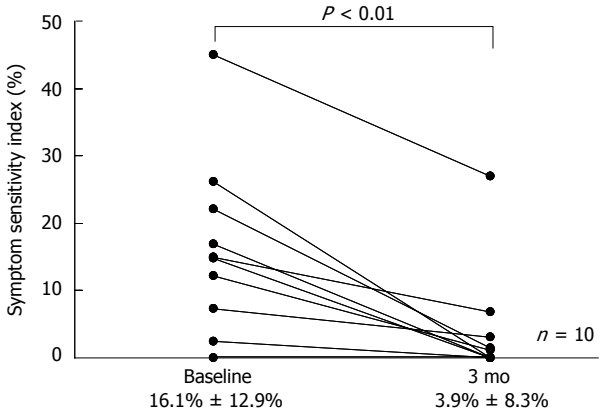

Figure 3 Symptom sensitivity index transition at baseline and 3 mo after endoluminal gastroplication.

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 H_2RA 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 findings 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 intraesophageal 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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BRIEF ARTICLE

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.

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 dysrythmia 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, prokintics 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

	Itopide (*n* = 24)		Mosapride (*n* = 28)		Levosulpiride (*n* = 22)	
Male:female (*n*)	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. Dysrythmia 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-prandial 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

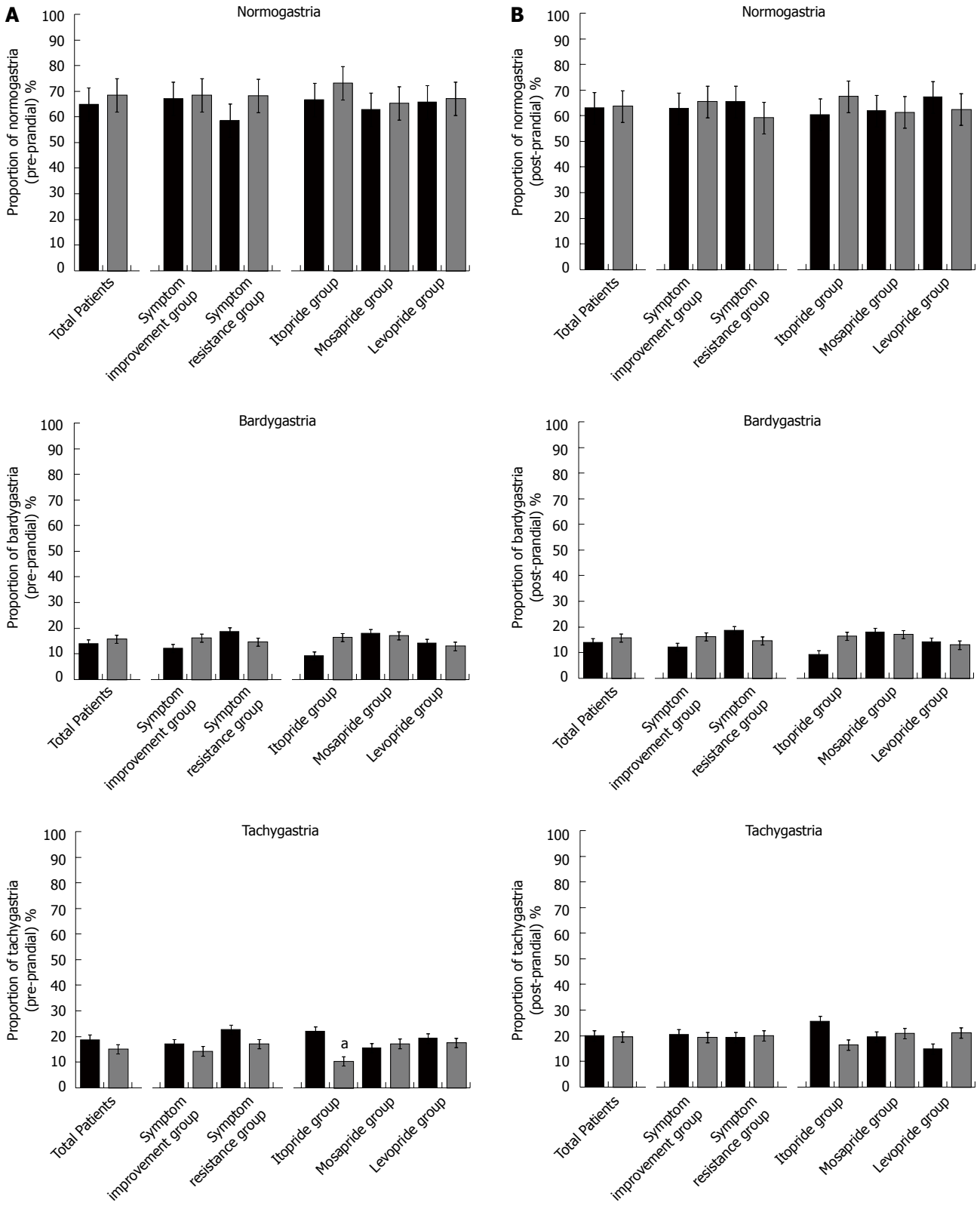

Figure 1 Proportion of gastric slow waves on electrogastrography. A: Pre-prandial; B: Post-prandial. [a]$P < 0.05$ *vs* pre-treatment.

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].

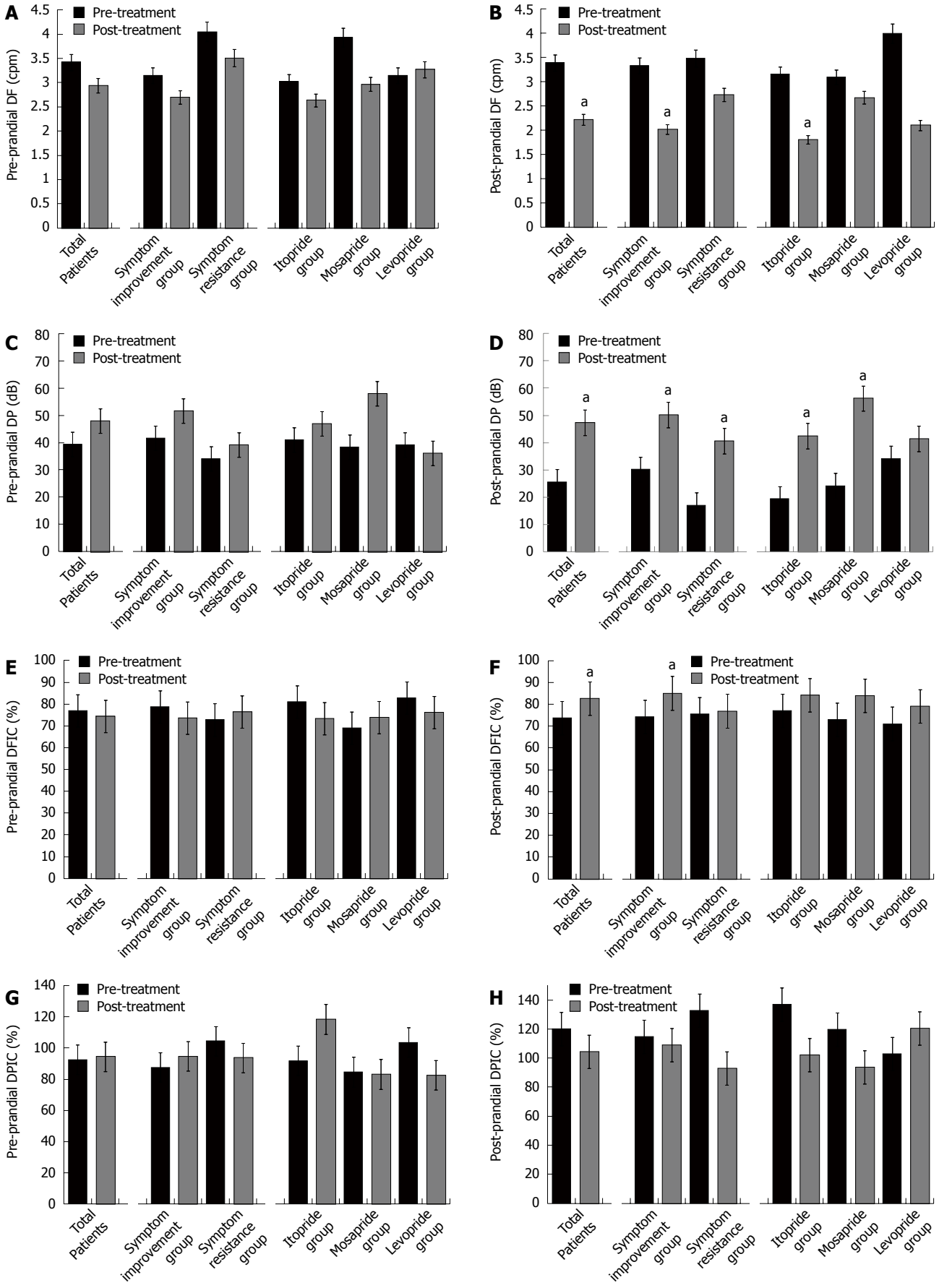

Figure 2 Changes in dominant frequency and dominant power, dominant frequency instability coefficient and dominant power instability coefficient after prokinetic treatment. A: Dominant frequency (DF) in pre-prandial electrogastrography (EGG); B: DF in post-prandial EGG; C: Dominant power (DP) in pre-prandial 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.

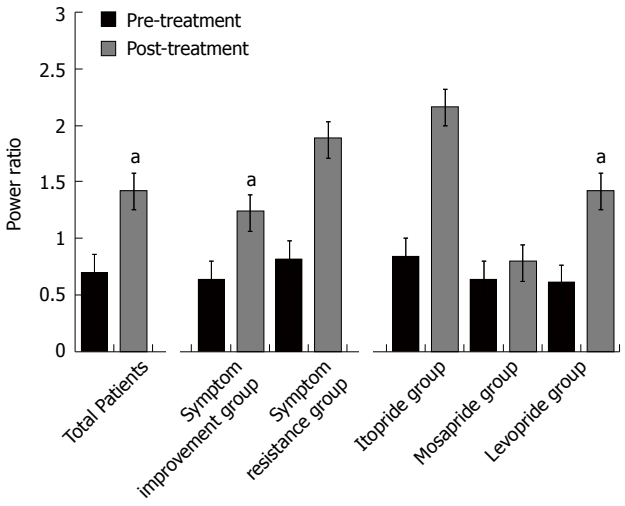

Figure 3 Power ratio before and after the 8-wk course of treatment. $^{a}P < 0.05$ *vs* pre-treatment.

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-HT_4 agonist/weak 5-HT_3 antagonist) and domperidone (D_2 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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BRIEF ARTICLE

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.

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 cholangiography 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.

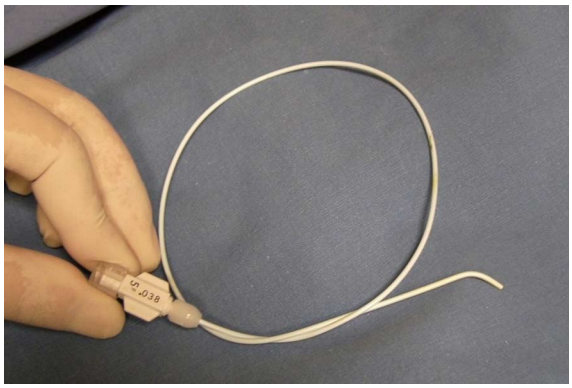

Figure 1 Kumpe catheter (5F, 40 cm).

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

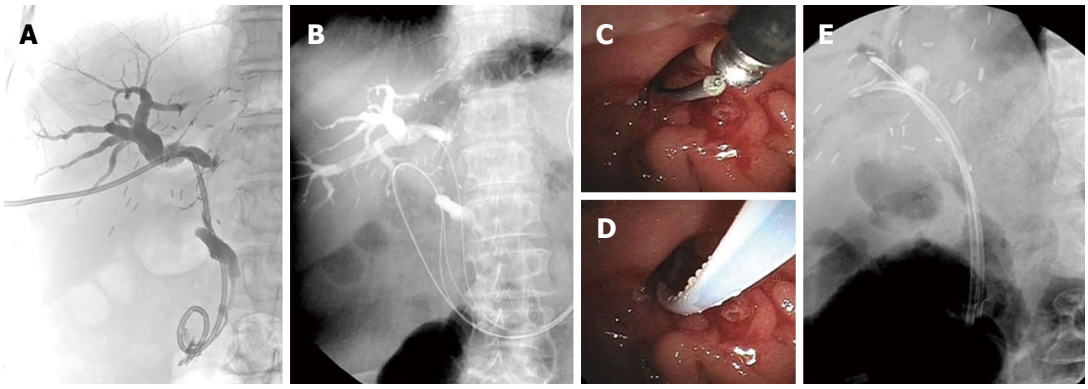

Figure 2 Guidewire technique. A: The percutaneous transhepatic biliary drainage (PTBD) catheter was located over the anastomotic stricture into the duodenum. The angle between the right hepatic duct and the common bile duct was steep (100°); B: The 0.035 inch guidewire was inserted through the PTBD catheter, 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

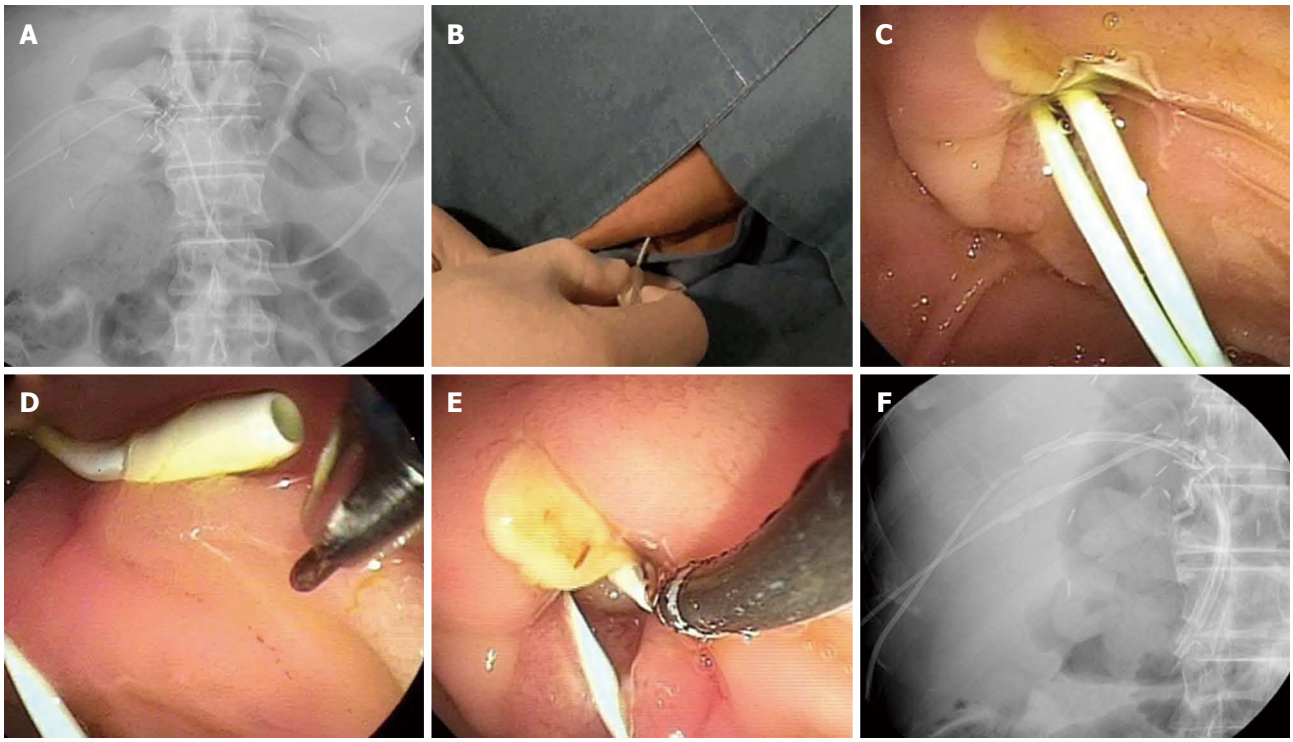

Figure 3 Kumpe catheter technique. A: Two Kumpe (KMP) catheters were placed along the previous percutaneous transhepatic biliary drainage tracts; B, C: The KMP catheters were located out of the major ampulla in the duodenum; D, E: The KMP catheter was pulled back and rotated to approximate the slightly 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	

[1]Between the right hepatic duct of the donor and the common bile duct of the recipient; [2]One day before the rendezvous technique; [3]Time after positioning the end of the duodenoscope in front of the major ampulla until placement of the inside stents; [4]One 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 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.

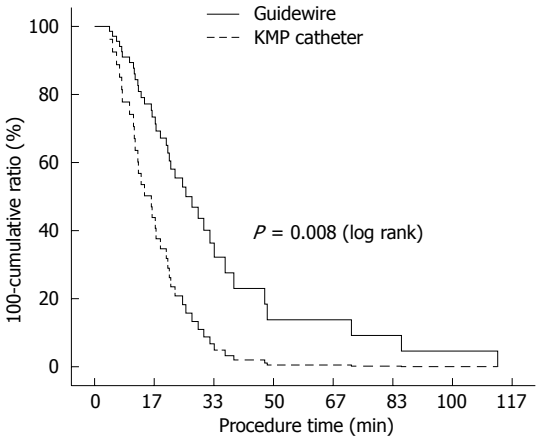

Figure 4 Cumulative probability of rendezvous procedures corresponding to procedure time. Kumpe catheter group *vs* guidewire group.

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 Stone	Present	2	1517 (1202-1831)	0.513
	Absent	36	1525 (251-6758)	
No. of inside stents	One	19	1198 (251-5144)	0.04
	Two	19	1851 (666-6758)	
Operator	Lee IS	31	1292 (251-6758)	0.024
	Chang JH	7	2555 (735-5144)	
Balloon dilation of the stricture	Yes	4	2568 (2189-2895)	0.008
	No	34	1402 (251-6758)	
Age	< 60	29	1571 (251-6758)	0.904
	> 60	9	1373 (301-2895)	
Procedure Familiarity[1]	First half[2]	15	1009 (368-1934)	0.635
	Second half	16	1557 (251-6758)	

[1]Performed by Lee IS; [2]The rendezvous procedures were performed for the first half of each rendezvous group. KMP: Kumpe; CBD: Common bile duct; IHD: Intrahepatic duct.

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 *vs* rendezvous)	0.010	2.663 (1.258-5.637)
No. of inside stents (1 *vs* 2)	0.067	
Operator (Lee IS *vs* Chang JH)	0.195	
Balloon dilation (yes *vs* 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]. Although 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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BRIEF ARTICLE

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.

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 ℃ 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

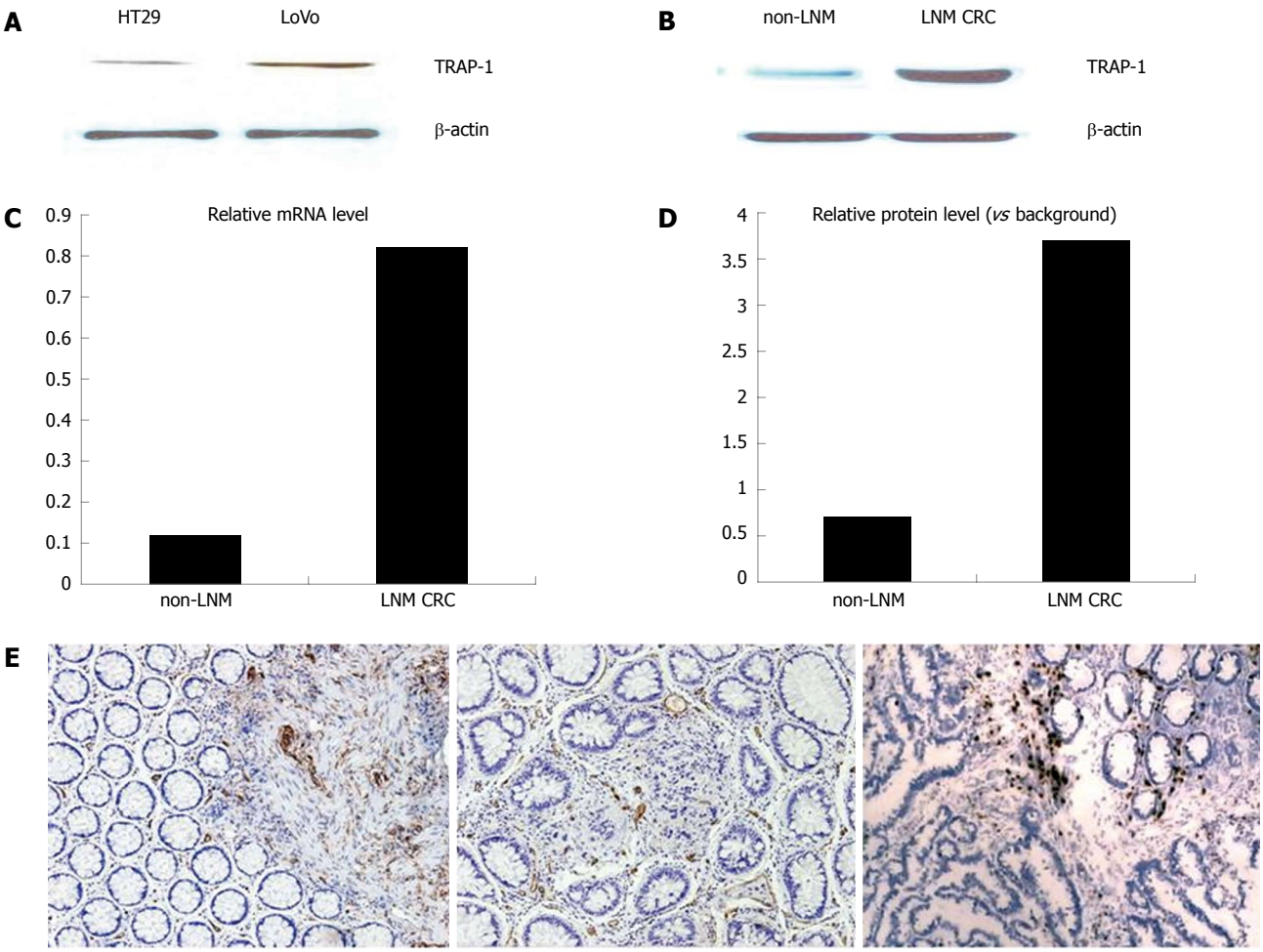

Figure 1 Confirmation of the overexpression of tumor necrosis factor receptor-associated protein-1 in colorectal cancer. A: Western blotting analysis for tumor necrosis factor receptor-associated protein-1 (TRAP-1) expression in different metastatic potential LoVo cell and HT29 cell. β-actin was used 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 ± 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 ± 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% CO_2.

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 μg 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-μL 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'-TGACGTGGACATCCGCAAAG-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-μg 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	

[1]Statistical analysis was performed with χ^2 test; [2]Grading 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).

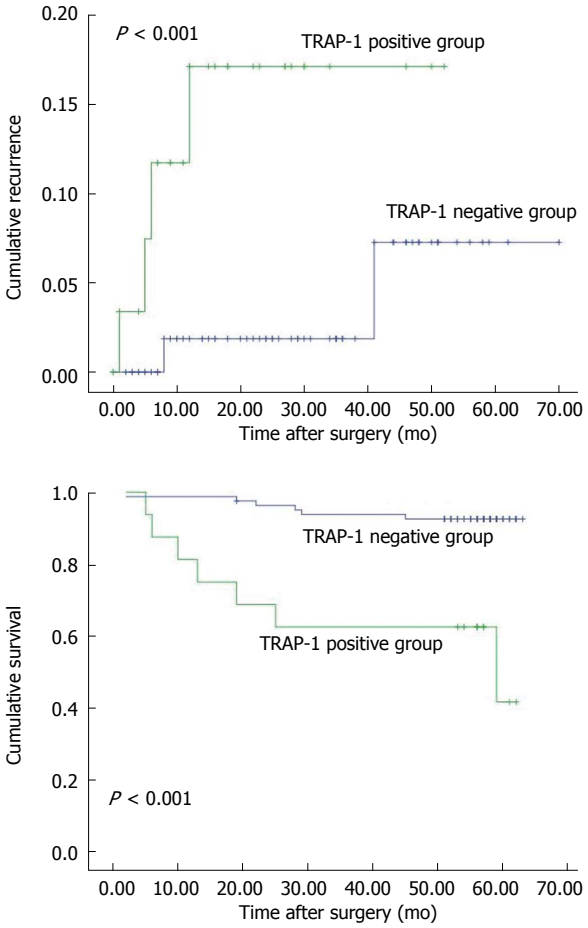

Figure 2 Overexpression of tumor necrosis factor receptor-associated protein-1 correlated with poor prognosis in 96 colorectal cancer patients. A: Cumulative recurrence; B: Cumulative survival. The tumor necrosis factor receptor-associated protein-1 (TRAP-1)-positive *vs* TRAP-1-negative groups ($P < 0.001$, log-rank test).

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-μm-thick tissue sections were heated in 6.5 mmol/L citrate buffer (pH 6.0) at 100 ℃ 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 case was considered negative if the final score was 0 or 1 (-) or 2 or 3 (±), 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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BRIEF ARTICLE

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.

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 proportion 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 > 1×10^3[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 $\geqslant 5$ (ALT and AP expressed as multiples of their upper normal limit) were classified as hepatocellular, cases with ALT/AP ratio $\leqslant 2$ 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 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.

Table 1 Characteristics of patients with alanine aminotransferase > 10 upper limit of normal range (mean ± SD)

Causes	*n* (%)	Age (yr)	Female *n* (%)	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
P (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.

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 cefoxitin ($n = 1$). 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 (*n*)	Specific drugs (*n*)
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), cefoxitin (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), ademetionine 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 pancreatobiliary 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 occured 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, hepatoxicity 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 hepatic 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 $\leq$ 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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CASE REPORT

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.

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-

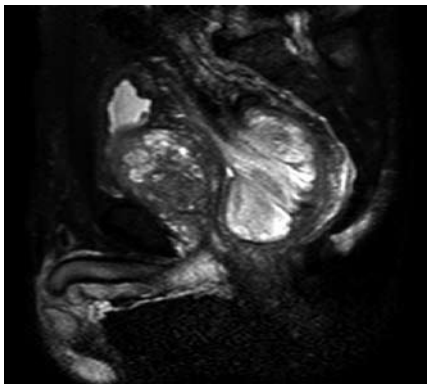

Figure 1 T2-weighted magnetic resonance image demonstrating a high-intensity mass in rectum with prostatomegaly.

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 continuous 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.

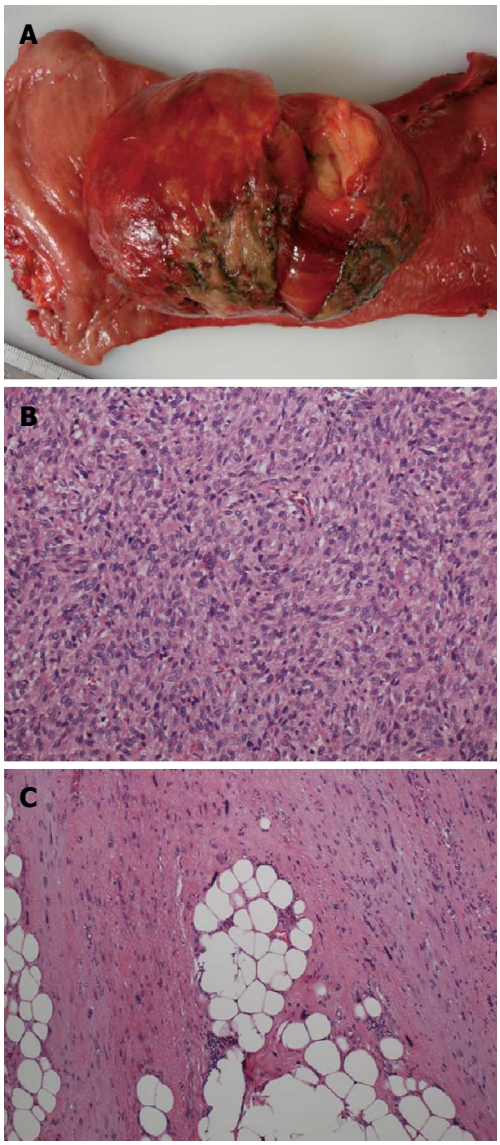

Figure 2 Dedifferentiated liposarcoma in rectum. A: Gross picture showing a huge dedifferentiated liposarcoma in rectum; B: Dedifferentiated component identified in polypoid lesion at rectum [hematoxylin and eosin (HE), × 400]; C: Well-differentiated liposarcoma at mesorectum (HE, × 400).

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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CASE REPORT

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 tuberous 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.

Key words: Bile duct cancer; Synchronous double cancer; Common bile duct; Pancreaticobiliary maljunction; Two primary cancers

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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-

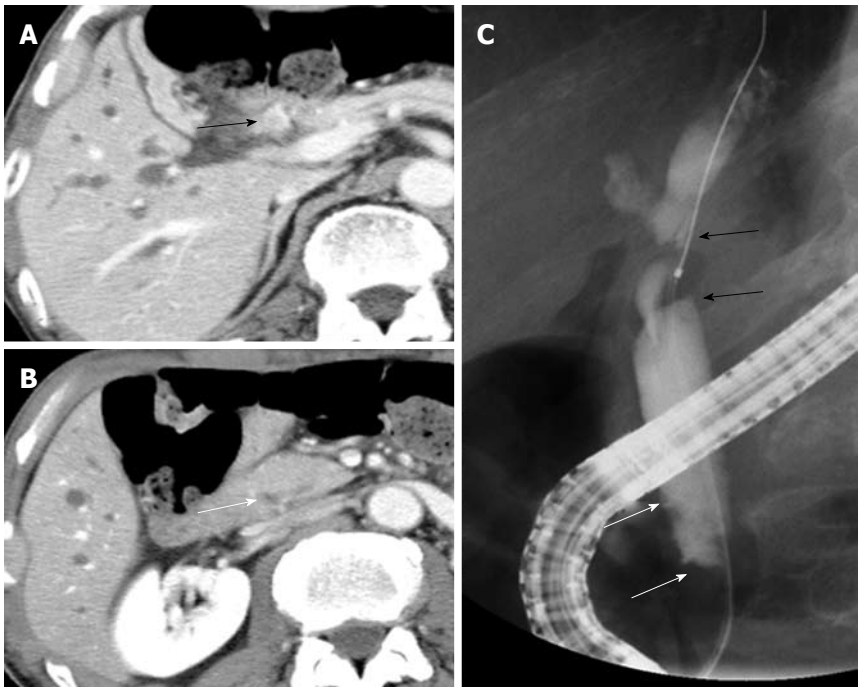

Figure 1 Imaging findings before the first surgery. A: Computed tomography showed a well enhanced mass in the middle and superior parts of the bile duct (black arrow); B: No mass was detected in the inferior part of the bile duct (white arrow); C: Endoscopic retrograde cholangiopancreatography revealed a tuberous filling defect in the middle and superior parts of the bile duct (black arrows) and mild stenosis in the inferior part (white arrows).

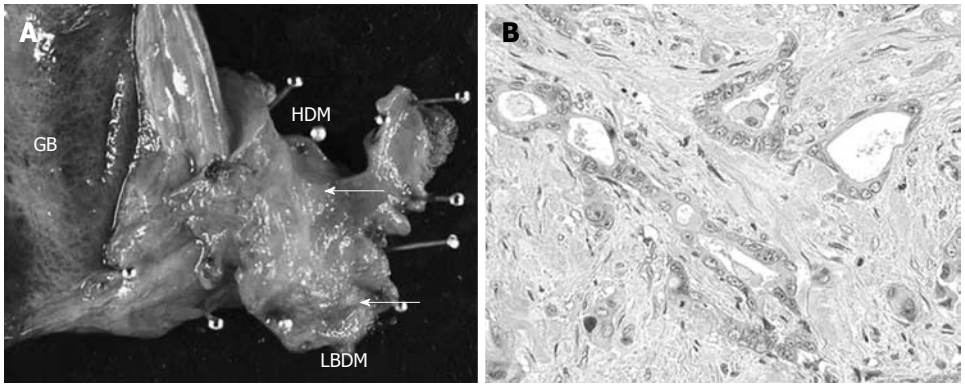

Figure 2 Macroscopic and microscopic findings of resected specimen at the first surgery. A: Resected specimen of the extra-hepatic bile duct showed whitish tuberous tumor in the middle and inferior parts of the bile duct (cancer marked by the white arrows); B: Microscopically, the tumor was moderately differentiated tubular adenocarcinoma with invasive growth (hematoxylin-eosin; magnification; × 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 tuberous 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

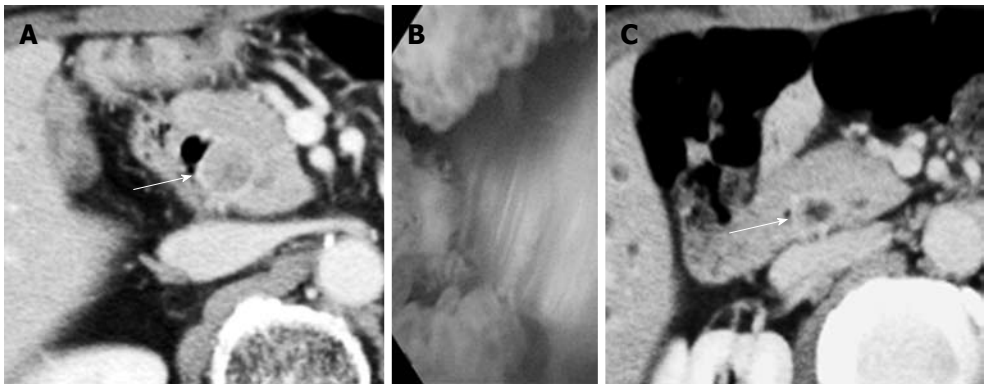

Figure 3 Imaging findings before the second surgery and retrospectively reviewed computed tomography finding before the first surgery. A: Computed tomography (CT) taken 11 mo after the first surgery showed enhanced inferior bile duct wall (white arrow) and slightly enhanced tumor within the duct; B: 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.

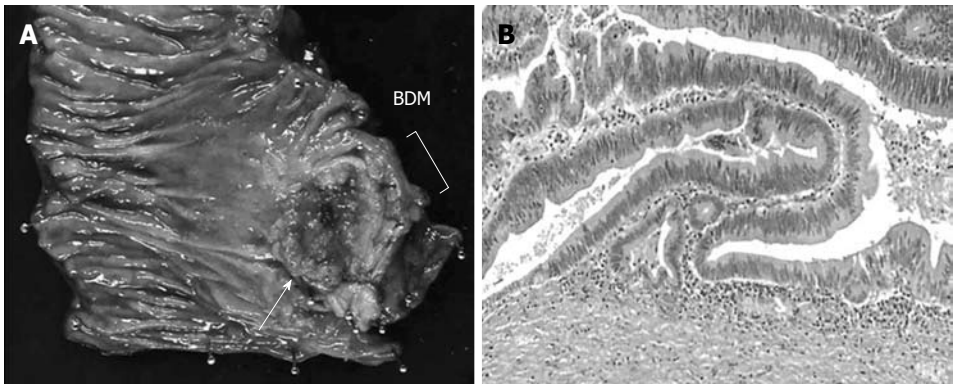

Figure 4 Macroscopic and microscopic findings of resected specimen at the second surgery. A: Specimen resected during pancreaticoduodenectomy. Note the papillary tumor in the inferior bile duct (white arrow); B: Microscopic examination showed papillary adenocarcinoma with expansive growth (hematoxylin-eosin, magnification; × 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 preoperative 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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CASE REPORT

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.

Key words: Myopathy, encephalopathy, lactic acidosis, and stroke-like episodes syndrome; Acute mesenteric ischemia; Immunohistochemistry; Anti-mitochondrial antibody; Pathology

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Fukuyama K, Ishikawa Y, Ogino T, Inoue H, Yamaoka R, Hirose T, Nishihira T. Mucosal necrosis of the small intestine in myopathy, encephalopathy, lactic acidosis, and stroke-like episodes syndrome. *World J Gastroenterol* 2012; 18(41): 5986-5989 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i41/5986.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i41.5986

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 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

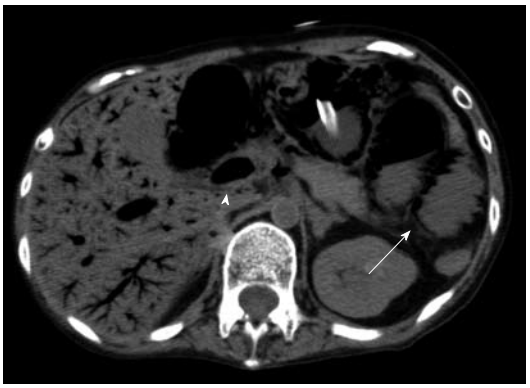

Figure 1 Preoperative computed tomography. There was a massive amount of portomesenteric venous gas (arrow head) and pneumatosis intestinalis in the small intestine (arrow).

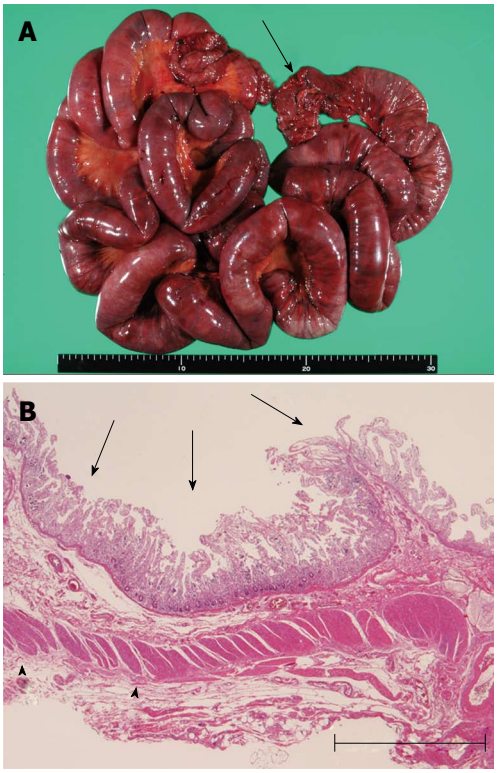

Figure 2 Macroscopic findings of resected tissue and specimen. A: Macroscopic findings of resected tissue. The specimen measured 150 cm in length. There was diffuse mucosal necrosis in two thirds of oral-sided area, and mottled necrosis in the remaining area. Arrow: Oral stump; B: Microscopic findings 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 μm.

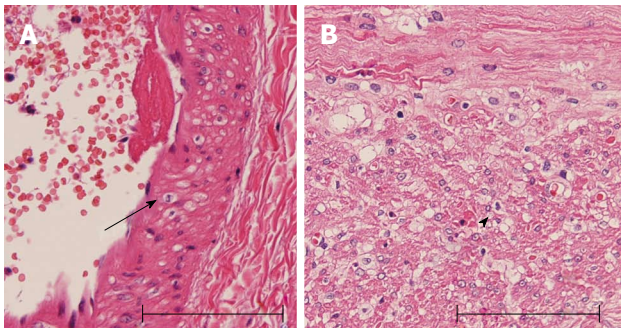

Figure 3 Vacuolar degeneration. A: Artery wall (arrow); B: Muscular layer (arrow head). Hematoxylin and eosin, Bar = 100 μm.

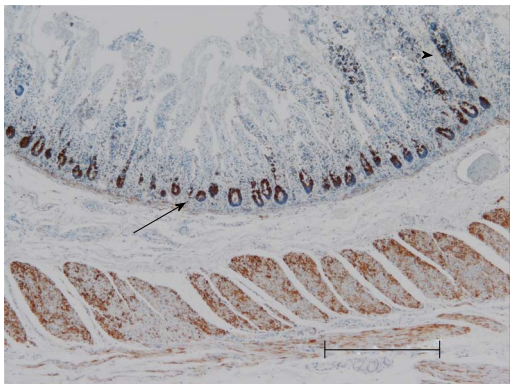

Figure 4 Immunohistochemical study. Anti-mitochondrial antibody is markedly expressed in the crypts (arrow) and preserved mucosa (arrow head). Bar = 500 μm.

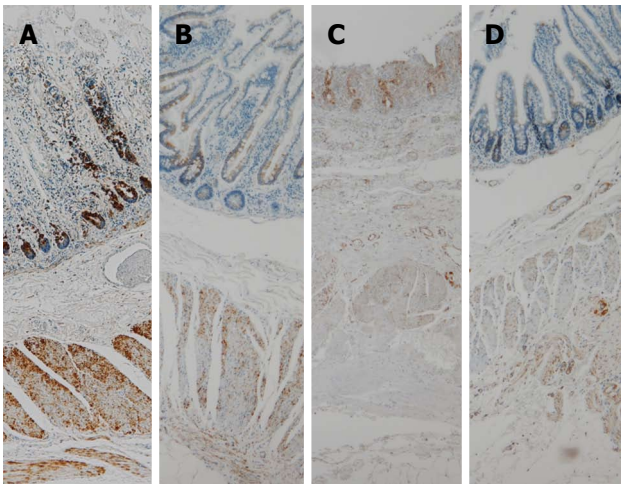

Figure 5 Expression of anti-mitochondrial antibody. A: Necrotic area of the present patient; B: Non-necrotic area of the present patient; C: Necrotic area of strangulated small intestine of a non-myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) patient; D: Non-necrotic c area in the strangulated small intestine of a non-MELAS patient. Bar = 500 μm.

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 in consideration of her general condition. She died of severe metabolic disorder due to MELAS 3 mo after discharge.

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.

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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CASE REPORT

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 uncinate 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.

Key words: Autoimmune disease; Pancreatitis; Cholangitis; Prednisolone

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Kim JH, Chang JH, Nam SM, Lee MJ, Maeng IH, Park JY, Im YS, Kim TH, Kim CW, Han SW. Newly developed autoimmune cholangitis without relapse of autoimmune pancreatitis after discontinuing prednisolone. *World J Gastroenterol* 2012; 18(41): 5990-5993 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i41/5990.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i41.5990

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/mm^3, 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

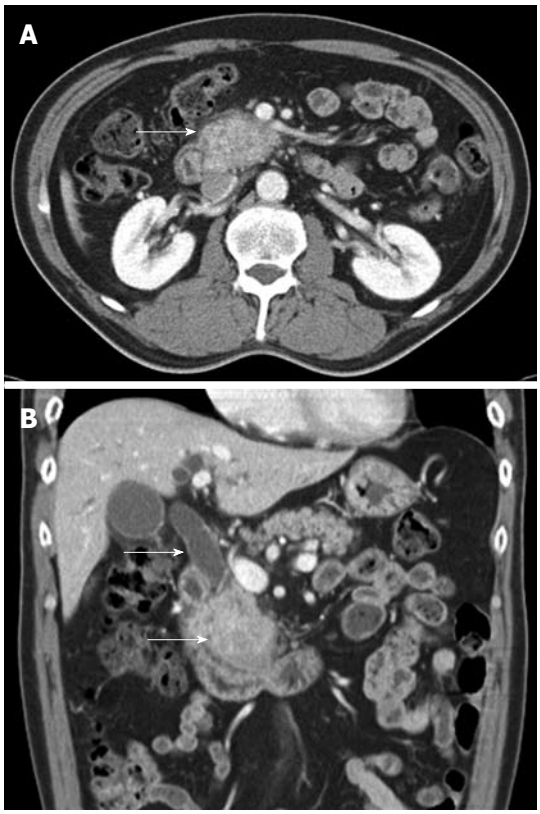

Figure 1 Initial abdominal computed tomography scan revealed an ill-defined enhancing mass-like lesion in the uncinate process of the pancreas with a dilatation of common bile duct. A: The uncinate process of the pancreas (white arrow) measuring 5.7 cm × 3.2 cm with regional infiltrations; B: In addition to this, there is small amounts of fluid collections around the pancreas head (white arrows).

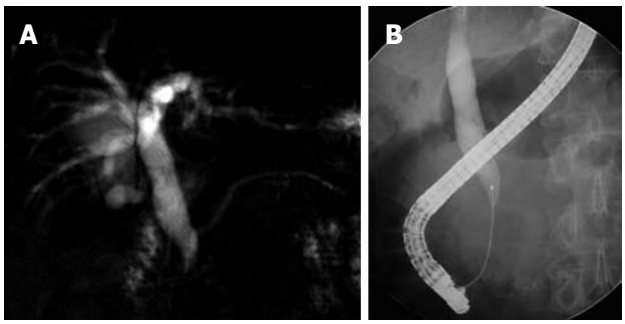

Figure 2 Magentic resonance cholangiopancreatography and endoscopic retrograde cholangiography. A: Magentic resonance cholangiopancreatography showed moderate dilatation of the intrahepatic and common bile duct. The distal common bile duct had an abrupt narrowing. The pancreatic duct was unremarkable; 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-

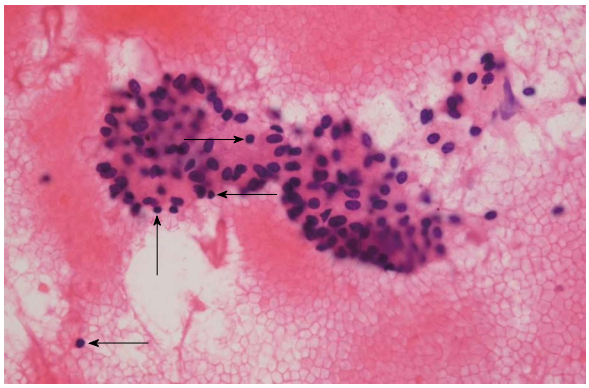

Figure 3 Photomicrograph of cytologic specimen obtained by endoscopic ultrasound-fine needle aspiration, showing lymphocytes (arrows) and irregular sheets of bland ductal epithelial cells on the bloody background (hematoxylin and eosin, × 400).

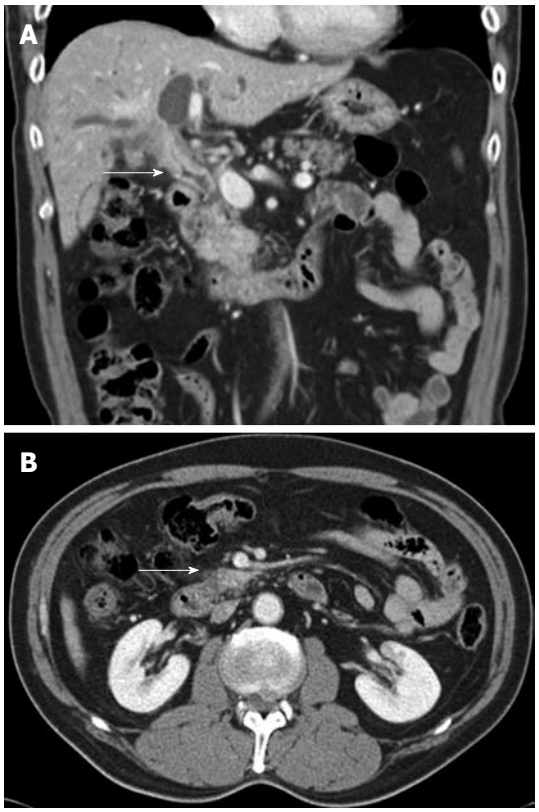

Figure 4 Follow up abdominal computed tomography scan. A: Coronal image revealed a stenosis of the common hepatic and the proximal common bile duct (white arrow) with significant thickening and inner wall enhancement of the bile duct; B: There was no pancreatic relapse (white arrow).

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.

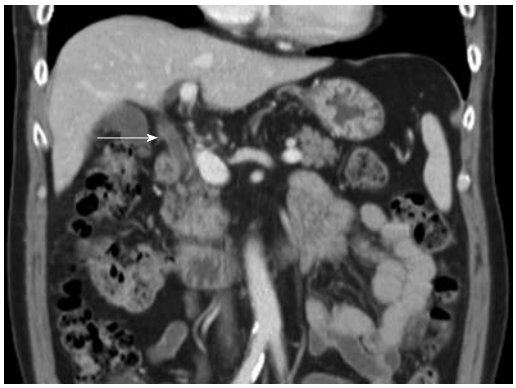

Figure 5 Abdominal computed tomography scan after retreatment with prednisolone showed resolution of the thickening of the bile duct (white arrow).

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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CASE REPORT

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.

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 antacid 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 ℃. 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/μL, neutrophils of 82.2%, lymphocytes of 12.1%, hemoglobin of 14.8 g/dL, and platelet count of 210 000/μL. 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.

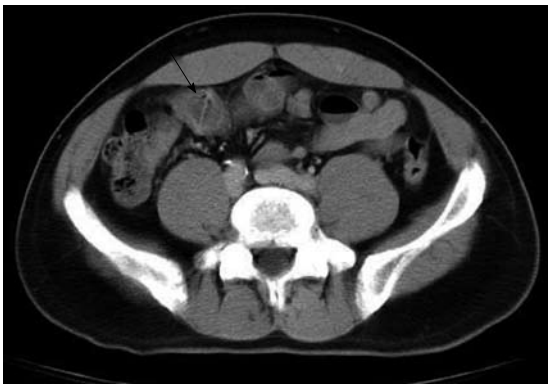

Figure 1 An unenhanced abdominal computerized tomography image reveals a 26 mm in length radiopaque linear shadow in the distal ileum lodged into the thickened intestinal wall at both ends (black arrow). Minimal peritoneal contamination without pneumoperitoneum, or abscess formation is noted, which is 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 ℃ 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/μL, neutrophils of 49.7%, lymphocytes of 37.3%, hemoglobin of 14.1 g/dL, platelet count of 227 000/μL 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

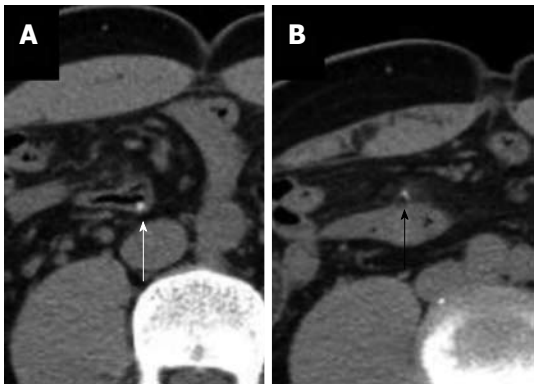

Figure 2 Two follow-up unenhanced abdominal computer tomography images, which reveal the radiopaque shadow still lodged in the intestinal segment. The fish bone rotates and becomes parallel to the distal ileum lumen. A: Most of the fish bone is still inside the intestinal lumen (white arrow). One end 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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CASE REPORT

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.

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 nonspecific 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

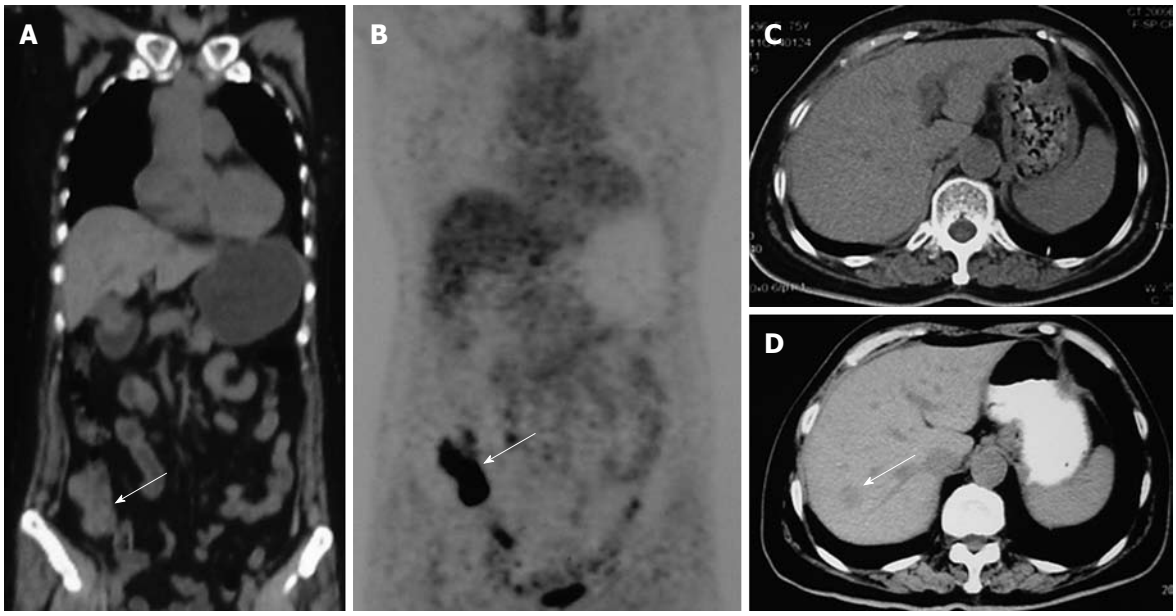

Figure 1 Positron emission tomography plus computed tomography and computed tomography scan findings in our patient. A: Positron emission tomography plus computed tomography (PET/CT) scan showed thickening of the ileocecal region (white arrow), and no liver lesions; B: PET/CT scan showed increased metabolic 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 μmol/L, direct bilirubin (DB) 4.5 μmol/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 performed 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

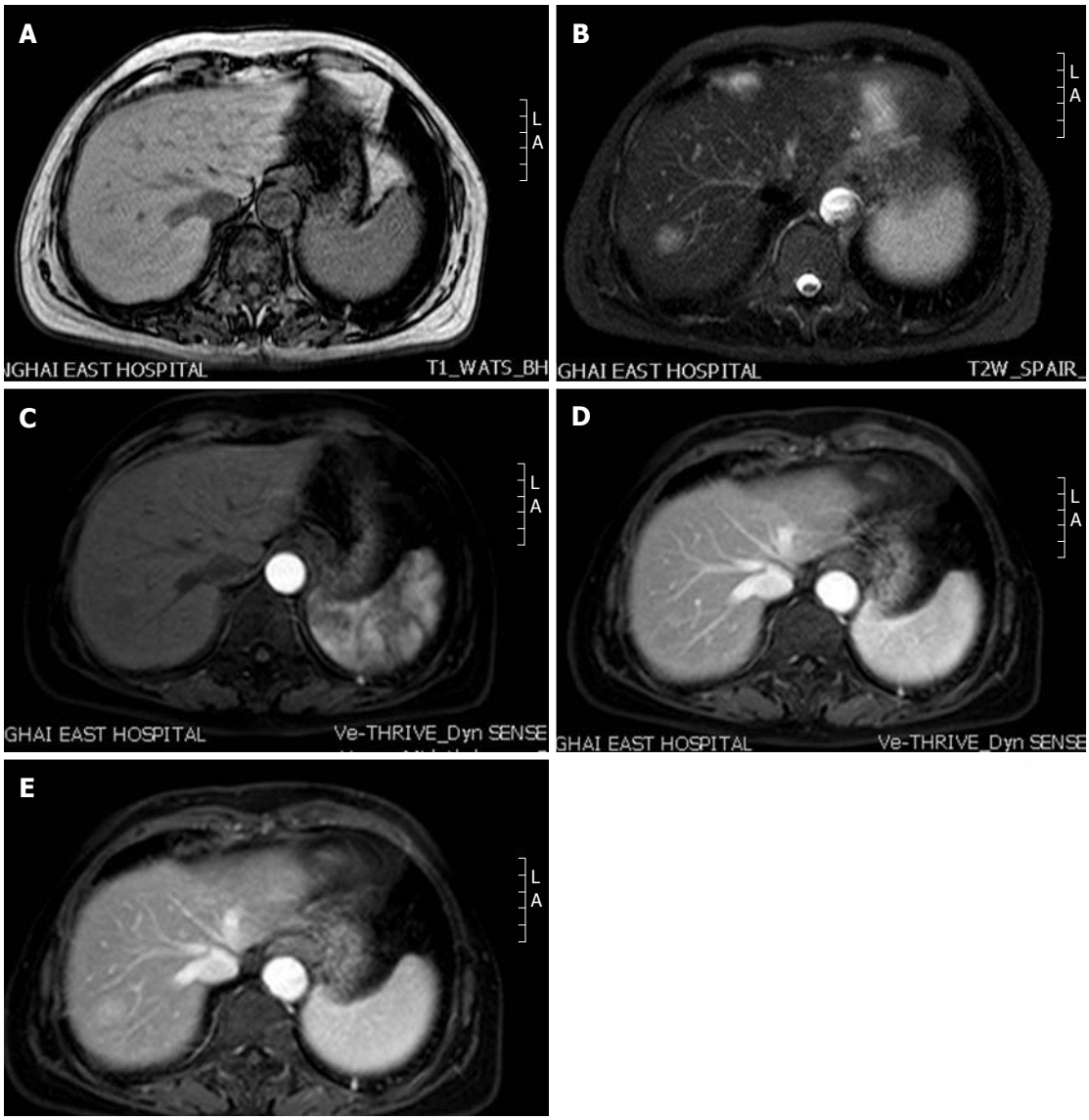

Figure 2 Magnetic resonance imaging scan findings in our patient. A: On T1-weighted images, the hepatic lesion shows low signal intensity; B: On T2-weighted images, the lesion shows hyperintense signal intensity; C: In the arterial phase, the lesion remains unenhanced; D, E: During the portal and delayed 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 progression 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, hemangiomatosis, 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-

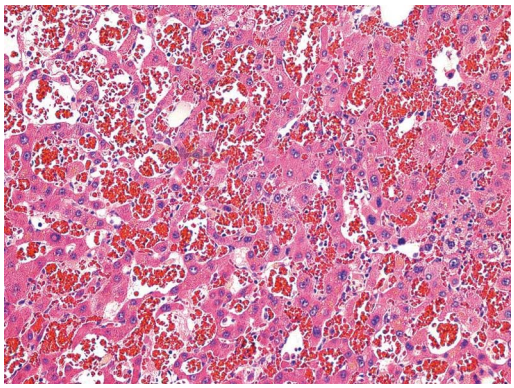

Figure 3 Photomicrograph of a liver section from our case showing variable-sized, blood-filled cystic spaces (hematoxylin and eosin, 200 ×).

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 ± SD, 60.5 ± 334.1 frames, range: 0-3329 frames) and in 66% of MiroCam® cases (mean ± 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®, $P = 0.0002$. This information might be useful when performing SBCE in patients with high risks for aspiration.

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 × 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 × 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 ± 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 ± SD, 60.5 ± 334.1 frames, range: 0-3329 frames) and in 66% of MiroCam® cases (mean ± 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 underwent 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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London, United Kingdom

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London, United Kingdom

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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
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San Diego, CA 92121, United States

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Omaha, NE 68197, United States

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Mayo Clinic Gastroenterology and Hepatology
Orlando, FL 32808, United States

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Milwaukee, WI 53202, United States

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Singapore, Singapore

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Prague, Czech

April 18-20, 2012
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New Orleans, LA 70166, United States

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Diffuse Small Bowel and Liver Diseases
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April 22-24, 2012
EUROSON 2012 EFSUMB Annual Meeting
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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar 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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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

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