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EDITORIAL

Angiogenic inhibitors for older patients with advanced colorectal cancer: Does the age hold the stage?

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Abstract

Although major progress has been achieved in the treatment of advanced colorectal cancer (CRC) with the employment of antiangiogenic agents, several questions remain on the use of these drugs in older patients. Since cardiovascular, renal and other comorbidities are common in the elderly, an accurate assessment of the patients' conditions should be performed before a treatment decision is made. Since most CRC patients enrolled in clinical trials testing antiangiogenic drugs were aged < 65 years, the efficacy and tolerability of these agents in elderly patients has not been adequately explored. Data suggest that patients with advanced CRC derive similar benefit from bevacizumab treatment regardless of age, but the advantage of other antiangiogenic drugs in the same class of patients appears more blurred. Literature data suggest that specific antiangiogenic-related toxicities such as hypertension or arterial thromboembolic events may be higher in the elderly than in the younger patients. In addition, it should be emphasized that the patients included in the clinical studies discussed herein were selected and therefore may not be representative of the usual elderly population. Advanced age alone should not discourage the use of bevacizumab. However, a careful patients' selection and watchful monitoring of toxicities are required to optimize the use of antiangiogenics in this population.

Key words: Advanced colorectal cancer; Bevacizumab; Elderly; Antiangiogenesis; Chemotherapy

Core tip: Although promising, limited evidence supports the use of antiangiogenic drugs to treat elderly colorectal cancer patients, that also may have increased toxicities compared to younger subjects. However, advanced age *per-se* should not discourage the use of these drugs. Since older patients constitute a heterogeneous population in terms of overall health status and comorbid conditions, a careful patients' selection and a watchful monitoring of potential treatment-related side effects are recommended to optimize the use of angiogenesis inhibitors in this population.

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INTRODUCTION

Whilst most of cancer diagnosis and deaths occur in

older subjects[1,2], three major factors are shaping the scenery in which the advanced colorectal cancer (CRC) is managed in all developed countries. Firstly, people are steadily aging and cancer incidence and prevalence are rising among senior citizens[3,4]. Secondly, the incorporation of new drugs within more complex treatment strategies has raised the median survival of CRC patients to unprecedented figures of 30 mo[5]. Lastly, more often than before, aggressive surgery and other regional approaches are performed with curative intent in older oligometastatic patients. As a result, the soaring demand for care of senior with CRC is likely to further increase. Although many elderly cancer patients have concurrent chronic disorders or morbidities requiring medical treatment and present with diminished organ functions, impairment of daily vital activities or minor cognitive deficits, the majority of them are treated with systemic chemotherapy and/or biologics[6,7]. Bevacizumab, a humanized vascular endothelial growth factor (VEGF) inhibitor, has proven efficacy when added to systemic chemotherapy regardless CRC patients' age in first or subsequent lines of therapy[8]. Specific data regarding its use in the older population are limited. Nevertheless, one out of three patients receive bevacizumab beyond 65 years of age[9]. Chronological age is still a major barrier that limits the proposal of standard treatment options to the elderly and the harm-to-benefit risk is particularly challenging when treating with noncurative intent[10]. However, patients' chronologic age does not always reflect their overall health status and older patients are highly heterogeneous because of dissimilar types and grades of concurrent morbidities. All these reasons may increase the difficulty in choosing the most appropriate treatment. Besides, advanced age is a common exclusion criteria to be recruited in clinical trials so that elderly patients have been underrepresented in CRC studies and the few included, usually representing less than 15% of the whole trial population, are highly selected. Despite recent studies have demonstrated the usefulness of a comprehensive geriatric assessment, its adoption in the clinical practice is still limited. Herein, we present the latest data regarding the use of antiangiogenic drugs in older CRC patients, specifically focusing at safety issues and efficacy results of landmark clinical studies.

THE IMPORTANCE OF ANGIOGENESIS IN COLORECTAL CANCERS

Angiogenesis is a cornerstone of tumor mass expansion. In response to hypoxia, the activation of hypoxia-inducible factor (HIF) triggers the expression of VEGF, one of the most important proangiogenic molecules[11], and its numerous isoforms[12]. In order to grow, CRCs need to continually acquire new blood supplies throughout the neoangiogenetic process, the formation of new capillaries rising from the splitting of existing ones. In the same way as in other solid tumors, angiogenesis plays an important role in CRC progression and metastatization, and its therapeutic inhibition has become a key component of anticancer treatment. Bevacizumab, the first Food and Drug Administration-labeled antiangiogenic antibody, was been approved for clinical use after showing efficacy in combination with chemotherapy in CRC patients. Still, many issues are unresolved, such as the lack of validated predictive biomarkers[13], the reasons for initial or acquired resistance to VEGF-inhibitors, and the uncertainty surrounding the opportunity for further antiangiogenic treatment beyond tumor progression. The study of non-endothelial cells involved in the neoangiogenesis through the production of growth factors or the modulation of cell-matrix interactions is of interest[14]. For example, pericyte recruitment, a key phenomenon in the neovascular formation that is regulated by platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β) and angiopoietin/Tie2, may be blocked by a number of novel antiangiogenic multitarget tyrosine kinase inhibitors (TKI), including sunitinib, sorafenib, and regorafenib.

ANTIANGIOGENIC DRUGS IN OLDER CRC PATIENTS: FRIENDS OR FOES?

Elderly patients who received 5-FU either alone[15] or in combination with irinotecan[16] or oxaliplatin[17] had similar survival benefits when compared to younger patients, although they may suffer higher rates of specific toxicities[18]. Despite these reassuring data, clinicians tend to be conservative when considering systemic therapy in the elderly, either not proceeding or upfront reducing chemotherapy doses[19]. The use of antiangiogenic drugs in patients with advanced CRC is supported by strong scientific evidence and common bevacizumab-related side-effects have been extensively described. Although its treatment effect does not seem to be influenced by patients' age, specific outcome data on the use of bevacizumab in elderly patients derive from retrospective subpopulation analyses of large randomized controlled trials[20], small phase 2 studies[21-25], non-randomized community-based registries[26-29], or cohort studies[30,31], and have been summarized elsewhere[32].

In all, available data suggest that medically fit older CRC patients exposed to bevacizumab achieved the same benefits compared to younger patients[33,34], with a similar toxicity profile, except for a significant increase in arterial thrombosis[20].

More recently, the randomized phase Ⅲ AVEX study has prospectively evaluated the additive effect of bevacizumab in the older CRC population. In this trial, 280 elderly patients (median age 76 years, range 70-87 years) received either capecitabine alone (1000 mg/sqm *bid* days 1-14, q21) or combined with bevacizumab (7.5 mg/kg). Over 90% of the enrolled patients had ECOG PS ≤ 1. Clinically significant cardiovascular disease was among exclusion criteria. The simultaneous use of bevacizumab produced significant increase of median PFS (9.1 mo *vs* 5.1 mo, HR = 0.53). Interestingly, the RR was twice as high in the combination arm (19.3% *vs* 10.0%) while the safety profile was similar to that previously reported

when testing the combination of capecitabine and bevacizumab. Although the trial was underpowered to detect differences in survival, median OS was longer in the experimental arm (20.7 mo *vs* 16.8 mo, HR = 0.79)[35]. Nevertheless, senior patients are usually underrepresented in well-designed randomized clinical trials and those enrolled, with good PS and few comorbidities, may not represent the average elderly population. This is the main reason why although skilled in dealing with hypertension, proteinuria, vascular thromboembolic events, bleeding, congestive heart failure, gastrointestinal perforation, and wound-healing complications, most oncologists fear that frequency and intensity of those side-effects might be greater in older CRC patients and the benefit-to-risk ratio less favorable in the general practice.

ANTIANGIOGENIC-INDUCED HYPERTENSION IN OLDER CRC PATIENTS

Epidemiological data collected over the last 30 years have demonstrated that the increasing prevalence of hypertension with age is linked to the combination of increased arterial stiffness, neurohormonal and autonomic dysregulation, and the progressive decline of renal function[36-38]. In the elderly, hypertension *per-se* is a significant risk factor for cardiovascular morbidity and mortality. The increase of blood pressure, the most frequent side-effect of systemic inhibition of VEGF signaling, may occur at any time during therapy and it is often associated with asymptomatic proteinuria that spontaneously resolves as soon as treatment ends[39-41]. According to a retrospective review that found an incidence of increased blood pressure of 29% in patients aged > 75 years *vs* 11% in those aged 65 to 75 years[42], advanced age has to be considered a risk factors for the development of bevacizumab-induced hypertension.

Since hypertension-related disorders, such as stroke or myocardial infarction, have been reported with a higher incidence in older patients, a careful home-based daily blood pressure monitoring is suggested during the whole treatment period[43,44]. Older patients developing elevated systolic blood pressure may be at particular risk for complication, since this event is even more associated with cardiovascular morbidity and mortality than diastolic hypertension[45].

Management of antiangiogenic-induced hypertension in older patients usually requires standard treatment and should be promptly adopted[46]. An 1.5%-3.4% 60-d mortality rate has been reported for CRC patients older than 65 years who developed or worsened preexisting hypertension during exposure to bevacizumab in the BRITE trial[26]. How to manage this side-effect has been largely discussed. The upfront use of angiotensin-converting enzyme inhibitors is supported by their ability to counteract bevacizumab-induced plasminogen activator inhibitor-1 and this intervention is widely adopted in the general population[47]. However, the optimal treatment strategy in the elderly population is unconfirmed and the use of diuretics may be preferred. The JNC-7 hypertension guidelines suggested the use of thiazidic diuretics either alone or in combination as initial therapy for older patients[48]. Importantly, the Hypertension in the Very Elderly Trial study showed that the use of indapamide, either alone or combined with perindopril,significantly reduced the incidence of stroke and heart failure even in patients aged ≥ 80 years[49,50]. Although the long term benefits from antihypertensive drug treatment may be relevant for elderly subjects, fit octogenarians with bevacizumab-induced hypertension and a reduced life-expectancy should achieve benefits from intervention as soon as possible. Actually, immediate treatment compared with delayed treatment reduced the occurrence of stroke by 28% and cardiovascular complications by 15% in the Systolic Hypertension in Europe extension trial[51].

Interestingly, retrospective studies have consistently reported a better survival outcome for patients who had developed bevacizumab-induced hypertension[52,53]. Inhibition of VEGF signaling may induce a rapid increase in blood pressure, suggesting that hypertension could be a useful pharmacodynamic surrogate marker of VEGF activity[54]. However, a retrospective analysis of seven randomized phase Ⅲ trials with bevacizumab in different types of metastatic cancers, showed that the correlation between the vascular side-effect and the clinical outcome was shaggy, since the development of bevacizumab-induced hypertension inconsistently predicted longer PFS and OS[55].

OTHER CARDIOVASCULAR SIDE-EFFECTS: VENOUS THROMBOEMBOLIC EVENTS, ARTERIAL THROMBOEMBOLIC EVENTS, BLEEDING, AND HEARTH FAILURE

Older cancer patients are at increased risk for vascular thrombosis[56-59], More specifically, placebo-controlled trials confirmed that the risk for venous thromboembolic events (VTE) is higher when the patient is aged ≥ 65, diagnosed with gastrointestinal malignancies, or receiving antiangiogenic drugs[60]. The average risk for VTE among ambulatory patients undergoing chemotherapy exceeds 12% over one year after treatment initiation, being the use of bevacizumab a potential risk factors[61,62]. Nevertheless, a pivotal randomized trial enrolling over 800 CRC patients showed similar VTE incidences (19.4% *vs* 16.2%) regardless of bevacizumab exposure[63]. In addition, a large pooled analysis showed similar incidence of all-grade VTE among CRC cancer patients exposed to bevacizumab (10.9%) compared to the control group (9.8%), with a similar median time to VTE of 2.2 mo *vs* 1.7 mo[64]. Moreover, a real-practice observational study enrolling 637

advanced CRC patients reported a VTE incidence rate of only 4% in those aged over 65 years[9]. Taking into account these data, it remains to be clarified if thromboprophylaxis should be considered for all cancer patients[65,66], or limited to older patients with limited mobility[67].

Some concerns surround the use of antiangiogenic drugs and the risk of arterial thromboembolic events (ATE) in elderly patients, many of whom may have preexisting cardiovascular risk factors or known cardiovascular disease. Although the event-related death rate remained low, the overall incidence of ATE is close to 4% for advanced CRC patients receiving bevacizumab, and less than 2% in those receiving chemotherapy alone. Significant risk factors for ATE are the history of previous VTE (HR = 2.17) and the older age (HR = 3.65)[43]. In the BRITE (Bevacizumab regimens: investigation of treatment effects and safety) study, the rate of ATE was identical in patients aged < 65 years old (1.4%) compared with those aged between 65-74 years (1.4%), but it was significantly higher in patients aged > 75 years (4.8%). The analysis of the MAX AGTGC showed that bevacizumab was associated with a modestly higher risk of ATE, but the safety profile was similar regardless of age, previous history of ATE or other vascular risk factors[68]. Whether the use of low-dose aspirin may be beneficial in reducing the rate of cardiovascular events in cancer patients as well as in the general population[69] is plausible but unproven.

Atrial fibrillation and coronary artery disease are prevalent with increasing age. Patients on antithrombotic treatment for those conditions should be carefully monitored since bleeding is another potentially severe bevacizumab-induced adverse event[70]. Whether patients on anticoagulant or antiplatelet therapy could be safely treated with bevacizumab is unclear[71]. Patients receiving full-dose anticoagulants have a limited risk of severe bleeding (< 1%) regardless concomitant antiangiogenic exposure[64] and advanced CRC patients treated with bevacizumab while on low-dose acetylsalicylic acid experienced similar rates of bleeding compared to the others (11% *vs* 14%, $P = 0.13$)[72]. Nonetheless, because of the retrospective nature of the data, a note of caution should be used in patients who are candidates for bevacizumab and are receiving full-dose anticoagulation or antiplatelet therapy.

A large population-based study evaluated the risk of cardiovascular events (ATE, cardiac death, cardiomiopathy or congestive hearth failure) among 6803 older CRC patients receiving bevacizumab and chemotherapy[73]. Median age of included patients 73 years and a fifth were 80 years or older. The cohort study confirmed that the cardiovascular risk of bevacizumab use is modest, reporting no clear association between bevacizumab use and cardiovascular events and a lower than expected increased risk for ATE (HR = 1.82). Accordingly, a large Surveillance, Epidemiology and End-Results Medicare analysis suggested that older CRC patients treated with bevacizumab do not experience an increased risk of cardiovascular adverse events compared with patients not treated with bevacizumab[74]. Nevertheless, in the presence of ECgraphic signs of asymptomatic ischemia or in the case of angina or myocardial infarction, antiangiogenic treatment should be immediately discontinued[75].

ANTIANGIOGENIC-INDUCED PROTEINURIA AND THE AGING RENAL FUNCTION

Animal models showed that VEGF is critical in the regulation of renal vascular network and that perturbations of VEGF expression may damage cellular architecture and function, leading to hypertension and proteinuria[76]. Clinical data confirmed that bevacizumab may induce thrombotic microangiopathy by reducing glomerular VEGF, and the presence of podocyturia in patients treated with antiangiogenic drugs suggested that to quantify urinary podocyte excretion may be a highly sensitive indicator of glomerular damage[77]. A retrospective chart review showed that only 1.6% of patients developed severe proteinuria during bevacizumab administration; baseline chronic kidney disease and the development of hypertension significantly correlated with its occurrence ($P < 0.01$)[78]. Indeed, a number of factors may increase the chance for antiangiogenic-induced renal toxicity among elderly patients, including age-related renal structural changes and limited nephron reserve, baseline comorbid conditions such as hypertension, diabetes, or cardiovascular diseases, and the use of polypharmacy or potentially nephrotoxic agents[79]. Since renal failure is initially asymptomatic, a decreased glomerular filtration rate (GFR) or/and an increased albumin-to-creatinine ratio (albuminuria > 30 mg/g of creatinine) may suggest initial kidney damage and forecast later kidney failure[80]. Therefore, an accurate assessment of renal function is essential during antiangiogenic therapy, especially for elderly people at risk of developing renal dysfunctions. In the clinical practice, elderly CRC patients should be accurately screened for proteinuria before starting bevacizumab or other antiangiogenic drugs by dipstick urine analysis, and a 24-h urine collection is suggested when a 2+ or greater urine dipstick reading is detected. The frequency of the test during the course of therapy should be customized.

THE ISSUE OF THE INTACT PRIMARY TUMOR IN THE ELDERLY

Metastatic CRC patients with intact primary tumor seldom require palliative treatment while on systemic upfront chemotherapy[81,82]. Although bevacizumab has been associated with a 2% incidence of bowel perforation and a possible increased risk may exist in those with intact primary tumor, upfront noncurative intestinal resection of asymptomatic metastatic CRC patients may be avoided[83,84]. Among 1953 bevacizumab-treated patients included in the BRITE study, 37 (1.9%) developed gastrointestinal perforation[85]. Twenty-six of these cases (70%)

occurred within the first 6 mo since treatment start, with a median time to event of 3.5 mo. The presence of an intact primary tumor (HR = 2.0) or having received radiotherapy (HR = 2.1) were significant risk factors for perforation. Interestingly, the study failed to show higher rates of perforation in patients with history of peptic ulcer disease, diverticulosis, or in those who chronically used aspirin (≥ 325 mg/d) or other anti-inflammatory drugs. Moreover, the event was less frequently reported among those aged > 65 (1.1%) compared to those younger than 66 (2.6%) with an HR of 0.49. Similarly, in the MAX AGTGC trial, no gastrointestinal perforations were reported in CRC patients aged over 75 years exposed to bevacizumab, but 4 cases noted in the younger cohort[34]. Alongside, the rate of intestinal perforation was 3.6% among 223 patients with unresected primary tumor compared to 1.2% among 1373 patients who had been previously resected in the First BEAT study, although an age breakdown was not available[86].

IS MAINTENANCE WITH BEVACIZUMAB USEFUL IN THE ELDERLY?

Showing a greater benefit if bevacizumab was given until disease progression, results of No. 19966 trial suggested a possible role of antiangiogenic drugs in the maintenance phase[87]. In addition, a number of randomized studies have been conducted to formally assess the role of bevacizumab as maintenance agent[88]. In the MACRO (Maintenance in Colorectal) trial, 480 CRC patients were randomly assigned to receive six cycles of bevacizumab, capecitabine, and oxaliplatin every 3 wk followed by bevacizumab either alone or combined with the same chemotherapy regimen until progression[89]. A slightly longer median PFS was reported in the combination arm (10.4 mo *vs* 9.7 mo), although burdened by a higher rate of severe sensory neuropathy (26% *vs* 8%) and HFS (13% *vs* 7%). Up today, the role of bevacizumab as maintenance therapy is still controversial[90] and additional randomized maintenance studies, such as the AIO KRK0207, the CAIRO-3, the FFCD Prodige 9, and the SAKK 41/06 trial, will soon clarify the point. Waiting for more substantial data, small non-randomized studies have investigated the role of bevacizumab as maintenance therapy. In the BOXE study, 44 elderly CRC patients with median age of 74 years (range 70-84 years) received XELOX and bevacizumab at the dose of 7.5 mg/kg every 3 wk for up to 8 cycles followed by maintenance with single-agent bevacizumab at the same dose[23]. The trial suggested that the combination is feasible and safe in the elderly population and a maintenance with bevacizumab may be offered to responding patients with the intent to prolong PFS.

NOVEL ANTIANGIOGENIC ANTIBODIES: DO THEY FOSTER HOPE FOR OLDER PATIENTS?

Among the more promising novel drugs, aflibercept and ramucirumab deserve to be presented. Aflibercept, a humanized protein composed of the extracellular domains of VEGFR-1/2 fused onto the constant region of human IgG, was specifically designed to bind VEGF-A, VEGF-B, and PIGF. VELOUR is a phase Ⅲ placebo-controlled trial that tested the combination of FOLFIRI and aflibercept for advancer CRC patients that had failed an oxaliplatin-based first-line therapy[91]. The primary endpoint of the trial was OS; secondary endpoints included PFS, overall response rate, and safety. Median age of patients treated with aflibercept was 61 years (range 21-82 years). Patients exposed to aflibercept had longer median OS (13.5 mo *vs* 12 mo, HR = 0.81) and PFS (6.9 mo *vs* 4.6 mo, HR = 0.75) compared to those who were not. However, the toxicity profile was not negligible. While the increases in hypertension and proteinuria were expected as class side-effects, patients receiving aflibercept reported unforeseen significantly higher rates of severe diarrhea (19.3% *vs* 7.8%), fatigue (16.9% *vs* 10.6%), stomatitis (13.7% *vs* 5.0%), and neutropenia (36.7% *vs* 29.5%). This should be considered when offering the treatment to older subjects because they may have increased toxicity when treated with second-line FOLFIRI. Ramucirumab, a VEGFR-2 inhibitor that has shown efficacy in second line gastric cancer, is being studied combined with FOLFOX in the RAISE trial[92]. In this phase 3 study, over 1000 CRC patients that have previously failed first-line FOLFIRI and bevacizumab are randomized to FOLFOX or FOLFOX plus ramucirumab (8 mg/kg) every 2 wk. Results are eagerly awaited.

IS THERE A ROLE FOR ORAL TKI IN CRC?

In the last few years a number of small molecule inhibiting the VEGF pathway have been tested for advanced CRC patients with disappointing results. A phase Ⅲ randomized trial compared FOLFIRI plus sunitinib (37.5 mg every 4 out of 6 wk) to FOLFIRI alone in 768 patients with advanced disease[93]. At the second planned interim analysis the trial was stopped because the data monitoring committee found that the futility boundary had been crossed and more toxicity events were reported in the experimental arm, including neutropenia and severe diarrhea. Final results confirmed no differences in median PFS (7.8 mo *vs* 8.4 mo, HR = 1.05) and a more severe toxic profile.

Vatalanib (PTK787/ZK222584) is an antiangiogenic TKI that blocks VEGFR-1, 2, and 3 by acting as a competitive inhibitor at the adenosine triphosphate–binding site of the receptor kinase. Two randomized, placebo-controlled, large phase 3 trials studied the role of vatalanib in CRC patients treated upfront or in second-line setting[94,95]. The CONFIRM-1 study showed that the addition of vatalanib to FOLFOX-4 had no impact on median PFS (7.7 mo *vs* 7.6 mo, HR = 0.88) or OS (21.4 mo *vs* 20.5 mo, HR = 1.08) compared with FOLFOX-4 alone as first-line treatment. Similarly, the CONFIRM-2 trial compared FOLFOX plus vatalanib or placebo in

855 advanced CRC patients after the failure of a first-line treatment. Again, marginal differences in terms of PFS (5.6 mo *vs* 4.2 mo, HR = 0.83) were registered with identical survival results (OS 13.1 mo *vs* 11.9 mo). In both trials, more gastrointestinal toxicities, dizziness, anorexia, pulmonary embolism and hypertension were reported in the vatalanib group. Taken together, the results of these trials suggested the uselessness of vatalanib for CRC patients, although a PFS advantage (HR = 0.65) was noted in those patients with higher lactate dehydrogenase baseline values.

The combination of oxaliplatin-based chemotherapy and cediranib, a potent inhibitor of the VEGF family receptor tyrosine kinases with multitarget TKI properties, has been extensively tested. The HORIZON Ⅲ trial compared FOLFOX plus cediranib (20 mg, daily) or bevacizumab in over 1400 advanced CRC patients in first-line setting[96]. The study did not meet its primary endpoint and the group exposed to cediranib experienced more toxicity. In addition, the randomized HORIZON Ⅱ trial showed a marginal improvement in median PFS (8.6 mo *vs* 8.3 mo) when cediranib was added to FOLFOX or XELOX (*vs* placebo), without overall survival differences[97]. A third randomized trial compared the outcome of advanced CRC patients receiving FOLFOX combined to bevacizumab or cediranib at two different daily doses (20 or 30 mg)[98]. The trial revealed reduced median PFS for the low-dose cediranib group compared to the standard arm (5.8 mo *vs* 7.2 mo). Similar outcome results and increased toxicity rates were noted when comparing the high-dose cediranib arm to the standard arm.

After all these unsatisfactory data, regorafenib renewed the interest in oral VEGF inhibitors for CRC patients. Regorafenib is an oral multi-kinase inhibitor which targets angiogenic, stromal and oncogenic receptor TK[99]. In the randomized double-blind, placebo-controlled CORRECT study 760 advanced CRC patients received regorafenib or placebo plus best supportive care after progression to all approved standard therapies[100]. Overall survival, the primary endpoint, was significantly increased from 5 to 6.4 mo. The most common regorafenib-related AE included fatigue (47.4%), HFSR (46.6%), diarrhea (33.8%), anorexia (30.4%), voice alteration (29.4%), hypertension (27.8%), mucositis (27.2%), and rash/desquamation (26.0%).

Currently, there are no available data on the specific use of these new drugs in the elderly, and trials designed specifically for older patients are strongly desirable.

CONCLUSION

There is strong evidence for efficacy of bevacizumab and other antiangiogenic drugs in the treatment of advanced CRC. Older age *per-se* should not represent a stringent limit for the employ of these agents. However, the widespread clinical use of antiangiogenetics to treat elderly CRC patients should be cautious and always deserves a personalized benefit-to-risk evaluation along with a careful monitoring of cardiovascular and renal potential side effects.

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EDITORIAL

Sorafenib and entecavir: The dioscuri of treatment for advanced hepatocellular carcinoma?

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Abstract

Hepatitis B virus (HBV) is responsible for 50%-80% of cases of hepatocellular carcinoma (HCC) worldwide. Entecavir (ET) is a potent inhibitor of chronic HBV-DNA polymerase, inhibiting both the priming and elongation steps of viral DNA replication. Sorafenib (SO) has proven efficacy in prolonging survival in patients with advanced HCC. In this frontier report we discuss a possible way to optimize treatment outcomes in patients with HBV and HCC by treatment with ET and SO, on the basis of our practice and published evidence from the literature.

Key words: Entecavir; Hepatocellular carcinoma; Hepatitis B virus; Liver function; Sorafenib

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INTRODUCTION

Over 350 million people globally are chronically infected with hepatitis B virus (HBV) and around 25% of these will develop hepatocellular carcinoma (HCC)[1,2]. HCC is the fifth most common malignancy with approximately 750 000 new cases occurring worldwide each year[3,4]. Overall 70%-90% of patients with HCC have liver cirrhosis caused mainly by HBV and hepatitis C virus[5,6]. HBV, an oncogenic virus, can cause HCC in the absence of cirrhosis and the risk of HBV-induced HCC varies depending on the presence or absence of concomitant cirrhosis. Chronic carriers of HBV have up to a 30-fold increased risk of HCC[7]. In areas of high HBV endemicity, persons with cirrhosis have an approximately 16-fold higher risk of HCC than the inactive carriers, and a 3-fold higher risk for HCC than those with chronic hepatitis but without cirrhosis[8]. While epidemiological studies provide strong evidence for a causal role of chronic HBV infection in the development of HCC, the pathogenesis of HBV infection and carcinogenesis of HBV-associated HCC are still not fully understood. It is thought that HBV exerts its oncogenic potential through both indirect and direct mechanisms that may act in synergy[9-11].

In this frontier report we discuss a possible way to optimize treatment outcomes in patients with HBV and HCC by treatment with entecavir (ET) and sorafenib (SO), on the basis of our practice and published evidence from the literature.

POTENTIAL ROLE OF ET AND SO

The most effective way to prevent HBV-related HCC is by vaccination but in patients already infected with HBV, antiviral therapy is the best strategy[9]. ET, a cyclopentyl guanosine analog, is a potent inhibitor of chronic HBV-DNA polymerase, inhibiting both the priming and elongation steps of viral DNA replication. In clinical trials, ET was superior to lamivudine for all primary end points

evaluated in both nucleoside-naive and lamivudine-resistant patients as well as being effective in both hepatitis B "e" antigen-positive and -negative nucleoside-naive patients. Antiviral therapy can reduce, but not eliminate the risk of HCC especially in patients with pre-existing cirrhosis and it is therefore important to maintain virological remission. The use of ET allows long-term HBV-DNA suppression with a low risk of resistance.

SO, a tyrosine kinase inhibitor, has been demonstrated in two large scale randomized, double-blind, placebo-controlled, multicentre, phase Ⅲ trials (the SHARP trial and the Asia-Pacific trial) to prolong median overall survival and delay the median time to progression in patients with advanced HCC[12,13]. The SHARP study was the first to show an overall survival benefit for SO in patients with advanced HCC, in which the overall survival was 10.7 mo[14]. Subanalyses of the SHARP population based on a range of parameters including aetiology (hepatitis B virus present/absent); tumour burden (macroscopic vascular invasion and/or extrahepatic spread present/absent); presence or absence of either lung or lymph node metastasis at baseline, confirmed the efficacy and safety of SO in these subpopulations indicating that SO is effective for patients from the AP region with advanced HCC, irrespective of baseline status[15,16].

Individually ET and SO have been demonstrated to have important roles in the management of patients with HBV and HCC but how best should we use these agents - in combination or as a sequential strategy. The problem is that although there are a number of published guidelines on the treatment of patients with HBV there are no precise indications on the use of antiviral agents in patients with HBV-related HCC, however it is recognized that the goal of antiviral therapy for HBV is to preserve liver function and prevent the development of cirrhosis and HCC. Early intervention is therefore necessary to prevent liver cell damage and decrease viral genome integration. We believe that it is vital to prevent the deterioration of liver function as modulation of liver function may affect survival directly and indirectly but also it may have an impact on the patient's ability to tolerate subsequent treatments.

In a study by Jin *et al*[17], first-line ET monotherapy was effective in HBV patients (with and without HCC), improved hepatic function and importantly was associated with increased survival after eradication of HCC - confirming previous results that it improved liver function in patients with decompensated cirrhosis[18,19]. Considering that liver function is a key factor in deciding treatment options for a given patient and concomitant liver dysfunction often hampers both curative and palliative therapies, the fact that ET can improve hepatic function is decisive in the clinical scenario[20]. Furthermore, in a study by Chang *et al*[21] the majority of nucleoside-naive patients with HBV who were treated with long-term ET achieved substantial histological improvement together with regression of fibrosis or cirrhosis. SO has also shown promising antifibrotic activity with efficacy at relatively low doses at the early stage of liver fibrosis[22].

Table 1 Baseline characteristics and main treatment outcomes of our cohort (*n* = 15) *n* (%)

Baseline characteristics	Value
Characteristic	
Male	1 (6.7)
Age, yr (range)	67 (62-76)
BCLC stage	
B - intermediate	10 (66.7)
C - advanced	5 (33.3)
Child-Pugh score	
5	6 (40)
6	9 (60)
Treatment outcomes	
Overall survival, mo (range)	26.5 (10-36)
Liver decompensation	4 (26.7)
Hepatocellular carcinoma progression	3 (20.0)
Interruption of sorafenib therapy due to adverse events	0 (0)

All subjects achieved viral clearance following entecavir treatment before the initiation of sorafenib 800 mg/d.

OUR EXPERIENCE

In our unit, we treated a total of 15 patients (1 male; aged 62-76, median 67 years) with advanced HCC and a history of HBV cirrhosis from October 2008 to December 2011. Diagnosis of advanced HCC was made according to the Barcelona Criteria using contrast enhanced ultrasound, elevated values of alpha-fetoprotein and/or liver biopsy. Ten patients had intermediate BCLC stage B and 5 had advanced BCLC stage C and all had Child Pugh A (9 with an A6, 6 with A5). The baseline characteristics of patients are summarized in Table 1.

All patients achieved a complete clearance of HBV-DNA following the administration of ET (0.5 mg/d) before the initiation of SO. The dosage of SO was gradually increased over a 6-wk period to reach the recommended dosage of 800 mg/d.

The median survival in these patients with HCC and HBV was 26.5 mo (range 10-36 mo). No patient stopped therapy due to AEs (cardiac, gastrointestinal, haematological, neurological or dermatological, or endocrinological). All patients had blood pressure within the accepted recommend range, assumed regular cardiac medication as necessary and were negative for HBV-DNA. Four patients had liver decompensation and three had progression of HCC.

It must be emphasized that our experience is reported here in a very synthetic form, since this paper should be intended as a short commentary addressing how treatment with SO and ET might optimize treatment outcomes in patients with HBV and HCC. In addition, the data reported here present several limitations, which should be taken into account to put the above-mentioned findings in a proper framework. First, the sample observed in our experience is too limited to draw any conclusion. Second, the pure observational nature of our findings does not

allow to retrieve any definite cause-effect relationship.

These limitations taken into account, these results are somehow encouraging: this may be, at least in part, due to the viral clearance achieved by patients. We cannot rule out, however, that the longer survival observed in our patients can be attributed to the high proportion of subject with BCLC-B stage HCC.

CONCLUSION

On the basis of our experience and current literature, therefore, we propose that in patients with HBV monotherapy with ET should be given initially to reduce viral load and preserve liver function thereby allowing follow-up treatment with SO to treat HCC. We believe that this treatment approach may represent a potential improvement in the current management of advanced HCC in patients with concomitant HBV infection. However, further, well-designed studies are needed to investigate the efficacy and safety of this therapy in a large sample of patients. If such study will provide positive results, we feel that SO and ET will be considered the "Dioscuri", the warrior twins of the Greek mythology, of the treatment of advanced HCC.

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REVIEW

Diagnosis of bowel diseases: The role of imaging and ultrasonography

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Abstract

Examinations with a visualisation of the anatomy and pathology of the gastrointestinal (GI) tract are often necessary for the diagnosis of GI diseases. Traditional radiology played a crucial role for many years. Endoscopy, despite some limitations, remains the main technique in the differential diagnosis and treatment of GI diseases. In the last decades, the introduction of, and advances in, non-invasive cross-sectional imaging modalities, including ultrasound (US), computed tomography (CT), positron-emission tomography (PET), and magnetic resonance imaging, as well as improvements in the resolution of imaging data, the acquisition of 3D images, and the introduction of contrast-enhancement, have modified the approach to the examination of the GI tract. Moreover, additional co-registration techniques, such as PET-CT and PET-MRI, allow multimodal data acquisition with better sensitivity and specificity in the study of tissue pathology. US has had a growing role in the development and application of the techniques for diagnosis and management of GI diseases because it is inexpensive, non-invasive, and more comfortable for the patient, and it has sufficient diagnostic accuracy to provide the clinician with image data of high temporal and spatial resolution. Moreover, Doppler and contrast-enhanced ultrasound (CEUS) add important information about blood flow. This article provides a general review of the current literature regarding imaging modalities used for the evaluation of bowel diseases, highlighting the role of US and recent developments in CEUS.

Key words: Gastrointestinal tract; Bowel; Imaging; Ultrasound; Colour-Doppler; Contrast-enhancement; Time-intensity curve

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INTRODUCTION

Endoscopy remains the main technique for the diagnosis of gastrointestinal (GI) tract diseases because it allows a direct visualisation of the mucosa and the possibility of taking samples for histological analysis. Moreover, in recent years, improvements in endoscopic techniques have also made it possible to use endoscopy for interventions in some diseases of the GI tract. However, endoscopy has some limitations due to its invasiveness and the difficulty of examining the small bowel, and it does not allow the visualisation of extra-intestinal structures that may be involved.

For many years, traditional radiological techniques played a crucial role in the diagnosis of small bowel diseases. In the last decades, the introduction of, and improvements in, non-invasive cross-sectional imaging tech-

niques including ultrasound (US), computed tomography (CT), positron-emission tomography (PET) and magnetic resonance imaging (MRI), have changed the diagnostic approach to the GI tract[1]. The high resolution of imaging data, ability to acquire 3D images, enhancement of tissues and additional co-registration techniques (PET-CT, PET-MRI) have improved the diagnostic classification of tissue pathology and performance in terms of sensitivity, specificity and accuracy, depending on the specific method and equipment used, the section of the GI tract investigated, the patient's constitution and preparation, and the type of pathology being studied[2].

In the last two decades, among the cross-sectional imaging techniques, US has had a growing role in the development and application of techniques for the diagnosis of GI diseases because it is cheap, non-invasive, and more comfortable for the patient, and it has sufficient diagnostic accuracy to provide the clinician with high temporal and spatial resolution image data. Moreover, Doppler and contrast-enhanced ultrasound (CEUS) contribute important information about blood flow.

This article provides a general review of the current literature regarding imaging modalities used for the evaluation of bowel diseases, highlighting the role of US and recent developments in CEUS.

CONVENTIONAL RADIOLOGICAL EXAMINATIONS

Plain-film radiography remains the first-line of investigation in the acute setting. Non-contrast radiography is useful in the initial assessment of various GI diseases, including bowel perforation, obstruction, volvulus, and toxic megacolon[3].

When detailed luminal evaluation is required, fluoroscopic barium or water-soluble single- and double-contrast studies are the modalities of choice. These techniques are able to visualise transit time, peristalsis, luminal emptying and pathological changes such as stenosis, dilatation, luminal filling defects and external compression. Moreover, double-contrast examinations allow detailed visualisation of the mucosa and the detection of inflammatory and neoplastic changes in the intestine[4].

Barium swallow studies remain the main investigational tool for dysphagia, allowing direct evaluation and inspection of the oesophageal mucosa and gastro-oesophageal junction, an objective measurement of oesophageal contractibility, assessment of reflux and identification of the presence of strictures, pouches, and hiatal hernia[5]. With respect to the small intestine, fluoroscopic imaging techniques such as small bowel barium follow-through and conventional enteroclysis are able to detect subtle mucosal abnormalities such as fistulous tracts, adhesions and, more rarely, intraluminal lesions. Functional information about transit time and peristalsis can also be ascertained.

Water-soluble, single-contrast oral studies are generally performed in the immediate post-operative period to assess anastomotic integrity, due to the potential for free intra-abdominal barium to induce peritonitis[6].

However, fluoroscopic imaging has several disadvantages: first, it only allows indirect detection of alterations of the small bowel, with no information on deeper wall layers and extramural disease extension; and second, its sensitivity for detecting marginal changes is low compared to direct inspection of the mucosa.

CROSS-SECTIONAL IMAGING

Computed tomography

The development of multi-detector computed tomography (MD-CT) scanners with rapid acquisition of thin slices and multi-planar reconstructions allows a detailed investigation of intestinal loops[7]. In particular, non-contrast-enhanced CT scanning is replacing plain-film radiography in the evaluation of acute abdominal disease such as intestinal perforation or obstruction[8]. Intravenous contrast enhancement together with distension of the intestinal lumen by water or positive contrast agents is very useful in the detection of inflammatory and neoplastic intestinal pathologies (fistula, abscess, and phlegmon) as well as in the evaluation of extra-intestinal involvement (mesenteric lymph nodes)[9].

MD-CT colonography, also known as virtual endoscopy, is a new technique to study the large intestine that is able to detect colonic polyps greater than 6 mm with a similar accuracy to conventional colonography[10-12]. Similar to CT, it is also important in the detection of extra-colonic pathology[13,14].

For these reasons, this technique may replace traditional double-contrast examinations as a non-invasive screening test or in acute colonic inflammatory processes when other approaches are contraindicated due to the high perforation risk[2].

MRI

MRI is generally considered the gold standard examination for TNM staging of rectal cancers because it allows an exact visualisation of the rectal wall and perirectal fat infiltration[15].

Moreover, MRI is the preferred technique in inflammatory bowel diseases (IBD) because it is able to examine the entire small intestine without radiation hazards[9,16]. It can detect luminal (stenosis and fissures), mural (wall thickening and wall enhancement after gadolinium administration) and exoenteric (mesenteric inflammation, fibrofatty proliferation, lymph adenopathy, hypervascularity, abscesses and fistulas) pathologies[16-20]. In particular, MRI is more sensitive than other techniques in the evaluation of anorectal fistulas[20].

Finally, the administration of intravenous contrast agent and the consequent detection of hypervascular areas are useful in distinguishing between active and inactive disease[17,21,22].

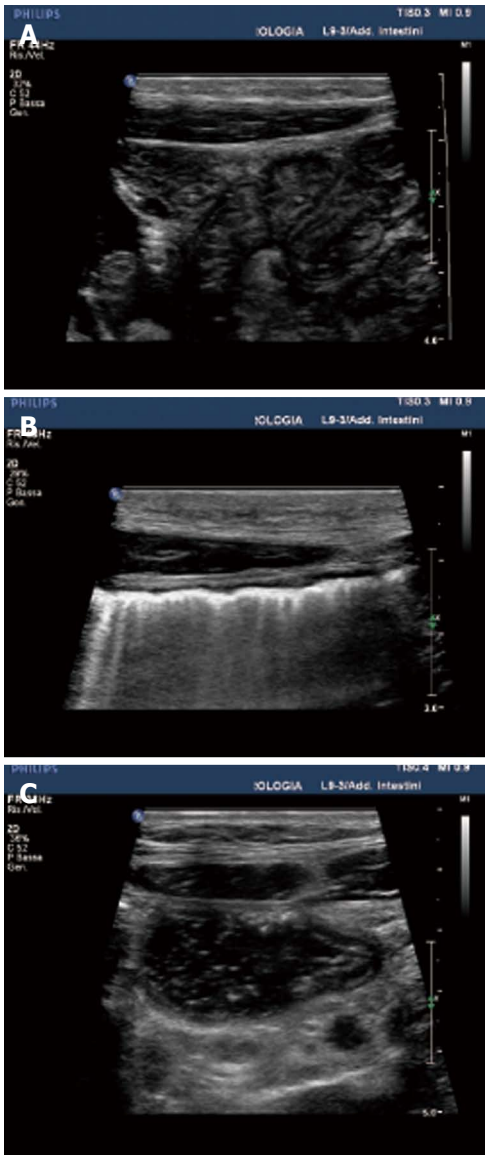

Figure 1 Sonographic appearance of normal bowel. A: Mucus pattern: collapsed bowel containing only a highly reflective core of mucus with target appearance on a transverse section; B: Gas pattern: only the proximal side of the bowel wall is visible due to beam attenuation by gas; C: Fluid pattern: the bowel is filled with fluid and faeces with a tubular appearance on a longitudinal section.

US

Among the cross-sectional imaging techniques, US is less invasive, more comfortable for the patient and has a significant diagnostic accuracy[23].

The normal bowel wall appears as a multi-layered area with hyperechoic bowel contents at the centre. Five distinct layers can be observed on sonography: an inner hyperechoic layer, the interface between the mucosa and the bowel contents; a second hyperechoic layer, the deep mucosa; a third hyperechoic layer, the submucosa; a fourth hypoechoic layer, the muscle proper; and a last outer hyperechoic layer, the serosa and the serosal fat[24].

The average wall thickness of the normal gut is 2-4 mm and the US appearance depends not only on the structure of the individual segment but also, more importantly, on its contents and degree of distension. The bowel may be collapsed, containing only a small amount of mucus (mucus pattern), or it may contain fluid or gas (respectively, fluid and gas patterns). The mucus pattern appears as a target with a highly reflective core of mucus. The fluid pattern gives a tubular appearance on a longitudinal section and a rounded pattern on a cross-section. In the gas pattern, only the proximal side of the bowel wall is visible due to beam attenuation by gas (Figure 1).

The jejunum has valvulae conniventes, which produce a ladder pattern, and the ileum has a smooth, featureless wall. The site of the studied bowel must also be inferred from the location of the bowel loop.

The large bowel wall thickness is < 4 mm; it has similar characteristics to the small bowel, but it can be distinguished by its location in paracolic regions and by the presence of haustra.

Similar to the other cross-sectional imaging techniques, US is able to evaluate intestinal findings, such as the bowel wall (in particular, its thickness, layers and perfusion), peristalsis, compressibility, rigidity and extra-intestinal structures, such as perienteric fatty tissues, mesenteric lymph nodes and adjacent organs[25-29].

US and bowel diseases

The most frequent pathological aspects found by sonography in intestinal diseases are wall thickening, mucosal abnormalities, the absence of peristalsis, mesenteric thickening, lymph node enlargement, vascular alterations, and extra-intestinal complications[30].

Morphological changes of the bowel wall

Bowel-wall thickening can be found in inflammatory, infectious, ischemic (but only in later stages) and neoplastic diseases. Usually, in inflammation and infections, the wall thickening is regular with preserved stratification, whereas in tumours, the thickness is irregular with loss of normal stratification[31] (Figure 2).

IBD: Crohn's disease and ulcerative colitis: The classic sonographic feature of Crohn's disease (CD) is the "target" sign on transverse images, which means a strong echogenic centre surrounded by a relatively sonolucent rim of more than 5 mm. In a longitudinal section, the sonographic feature is the "sandwich" sign. In CD, transmural inflammation or fibrosis can lead to complete circumferential loss of the typical gut wall layers, which results in a thick hypoechoic rim more than 5 mm. Strictures appear as marked thickenings of the gut wall with a fixed hyperechoic narrowed lumen, dilatation and hyperperistalsis of the proximal gut[32] (Figure 3).

In expert hands, the distribution of frank lesions of inflammatory bowel disease can be determined with a sensitivity of 73%-87%[33]. In ulcerative colitis, the sensitivity reaches 89%, and the specificity reaches 100%[34].

Differentiation between CD and ulcerative colitis based on sonographic findings is based on the location of the disease, the presence of skip lesions and the

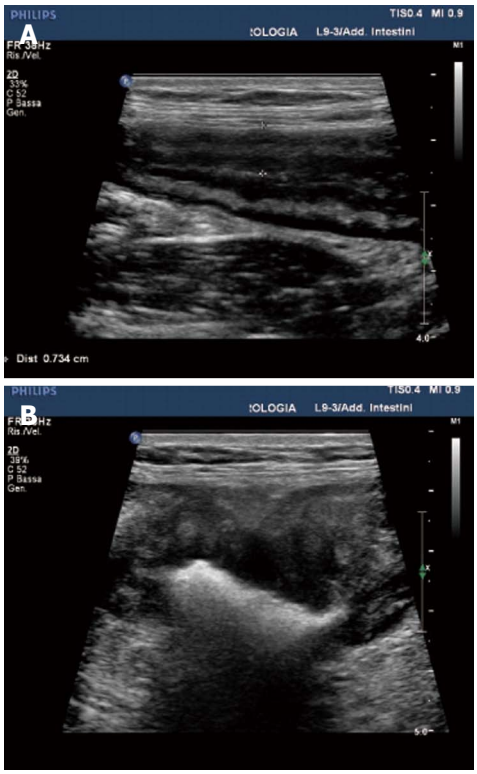

Figure 2 Wall thickening. A: Inflammatory thickening: regular, with preserved wall stratification; B: Neoplastic thickening: irregular with "pseudokidney appearance".

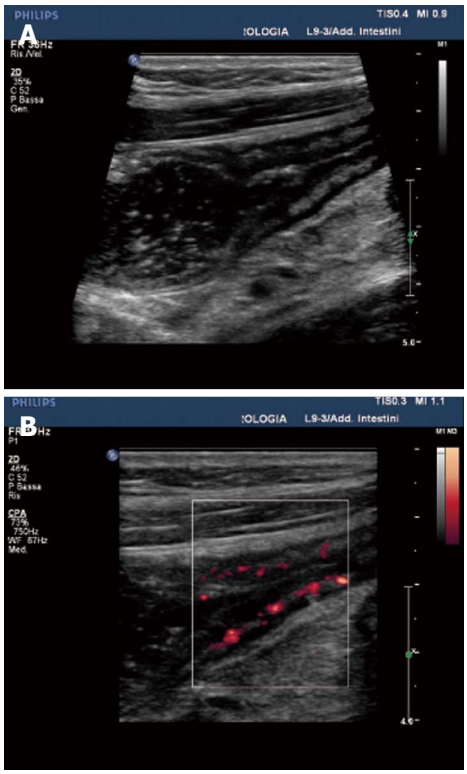

Figure 3 Stenosis in patients with Crohn's disease. A: B-mode aspect: narrow lumen with dilatation of the upstream segments; B: The presence of vascular signals on power Doppler indicates the inflammatory nature of stenosis.

presence of pericolic abscesses. Bowel-wall thickening is usually less marked in ulcerative colitis with preserved stratification. However, definitive differential diagnosis is difficult on transabdominal sonography[35-37].

Acute terminal ileitis: Acute terminal ileitis is frequently caused by Yersinia species but also by Campylobacter and Salmonella. Tuberculous enteritis and Behcet's disease may also affect the ileo-caecal region.

The reported sonographic features include hypoechogenic mural thickening of the terminal ileum and caecum between 6 and 10 mm, with hypoechoic swollen ileal folds in the edematous mucosa, and these findings should be related to clinical and laboratory data[38,39].

Appendicitis: The typical finding of acute appendicitis on a transverse cross-section is the target sign with a hyperechoic centre, an inner hyperechoic ring and an external, thicker hypoechoic ring. In sagittal images, the inflamed appendix is seen as a blind-end, non-compressible tubular structure. Focal or circumferential loss of the inner layer of echoes usually indicates gangrenous inflammation and ulceration of the submucosa. Several studies achieved sensitivities of 80%-93% and specificities of 94%-100% in the sonographic workup of acute appendicitis[40,41].

Graded compression sonography has gained widespread acceptance as a useful technique for the examination of patients with atypical signs of appendicitis[42].

The diagnosis can be established with confidence if the appendix is non-compressible, shows no peristalsis, and measures more than 6 mm in diameter on axial images, and if compression leads to a localised pain response[43].

A statistically significant association has been found between perforation and two sonographic findings: loculated pericaecal fluid and loss of the echogenic submucosa[44].

Small bowel tumours: The gut is the most common extranodal site of lymphoma after the stomach[45]. Eighty percent of gastrointestinal lymphomas have B-cell origins. In patients with underlying coeliac disease, however, a T-lymphocyte origin predominates. In most patients, the US appearance is characterised by transmural hypoechoic wall thickening up to 4 cm in diameter with loss of normal stratification and a central hypoechoic region. This pattern is known as the "pseudokidney" sign[46,47].

Isolated mucosal involvement is rare and leads to hyperechoic thickening of the mucosa. Sonographic patterns favouring the diagnosis of a non-Hodgkin's lymphoma over adenocarcinoma are transmural, circumferential, hypoechoic wall thickening with preserved peristalsis, lack of intestinal obstruction, involvement of a long stretch of the gut and the presence of multiple prominent lymph nodes[48].

Carcinoid is the most frequent small bowel tumour and occurs in 80% of cases in the distal ileum. Usually,

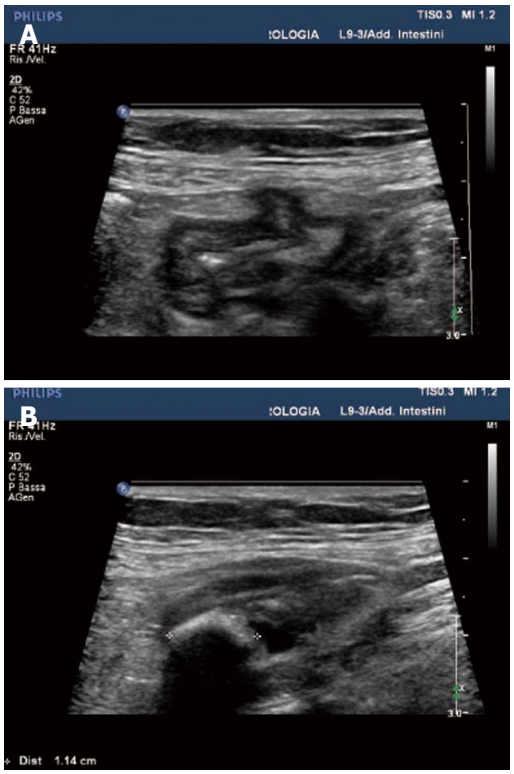

Figure 4 Diverticular disease. A: Reflective outpouchings adjacent to the colonic wall; B: Acoustic shadowing outside the lumen indicating the presence of a coprolith.

small bowel carcinoids appear as hypoechoic, homogenous, predominantly intraluminal masses with smooth intraluminal contours. The tumour is attached to the wall with a broad base, leading to interruption of the submucosa and thickening of the muscularis propria[49].

Pseudomembranous colitis: The sonographic findings of pseudomembranous colitis (PC) have been described in a number of reports. Striking thickening of the colonic wall with a wide inner circle of heterogeneous medium echogenicity surrounded by a narrow hypoechoic muscularis propria is found in all patients, reflecting the submucosal oedema. The lumen of the colon is almost completely effaced by the mural oedema, and 64%-77% of patients have ascites[50,51].

Diverticulitis: The sensitivity of US in the diagnosis of acute colonic diverticulitis ranges from 84% to 100% in different studies and is similar to the sensitivity of CT. US features of diverticulitis are the presence of colonic outpouchings associated with bowel-wall thickening and severe local pain induced by graded compression.

Diverticula are round or oval echogenic foci observed in or next to the gut wall, mostly with internal acoustic shadowing[52-56] (Figure 4).

Colonic carcinoma: There are two possible sonographic appearances of colonic carcinoma. The first is a localised hypoechoic mass up to 10 cm or more with an irregular shape, lobulated contours and a cluster of high-amplitude echoes (the intramural gas) located eccentrically. The second appearance is a segmental and irregular thickening that could be eccentric or circumferential but is less evident than the first type. The central echo clusters are small because the diseased lumen is usually narrow. This type frequently leads to colonic obstruction. Rectal carcinoma is observed only when the bladder is well-filled[57-60].

Shirahama *et al*[61] described four sonographic findings associated with colonic carcinoma in 90% of patients: localised colonic wall thickening with heterogeneous low echogenicity, irregular contour, lack of movement on real-time scanning, and the absence of the layered appearance of the colonic wall. However, negative findings during sonographic examination do not rule out the diagnosis of colonic carcinoma because small masses and overlying bowel gas can lead to false-negative results. Because of these limitations, abdominal sonography cannot be an effective screening technique in colon cancer[57,62].

Intussusception: Intussusception has a characteristic appearance, and it is usually not mistaken for other bowel abnormalities. Transverse sections reveal a swirled pattern of alternating hyperechogenicity and hypoechogenicity, representing alternating layers of mucosa, muscularis, and serosa: the "doughnut" or "bull's eye" sign[63,64]. On longitudinal sections, alternating loops of bowel and a loop-within-loop have a sandwich-like appearance (pseudokidney sign). The outer hypoechoic ring is formed by the intussuscipiens and the everted returning limb of the intussusceptum with their mucosal surfaces face-to-face. The centre of the intussusception varies with the scan level. At the apex, the centre is hypoechoic because of the entering limb of the intussusceptum. At the base, the entering bowel wall forms a hypoechoic centre that is surrounded by the hyperechoic mesentery[65,66].

Perfusion of the bowel wall: The role of colour-power Doppler and CEUS

Colour and power Doppler techniques may provide additional information about the macrovascularisation of the bowel wall. In particular, colour and power-Doppler may be helpful in differentiating among ischaemia, inflammation and cancer neovascularisation. The differential diagnosis is possible because ischaemia is characterised by few or no signals, inflammation is characterized by several signals with low resistivity index (RI) (< 60) and symmetric thickening, and cancer neovascularization is characterised by several signals with a high RI (> 60) and asymmetric thickening[67].

CEUS has recently gained increasing attention because it clearly improves the visualisation of perfusion in various tissues. The development of second-generation, contrast-enhancing agents used in low-mechanical-index harmonic US has enabled real-time assessment of the microvascular circulation and quantification of bowel vascularity[68-70].

US contrast agents consist of micro-bubbles (1-7

micrometres), often made of a phospholipid shell with a gaseous content that are given intravenously and excreted through the lungs. Obviously, the individual capillaries cannot be discerned, but the micro-bubble content gives rise to a signal "wash" with an intensity that is proportional to the micro-bubble concentration and thus to the blood volume in the portion of the tissue[71]. This technique has led to important new applications for US. The essential tool is the transit or wash-in, wash-out curve, often referred to as a time-intensity curve (TIC), in which the time course of the transit of micro-bubbles is measured, hence the term "dynamic contrast-enhanced US" (DCE-US). Two categories of information are available from these TICs: results, that depend on timing events such as the arrival time and the time to peak enhancement, and results that depend on the amount of enhancement detected such as the peak enhancement and the area under the TIC.

Such micro-bubble studies have been used to assess inflammatory diseases, giving important information about the severity of the inflammation and its response to therapy[72-83].

IBD: CD and ulcerative colitis: IBD is associated with hypervascularity of the bowel wall during active disease.

In patients with CD, CEUS is useful for assessing the pattern of neovascularisation within the intestinal layers, allowing better discrimination between active and inactive disease, between inflammatory and fibrotic strictures, and between inflammatory pseudo-tumours and abscesses[84-89].

In particular, Serra *et al*[84] prospectively evaluated the vascularisation of the thickened terminal ileum in CD patients using CEUS and compared the clinical activity as measured by the CD activity index (CDAI) with the CEUS findings. They used two parameters to assess the vascularisation of the bowel wall: a semi-quantitative method, the pattern of enhancement; and a quantitative method, the E/W ratio, which is the ratio between the major thickness of the enhanced layer (E) and the thickness of the entire wall section (W). The results showed a significant correlation between CDAI and the pattern of enhancement. In particular, the frequency of active patients (CDAI > 150) was significantly related to the enhancement of the entire wall section and the submucosal enhancement. A positive correlation was observed between the E/W ratio and the CDAI values[84].

Migaleddu *et al*[90] demonstrated that DCE-US might help in characterising bowel-wall thickening by differentiating fibrosis, oedema and inflammatory neovascularisation and may help to grade disease activity by assessing the presence, initial site, direction and distribution of enhancement.

De Franco *et al*[91] assessed microvascular activation in the thickened terminal ileal wall in patients with CD using CE-US and evaluated its correlation with a composite index of CD activity (CICDA), the CDAI and the simplified endoscopic score for CD (SES-CD). In this study, unlike the two previously discussed studies, the authors evaluated the mural microvascularity with a quantitative method, analysing software-plotted time-enhancement intensity curves to determine the maximum peak intensity (MPI) and wash-in slop coefficient (β). The MPI and β coefficient were significantly increased in patients with CICDA, CDAI and SES-CD scores indicative of active disease[91].

The introduction of new drugs such as immunomodulators or biological therapies such as monoclonal anti-TNF alpha antibodies in the treatment of CD has led to a need for non-invasive methods to assess the efficacy of pharmacologic treatment. A recent study demonstrated that CEUS could be suitable for evaluating changes in bowel wall vascularisation during anti-inflammatory therapy[92]. In this study, all of the kinetic parameters (slope, time to peak, and area under the curve) developed from TICs showed significant changes after treatment and were correlated with the CDAI score.

Acute appendicitis, acute terminal ileitis, diverticulitis, colitis: In these inflammatory pathologies, especially in the early stages, it is possible to find increased vascularisation with both colour-Doppler and CEUS techniques. The presence of visible hyperaemia or increased flow in the hypoechoic muscular layer of the bowel wall may be a marker of appendicitis, whereas increased flow in the mucosal layer most likely represents enteritis. Increased flow in the fat surrounding the appendix is indicative of transmural extension of the inflammation with mesenteric response. The absence of blood flow indicates gangrenous change or paracolic abscess formation[93].

Ischaemic disease: In chronic ischaemia of the small bowel, stenotic or occlusive lesions in the coeliac and/or mesenteric arteries are found, and patients typically have postprandial epigastric pain and weight loss. In acute ischaemia, during the first hour, little or no signal from colour-Doppler or echo-enhancing contrast US can be observed. If the ischaemia has lasted a few hours, dilated bowel loops and a thickened bowel wall can be observed, but these signs are non-specific, and the examination is often made difficult by increasing amounts of intraluminal air.

However, Doppler scanning is not the method of choice for diagnosing acute ischaemia of the small bowel because it does not permit the evaluation of the compensatory collateral circulation and distal embolisation. Thus, angiography must be performed for a definite diagnosis[94,95].

Neoplastic disease: Colour-Doppler and CEUS are not the techniques of choice for the diagnosis of tumours or to differentiate between benign and malignant neoplasia, but, because the tumours are often highly vascularised, these techniques may be helpful to differentiate between tumours and other benign lesions such as abscesses, cysts, and haematomas.

A finding of arterial enhancement with rapid washout on CEUS or arterial signs with an RI > 60 on Doppler are highly indicative of a malignant lesion. DCE US with time-intensity curves has recently been used to evaluate tumour responses to anti-vascular therapy[83].

Extra-intestinal structures: perienteric fatty tissue, mesenteric lymph nodes and adjacent organs

Several intestinal pathologies may involve other structures around the diseased segment such as perienteric fatty tissue, mesenteric lymph nodes, and adjacent or distant organs. The discovery of these findings by US may be helpful for the correct diagnosis.

IBD: Peri-intestinal inflammation leads to the "creeping fat" sign, which appears as a uniform hyperechoic mass typically observed around the ileum and caecum. Mesenteric lymph adenopathy appears as multiple oval hypoechoic masses, usually in the right lower quadrant.

Some of the possible complications of CD are fistula, abscess formation, mechanical bowel obstruction and perforation. Abscesses appear as poorly defined, mostly hypoechoic focal masses that can contain hyperechoic gas. Fistulas are a hallmark of CD and appear in up to one third of patients with advanced disease as hypoechoic tracts with gas inclusion connecting bowel loops or adjacent structures (bladder, abdominal wall, vagina, or the psoas muscle). Detection of gas bubbles in abnormal locations raises the possibility of fistulous communication[96,97].

Appendicitis: The surrounding mesentery is often inflamed, which can appear as a hypoechoic diffuse halo sign around the appendix.

The presence of a generalised adynamic ileus associated with the presence of free fluid should raise suspicion of perforating appendicitis, even if the appendix has not been found to be enlarged.

Abscess formation is the major complication of a perforating appendicitis. Abscesses may extend into the pelvis or into the peritoneal spaces of the upper abdomen. They may appear as a complex inflammatory mass or localised complex fluid collection. This appearance is indistinguishable from perforated bowel neoplasm. Mesenteric lymph adenopathy may be visualised as multiple oval hypoechoic masses, usually in the right lower quadrant[98].

Diverticulitis: The sonographic features of acute colonic diverticulitis include inflammatory changes in the pericolonic fat that appear as ill-defined echogenic masses adjacent to the involved thick-walled colonic segments. The most common complication of acute colonic diverticulitis is perforation with abscess formation: this condition is suggested by the presence of an associated localised complex fluid collection.

It is important to note that although sonography can be used to diagnose uncomplicated diverticulitis with excellent sensitivity and specificity, CT remains the technique of choice for further evaluation of acute colonic diverticulitis, particularly for the assessment of complications such as abscess formation, fistulas, and perforations[52,56,99,100].

Neoplastic disease: Malignant neoplasia, especially at advanced stages, can extend beyond the intestinal wall to involve perienteric tissues such as in peritoneal carcinomatosis.

The presence of regional malignant lymph adenopathy is highly suggestive of malignant disease. Malignant lymph nodes are larger than 1 centimetre and can measure up to several centimetres. They are round but may colliquate to form large irregular masses with necrotic areas and internal calcifications[51].

CONCLUSION

In the last decade many cross-sectional imaging techniques have evolved as superior alternatives to fluoroscopic imaging in the examination of the small and large bowels. In particular, transabdominal US may be regarded as the first imaging procedure in the diagnostic work-up and follow-up of bowel diseases. US has gained acceptance, especially in IBD, because it can provide important information including the extent and activity of the disease and the presence of complications. New sonographic techniques combined with the application of intravenous contrast agents increase the accuracy of Doppler US in evaluating bowel wall vascularisation in a real-time manner. The quantitative assessment of bowel wall vascularity by CEUS could provide a useful and simple method to assess the effectiveness of medical treatment.

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ORIGINAL ARTICLE

Clinicopathological features and outcomes of patients with gastric cancer: A single-center experience

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Abstract

AIM: To evaluate the location, histopathology, stages, and treatment of gastric cancer and to conduct survival analysis on prognostic factors.

METHODS: Patients diagnosed with of stomach cancer in our clinic between 2000 and 2011, with follow-up or a treatment decision, were evaluated retrospectively. They were followed up by no treatment, adjuvant therapy, or metastatic therapy. We excluded from the study any patients whose laboratory records lacked the operating parameters. The type of surgery in patients diagnosed with gastric cancer was total gastrectomy, subtotal gastrectomy or palliative surgery. Patients with indications for adjuvant treatment were treated with adjuvant and/or radio-chemotherapy. Prognostic evaluation was made based on the parameters of the patient, tumor and treatment.

RESULTS: In this study, outpatient clinic records of patients with gastric cancer diagnosis were analyzed retrospectively. A total of 796 patients were evaluated (552 male, 244 female). The median age was 58 years (22-90 years). The median follow-up period was 12 mo (1-276 mo), and median survival time was 12 mo (11.5-12.4 mo). Increased T stage and N stage resulted in a decrease in survival. Other prognostic factors related to the disease were positive surgical margins, lymphovascular invasion, perineural invasion, cardio-esophageal settlement, and the levels of tumor markers in metastatic disease. No prognostic significance of the patient's age, sex or tumor histopathology was detected.

CONCLUSION: The prognostic factors identified in all groups and the proposed treatments according to stage should be applied, and innovations in the new targeted therapies should be followed.

Key words: Gastric carcinoma; Chemotherapy; Prognostic factors; Treatment; Survival; New agents

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INTRODUCTION

Despite the innovations in treatment, gastric cancer still remains a mortal disease[1]. Patient, tumor and treatment factors determine the prognosis. In recent years, when there has been an overall reduction in gastric cancer, a moderate increase in proximal stomach and esophagogastric junction region adenocarcinoma has been observed[2]. While the basic treatment of gastric cancer is complete resection and, following this treatment, if necessary, adjuvant chemoradiotherapy, the standard treatment in meta-

static patients is chemotherapy and palliative treatment. Currently, studies on neoadjuvant therapy are ongoing.

Adjuvant therapy approach

In the intergroup trial (INT 0116), which was a randomized phase Ⅲ trial, the effectiveness of adjuvant chemoradiotherapy was compared with the monitoring group. In that study, 556 patients were randomized to the adjuvant therapy group, in which the five-year survival rate was 50%, or the surgery group, in which it was 41% (HR = 1.35). That study established the standard adjuvant therapy in gastric cancers. After Macdonald's research[3], with close to ten years' follow-up demonstrating that survival was 41% after surgery and 50% after adjuvant chemoradiotherapy, this treatment approach has become the standard treatment. However, many studies have been conducted regarding systemic adjuvant treatment[4].

Neoadjuvant treatment approach

One of the most well-known randomized trials on neoadjuvant treatment for gastric cancer has been reported by Jackson *et al*[5] and Cunningham *et al*[6]. The MAGIC study comparing neoadjuvant treatment to surgery alone is the most important work demonstrating a survival advantage for the neoadjuvant treatment approach.

Advanced gastric cancer

Among the forms of treatment of advanced gastric cancer, the best supportive therapies are single-agent chemotherapy, combination chemotherapy and targeted therapies. The five-year survival for stomach cancer is approximately 78%-95% in stage Ⅰ A, 58%-85% in stage Ⅰ B, 34%-54% in stage Ⅱ, 20%-37% in stage ⅢA, 8%-11% in stage Ⅲb, and 5%-7% in stage Ⅳ. Wagner *et al*[7] demonstrated that combination chemotherapy is more beneficial than single-agent chemotherapy (HR = 0.82, 95%CI: 0.74-0.90) Survival with combination treatments *vs* single-agent chemotherapy is 6.7 mo *vs* 8.3 mo. Combination chemotherapies do not provide a significant increase in toxicity but do confer a slight difference in treatment-related mortality (1.1% *vs* 1.5%).

Cisplatin-fluorouracil

Cisplatin-fluorouracil (CF) is the most commonly used regimen for advanced gastric cancer. In 6 basic studies that investigated CF for gastric cancer, the response rate (RR), progression-free survival (PFS) and overall survival (OS) were similar between the CF groups and control groups. In these studies PFS was in the range of 3.7 to 4.1 mo, the median survival was 7.2 to 8.6 mo, and the 2-year survival was 7% to 10%. Addition of docetaxel to CF resulted in a survival advantage[8]. Kang *et al*[9] showed similar results for cisplatin ± capecitabine compared with CF. The REAL-2 study compared oxaliplatin combination regimens with regimens containing cisplatin and determined that the latter conferred the best median survival. In phase Ⅲ of the REAL-2 study, which analyzed the cisplatin ± 5-fluorouracil (5-FU) combination in advanced gastric cancer, the best median survival was 9.9 mo, and two-year survival was 15% [epirubicin-cisplatin-5-FU (ECF) 9.9 mo, cisplatin oxaliplatin 5-FU 9.3 mo, epirubicin oxaliplatin capecitabine cisplatin 9.9 mo and epirubicin-oxaliplatin-capecitabine 11.2 mo][10].

Docetaxel-cisplatin-fluorouracil

The TAX 325 study established the standard of phase Ⅲ trials in advanced gastric cancer. Randomized patients were divided into two arms[8]. The recurrence rate of the docetaxel-cisplatin-fluorouracil (DCF) arm was reduced approximately 32% compared to the CF arm, and time to progression was 5.6 mo in the CF arm *vs* 3.7 mo in the DCF arm ($P = 0.0004$).

Trastuzumab

HER2 overexpression or amplification is detected in 20% of all gastric cancers. In the ToGA trial in epidermal growth receptor-positive gastric cancer patients, in the first-line treatment, chemotherapy alone was compared with the use of trastuzumab + chemotherapy. Time to progression was 5.5 mo in the patients who received chemotherapy alone 6.7 mo in the chemotherapy + trastuzumab group ($P = 0.0002$). The median survival rate of the patients receiving chemotherapy alone was 11.1 mo *vs* 13.8 mo among patients receiving trastuzumab and chemotherapy together[11].

MATERIALS AND METHODS

Patients and follow-up

The records of patients with gastric cancer followed by the Department of Medical Oncology were analyzed retrospectively. Patients were recruited to the study if they were treated between 2000 and 2011 by the outpatient clinic. They were followed up by no treatment, adjuvant therapy, or metastatic therapy. We excluded from the study any patients whose laboratory records lacked the operating parameters. According to these criteria, the study sample consisted of the remaining 796 patients (552 male, 244 female, mean age at diagnosis: 58 years).

Patient age, sex, symptoms at diagnosis, localization of the tumor, operative details, histopathological features, AJCC 2010 TNM stage, treatment decisions, sites of metastasis, tumor marker levels at baseline, the presence of adjuvant radiotherapy, PFS, disease-free survival (DFS), and OS were recorded.

The type of surgery in patients diagnosed with gastric cancer was total gastrectomy, subtotal gastrectomy or palliative surgery. Patients with indications for adjuvant treatment were treated with adjuvant and/or radiochemotherapy. The number of patients who received adjuvant treatment was 352 (44.2%). Initially, 394 (49.4%) patients were admitted with metastases, and these patients received chemotherapy. No treatment was initially suggested for 48 patients (6.4%). Each series of chemotherapy treatments received by the patients was recorded.

Statistical analysis

Statistical analysis were performed with SPSS for Win-

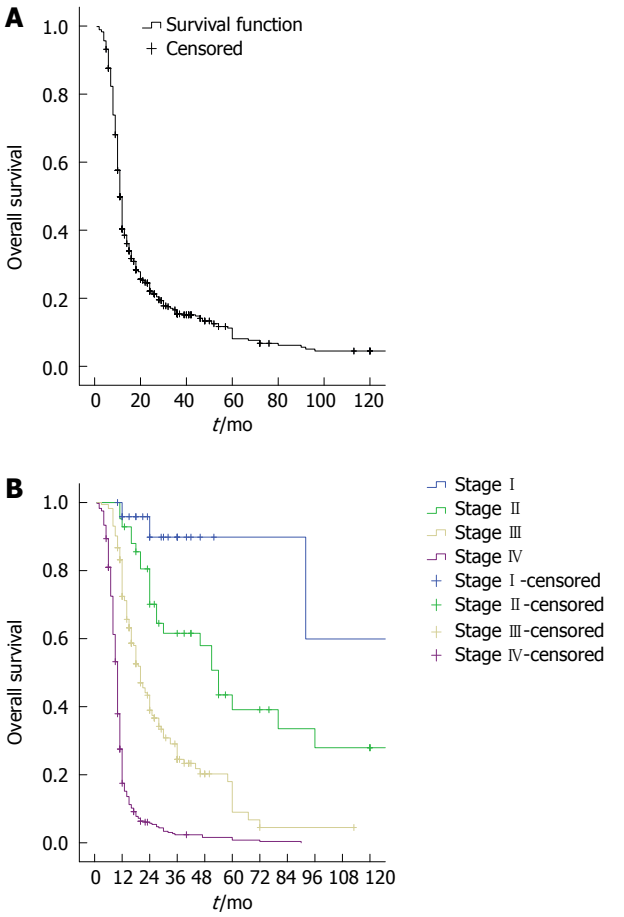

Figure 1 Overall survival in gastric cancer (A) and survival according to gastric cancer stage (B).

dows ver. 15.0 (standard version). Quantitative (numerical) data are reported as the mean ± SD. For two-group comparisons, we used the paired Student's *t*-test or, when necessary, the Mann-Whitney *U* test. For non-numerical data, when suitable for 2 × 2 contingency tables, Yates' corrected χ^2 test and Fisher's exact test were used. Correlations between numerical parameters were analyzed with Spearman's (*p*) correlation test. For the comparison of groups, Student's *t*-test or, when needed, one-way or multi-factor analysis of variance was used.

RESULTS

In this study, outpatient clinic records of patients with gastric cancer diagnosis were analyzed retrospectively. Demographic and clinical characteristics of the 796 gastric cancer cases included in the study were as follows: initial symptoms were dyspeptic symptoms, (39.3%), abdominal pain (24.8%), nausea and vomiting (16.3%), weight loss (7.5%), bleeding (6.4%) and acute abdominal pain (1.6%). The median follow-up period was 12 mo (1-276 mo), the median survival was 12 mo (11.5-12.4 mo), and the 5-year survival rate was 11%. The OS curve is given in Figure 1A, and the survival curve according to stage is given in Figure 1B. The median survival of metastatic patients was 10 mo, compared to 92 mo in stage I patients ($P <$ 0.0001). The demographic data of the 796 gastric cancer patients are given in Table 1. While the 5-year survival rate with lymphovascular invasion was 18%, this rate was 31% in the patients without lymphovascular invasion (LVI) ($P < 0.0001$). The 5-year survival of patients with perineural invasion (PNI) was 16%, compared to 33.6% without PNI ($P < 0.006$). The 5-year survival rate for patients with negative surgical margins was 28%, which was significantly higher than those with positive margins ($P <$ 0.0001). All patients with positive margins died within 5 years.

Table 1 Demographic data of the 796 patients with gastric cancer *n* (%)

Age (yr)	Median	58 (22-90)
Sex	Male	552 (69)
	Female	244 (31)
Median follow-up time	12 mo (range: 1-276 mo)	
Median survival	12 mo (range: 11.5-12.4 mo)	
Tumor location	Pyloric + antrum	362 (45.4)
	Large and small curvature	252 (31.6)
	Cardio-esophageal	97 (12.2)
	Diffuse	9 (1.1)
Stage	Stage Ⅰ	29 (3.6)
	Stage Ⅱ	43 (5.4)
	Stage Ⅲ	195 (24.5)
	Stage Ⅳ	393 (49.3)
Type of surgery	Total gastrectomy	265 (33.2)
	Subtotal gastrectomy	174 (21.8)
	Inoperable/palliative	341 (42.8)
Treatment	Adjuvant	352 (44.2)
	Metastatic	394 (49.4)
	Untreated follow-up	50 (3.9)
Histology	Adenocarcinoma (intestinal type)	493 (61.9)
	Signet ring cell (diffuse)	254 (31.9)
	Neuroendocrine	24 (3)
	Others	8 (1.1)
In metastasis	Peritonitis carcinomatosa	193 (24.2)
	Liver	169 (21.2)
	Lymphadenopathy	73 (9.2)
	Liver + peritoneum	35 (4.4)
	Lung	28 (3.5)
	Pleural effusion + acid	24 (3)
	Bone	23 (2.9)
	Others	17 (2.1)
Recurrence in	Peritonitis carcinomatosa	61 (40.1)
	Liver	36 (23.7)
	Lymphadenopathy	24 (15.8)
	Local	14 (9.2)
	Pleural/lung	12 (7.9)
	Others	5 (5)

While the 5-year survival of patients with initially normal crystalline egg albumen (CEA) level was 14.8%, patients with high CEA level all died within 5 years ($P <$ 0.012). Five-year survival among patients with initial normal carbohydrate antigen 19-9 (CA 19-9) level was 17.5% in all groups, but for the group with high CA 19-9, 5-year survival was 1.2% ($P < 0.2$). In the evaluation of only stage 4 patients, the tumor marker of high baseline CA 19-9 reached prognostic significance ($P < 0.03$). Gender ($P < 0.2$) and histological subtype had no effect on prognosis ($P < 0.5$). In multivariate analysis, tumor stage had significant effects on overall survival ($P < 0.0001$) and

Table 2 Treatment received by the patients with gastric cancer (*n* = 796) *n* (%)

	Treatment	
Adjuvant therapy	5-FU-LV	222 (27.9)
	5-FU-LV/cisplatin	43 (5.4)
	Untreated follow-up	58 (7.3)
	Others	17 (2.1)
Metastatic series 1	5-FU-LV	32 (4)
	DCF	152 (19.1)
	ECF	77 (9.7)
	5-FU-LV/cisplatin	121 (15.2)
	Palliative treatment	112 (14.1)
	Cisplatin/capecitabine	20 (2.5)
	Others	31 (3.9)
Metastatic series 2	DCF	31 (3.9)
	5-FU-LV/cisplatin	19 (2.4)
	ECF	14 (2.3)
	Irinotecan/cisplatin	17 (2.1)
	Supportive	267 (33.5)
	Others	32 (4)

5-FU-LV: 5-Fluorouracil plus leucovorin; CF: Cisplatin-fluorouracil; DCF: Docetaxel-cisplatin-fluorouracil; ECF: Epirubicin-cisplatin-5FU.

surgical margin ($P < 0.001$).

The approaches used for gastric cancer treatment are shown in Table 2. A group of patients with gastric cancer without metastasis was followed without medication, and chemotherapy was applied to the others. DFS for approaches to non-metastatic gastric cancer is given in Table 3. The mean survival of the non-treated follow-up group was significantly higher than other groups, primarily because of the survival of the stage I patients ($P = 0.007$). Table 4 shows the effects of chemotherapy or supportive treatment in patients with metastasis. Here, the time to the first progression after initial treatment was defined as PFS1, and the time to the second progression (after the second treatment) was defined as PFS2. PFS1 for patients receiving DCF was 6.56 mo, which was similar to other chemotherapy regimens. The first time to progression in patients receiving supportive therapy was 3.85 mo. After a second round of chemotherapy was started because of progression, DCF significantly prolonged PFS2. Eventually, DCF treatment of metastatic gastric cancer patients significantly prolonged time to progression compared to other approaches. Table 5 compares the results of the 1st and 2nd series of treatments for metastatic cancer. In the first metastatic series, DCF treatment was superior to all other treatments, and the greatest statistical superiority was to ECF and supportive care. DCF was therefore the preferred choice for first-line therapy in our study. A superior PFS was obtained with DCF compared to all other approaches. Supportive treatment was the preferred approach in the second series of our study. This was because of the frequent selection of DCF in the first series and the inability to repeat DCF after progression.

Our study population included 70 patients under age 40 (8.8%), 510 patients between 40 and 65 (64%), and 216 patients over the age of 65 (27.2%). A difference in survival according to age was not observed ($P = 0.8$). In the survival evaluation related to the tumor localization, patients with cardio-esophageal tumors ($P < 0.002$) and patients with linitis plastica ($P < 0.05$) showed the worst survival.

Table 3 Disease-free survival with chemotherapy and without chemotherapy in metastasis-free gastric cancer

Therapeutic approach	*n*	Average (mo)	Standard deviation	Minimum (mo)	Maximum (mo)
5-FU-LV	222	21.04[b]	19.912	2	120
Untreated follow-up	58	30.42	24.512	6	120
CF	43	19.00[b]	24.452	3	120
Others	17	21.00[b]	24.512	3	72

[b]$P < 0.01$ *vs* untreated follow-up. 5-FU-LV: 5-Fluorouracil plus leucovorin; CF: Cisplatin-fluorouracil.

Table 4 Time to first progression and time to 2nd progression according to treatment (chemotherapy or supportive) care in patients with metastatic gastric cancer ($P < 0.001$)

Therapeutic approach	*n*	Average (mo)	Standard deviation	Minimum (mo)	Maximum (mo)
First series of chemotherapy and time to progression					
DCF	152	6.56	2.869	1	18
ECF	77	4.56	9.021	1	48
CF	121	4.15	5.546	1	39
Supportive	112	3.85	9.951	2	60
Others	38	5.24	11.954	1	60
Second series of chemotherapy and time to progression					
DCF	31	4.38	3.921	2	15
ECF	14	3.71	2.443	2	10
CF	19	3.76	3.914	3	18
Supportive	267	3.39	1.871	1	12
Others	17	3.75	1.528	1	7

5-FU-LV: 5-Fluorouracil plus leucovorin; CF: Cisplatin-fluorouracil; DCF: Docetaxel-cisplatin-fluorouracil; ECF: Epirubicin-cisplatin-5FU.

DISCUSSION

This study was designed to determine the prognostic factors of gastric cancer based on tumor location, histological type, stage at diagnosis, and the phases of evaluation of treatment methods.

Talamanti *et al*[12] explored the relationship between tumor localization and prognosis. Because proximal tumors are more insidious, delay diagnosis, invade more deeply and metastasize to lymph nodes more frequently compared to distal tumors, Talamanti *et al*[13,14] reported a poorer prognosis for proximal tumors. Furthermore, they demonstrated that the placement of the disease in Caucasian populations significantly affects the prognosis and that tumors with this location show a poor prognosis. In our study, proximal tumors were associated with a worse prognosis than distal tumors, and the frequency of proximal tumors increased significantly after 2005. Proximal tumors required extended gastrectomy, D2 dissection and splenectomy. In this respect, patients with proximal tu-

Table 5 Comparison of treatment approaches in the first and second series of treatments in metastatic gastric cancer patients

1st-series treatment approach	*P* value	2nd-series treatment approach	*P* value
DCF *vs* 5-FU-LV	0.043	DCF *vs* ECF	0.050
DCF *vs* others	0.010	DCF *vs* Supportive	0.042
DCF *vs* Supportive	< 0.001	Supportive *vs* ECF	0.500
DCF *vs* ECF	< 0.001	DCF *vs* others	0.605
DCF *vs* CF	0.480	Irinotecan/Cisp *vs* ECF	0.423
ECF *vs* CF	0.960	Supportive *vs* Irinotecan/Cisp	0.100
Supportive *vs* ECF	< 0.01	DCF *vs* Irinotecan/Cisp	0.672

5-FU-LV: 5-Fluorouracil plus leucovorin; CF: Cisplatin-fluorouracil; DCF: Docetaxel-cisplatin-fluorouracil; ECF: Epirubicin-cisplatin-5FU.

mors are in serious danger of mortality and morbidity related to surgery as well as delayed diagnosis and increased depth of invasion.

Machara *et al*[15] and Persiani *et al*[16] demonstrated the relationship between young age and poor prognosis, but in our study there was no correlation between age and prognosis. In our series this rate was 56% *vs* 44%. In some studies, the depth of invasion, lymph node metastasis, and distant metastasis were the main prognostic factors[17]. In our study, the 5-year survival rate of 16% for patients with PNI was significantly lower than those without PNI. Although it is not lymph node metastasis, lymphovascular invasion is a poor prognostic parameter. Patients with LVI had significantly lower 5-year survival than patients without LVI. Ding *et al*[18] revealed that lymph node metastasis in gastric carcinoma is the most important prognostic factor. In our study, if the node period was increased, survival decreased, and in patients with N2 gastric cancer, 5-year survival decreased to 5%. In the german gastric cancer study, Siewert *et al*[19] demonstrated, by analyzing the 10-year results of 1654 patients with curative gastrectomy, that lymph node status, invasion depth, the development of postoperative complications, distant metastases and tumor size are associated with prognosis. Maruyama *et al*[20] showed in 4734 gastric cancer cases that depth of invasion, lymph node metastasis, macroscopic type, localization and histological type are the most important prognostic factors. In our study, while a correlation with the number of lymph no des removed was not detected, increased node stage affected survival.

The ratio of the number of metastatic lymph nodes to removed lymph nodes is an important prognostic factor. Ding *et al*[20] demonstrated that the increase of this ratio decreases survival. In our series, as the number of metastatic lymph nodes increased, the 1-year, 3-year, and 5-year survival rates were 97%, 74%, and 63% for N0; 87%, 34.8%, and 18.5% for N1; 73%, 16.4%, and 5% for N2; and 78%, 39% and 0% for N3. In addition, lymph node-negative patients, despite having better prognosis than lymph node-positive patients, experienced recurrence and short survival. After Lauren[21] demonstrated that gastric carcinoma has two separate histologies, an intestinal and a diffuse type, the distinct effect of tumor histology on prognosis was investigated. While the intestinal type shows a better prognosis, both histological types can cross the stomach wall and reach the serosal surface and may act metastatic. No difference in survival was observed in any of our patients according to histological type.

When the survival analysis was conducted separately according to the zone of metastasis, we found no differences in survival. However, if carcinoma peritonei was detected, survival averaged less than 8 mo. The role and value of metastasectomy for gastric cancer is not clear. Although there are too few data to draw conclusions about the effect of metastasectomy on survival, Kerkar *et al*[22] found 1-year, 3-year, and 5-year overall survival rates in 436 patients with liver metastasectomy of 62%, 30% and 26.5%, respectively. Our series included 8 gastric cancer patients with liver metastases who underwent metastasectomy, and the survival data obtained from these patients were consistent with that study. In another study, in the 23-mo follow-up of 43 patients with solitary pulmonary resection, 15/43 (35%) patients were without evidence of disease, and 5-year survival was reported as 33% for gastric cancer[23]. In our series, there were no cases of metastasectomy for pulmonary metastases of gastric cancer. Dewys *et al*[24] reported that the gastric cancer symptoms are often nonspecific but can include lumen obstruction, bleeding or acute abdominal pain. Seventy percent of patients initially had symptoms such as abdominal-epigastric pain or discomfort, followed by symptoms such as weight loss, nausea, vomiting, hematemesis and melena. The initial symptoms in our study were consistent with the literature.

In one study, serum CEA was elevated in one-third of gastric cancer patients at diagnosis. Although the CEA level in gastric cancers has low sensitivity as a prognostic marker, high levels are related to the phase of the disease. Higher levels of CA 19-9 and CEA are more sensitive as a combined prognostic factor[25]. Although in our study population, the initially determined marker values demonstrated no relationship with survival, the prognostic significance of high CA 19-9 at diagnosis in stage Ⅳ patients emerged. CA 19-9 was not correlated with the level of CEA-free survival. In gastric cancer, as the stage of the disease progresses, the level of CEA increases. In localized cases, CEA increases by 14%-29%, whereas in patients with metastatic cancer, this figure can reach 85%. Haglund *et al*[26] and Koga *et al*[27] reported a 48% sensitivity of CA 19-9 in predicting the prognosis of gastric cancer. Kago and colleagues found high levels of CA 19-9 in 20.9% of stomach cancer patients, including 37% of stage 4 patients and 69.2% of patients with liver metastases.

The median survival of patients with metastatic cancer in this study was 10 mo, and for stage I patients the median survival was 92 mo. We compared our data to the 1-year, 3-year, and 5-year free survival of gastric cancer according to the data Surveillance, Epidemiology and

End Results (SEER) study, covering the years 1975-2008 and a total of 10 601 patients with resected gastric cancer[28], and found that 1-year survival in stage Ⅰ, Ⅱ and Ⅲ patients of our series was greater, the life span of patients with stage Ⅳ; 3-year survival in stage Ⅰ, Ⅱ and Ⅲ patients in our series was greater, whereas stage Ⅳ patients showed a worse outcome in our series, and 5-year survival in stage Ⅰ, Ⅱ and Ⅲ patients in our series was better, whereas stage Ⅳ patients showed a worse outcome. Comparing all of our study population's survival data with data from the SEER study showed that stage Ⅳ patients showed similar survival rates, whereas stage Ⅰ, Ⅱ, and Ⅲ patients seemed to have longer survival times in this series. While local or locoregional recurrence after surgical resection of gastric cancer is a current problem, adjuvant treatment should be administered to patients. Adjuvant therapy, especially in node-positive disease, gives better results. Adjuvant radiotherapy and/or adjuvant chemotherapy has been designed for this purpose in phase Ⅲ trials.

In a randomized phase Ⅲ trial, the Intergroup trial (INT 0116), the effectiveness of adjuvant chemoradiotherapy was compared with the observation group and a group treated only with surgery. For resected stage Ⅰ B-Ⅳ (M0), a 5-treatment strategy was planned for gastric and gastroesophageal adenocarcinoma patients, and at the same time, radiotherapy was used. That study reported a statistically significant advantage in median survival. In the current study, 5-year survival for patients receiving adjuvant therapy was 50%, compared to 41% for the surgery group (HR = 1.35)[3]. In our study, 246 patients were evaluated in terms of the success of adjuvant treatment. A total of 199 patients received adjuvant therapy, but in 99 patients the indication for treatment had not been set. Comparing the types of treatment or follow-up in patients without metastasis at the beginning of the study, the non-treatment group had significantly longer survival than other groups, and significant differences were not found between the other groups. The reason for this most likely is that the patients who received non-adjuvant therapy were already in stage Ⅰ A, and a longer survival time was expected for these patients. For patients with an indication for adjuvant treatment who underwent a Macdonald regimen, 5-year survival rates were in 90% in stage Ⅰ, 50% in stage Ⅱ and 20% in stage Ⅲ, which are consistent with the literature. The local recurrence rate in the group receiving chemoradiotherapy was 19%. The regional relapse rate was 65% against the 72%. Patients tolerated the regime well. Other adjuvant therapies did not confer a significant increase in survival.

Although some studies have assessed preoperative chemoradiotherapy, the numbers of patients who received neoadjuvant therapy were not large enough for statistical analysis. Compared with general treatment forms in advanced gastric cancer, approaches such as single-agent chemotherapy, combination chemotherapy and targeted therapies can be considered the best adjuvant treatments. Wagner *et al*[29], in a meta-analysis, compared the best adjuvant treatment with chemotherapy regimens and evaluated the median and overall survival rates. Four quality-of-life questionnaires were used to compare chemotherapy with the best supportive care, and chemotherapy was considered better at 12 mo than at 6 mo. In our study, the chemotherapy regimens were superior to supportive care, in accordance with the literature. DCF was used as a metastatic first-line treatment and produced a PFS of 6.5 mo, compared to 4.5 mo using ECF, 4.1 mo using CF, and 3.8 mo using supportive care. In the evaluation of the effectiveness of treatment on survival, using DCF the overall survival was 9.5 mo, 6.5 mo using EC, 5.1 mo using CF and 4.8 mo in patients with only supportive treatment. Any progression under treatment with chemotherapy or supportive care in the second series of treatments was noted, and the PFS2 for DCF was 4.3 mo, for ECF was 3.7 mo and for supportive therapy was 3.3 mo. Considering the effect of combination chemotherapy on PFS, the DCF regimen was superior to all other treatments. Our study was consistent with the results of the TAX 325 study of Van Cutsem *et al*[8], which created the standard of advanced gastric cancer care. In their study, DCF was superior to CF in overall survival as well as in time to progression.

Our study evaluated patients treated with different chemotherapy regimens, and DCF showed superior efficacy in all arms in both PFS and overall survival.

The combination of cetuximab with docetaxel and cisplatin does not significantly affect time to progression or overall survival[30]. Lapatinib, the first dual inhibitor of human epidermal growth factor receptor (HER-1) and HER-2, has been investigated in two phase Ⅱ studies as a single therapeutic agent, but no survival advantage was observed[31]. Gefitinib and erlotinib, two tyrosine kinase inhibitors, have been used as a combination treatment for cancer, and in extensive studies, a RR of 9% was obtained[32]. Bevacizumab, a monoclonal antibody against vascular endothelial growth factor A, was investigated in the AVAGAST study. The combination of bevacizumab + CC conferred no significant survival advantage compared to CC alone. In another study, I C was combined with bevacizumab, and a significant advantage was not observed compared to I C alone[33]. Sunitinib is an oral inhibitor of VEGFR1, -2, and -3, PDGFR-α and -β and c-kit. Use of second-line sunitinib in phase Ⅱ trials produced an overall survival of 47.7 wk. In another phase Ⅱ study using sorafenib in combination with docetaxel and cisplatin, clinical activity was observed[34]. Everolimus, an oral inhibitor of mTOR, has been effective in gastric cancers in phase Ⅰ and phase Ⅱ trials[35].

Due to the limited number of patients with targeted therapy in this study, HER-2 status and the effectiveness of trastuzumab could not be assessed. Efficacy assessments could not be made also because targeted therapies such as trastuzumab were not used in our series. When HER-2 receptor status is analyzed routinely in stomach cancer patients, targeted therapy may be evaluated more completely.

COMMENTS

Background

In spite of the development of oncology treatments, gastric cancer still has a high mortality. All the prognostic factors should be evaluated before planning the treatment of gastric cancer. It should be kept in mind that there are new treatment modalities for gastric cancer.

Research frontiers

In this study, the authors retrospectively evaluated gastric cancer patients treated in our clinic during the last 10 years. The prognostic factors for these patients were identified and the treatment plan made according to these factors. The treatments of the patients and their survival were evaluated and compared with the literature. Additionally, the importance of targeted therapy is emphasized.

Innovations and breakthroughs

This study has provided new insight into gastric cancer. Properly identifying the prognostic factors and planning the treatment and follow-up according to these factors is suggested. This study has shown that mortality is high in metastatic patients and that clinicians should be more encouraged to use targeted therapy.

Applications

Based on the results, molecular features of metastatic patients, such as human epidermal growth factor receptor (HER-2) receptor status, will be identified and targeted therapy principles will be developed.

Terminology

HER-2 is a member of the epidermal growth factor family. It is involved in tumor proliferation, metastasis and poor prognosis. If a patient is HER-2 positive, then the anti-HER-2 antibody trastuzumab can be useful. Authors need further clinical studies to evaluate other targeted therapy modalities.

Peer review

The authors have identified the prognostic features of gastric cancer patients and compared the standard treatment modalities. They note the importance of molecular studies in gastric cancer patients, and they predict that targeted therapy will be a part of the standard treatment in the future.

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ORIGINAL ARTICLE

Modulatory effects of *Bifidobacterium longum* BB536 on defecation in elderly patients receiving enteral feeding

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Abstract

AIM: To investigate the effects of the probiotic *Bifidobacterium longum* BB536 on the health management of elderly patients receiving enteral feeding.

METHODS: Two double-blind, placebo-controlled trials were performed with long-term inpatients receiving enteral tube feeding at Kitakyushu Hospital Group, Fukuoka, Japan. BB536 was administered as BB536-L and BB536-H powders that contained approximately 2.5×10^{10} and 5×10^{10} cfu of BB536, respectively. In the first trial, 83 patients (age range: 67-101 years) were randomized into 2 groups that received placebo (placebo group) or BB536-H (BB536 group) powders. In the second trial, 123 patients (age range: 65-102 years) were randomized into 3 groups, and each group received placebo (placebo group), BB536-L (BB536-L group), or BB536-H (BB536-H group) powders. Each patient received the study medication for 16 wk after 1 wk of pre-observation. Fecal samples were collected from each patient prior to and after the intervention during Trial 2. Clinical observations included body temperature, occurrence of infection, frequency of defecation, and fecal microbiota.

RESULTS: No significant changes were observed in the frequency of defecation for either treatment in Trial 1. However, a significant change was noted in the BB536-L group ($P = 0.0439$) in Trial 2 but not in the placebo or BB536-H groups. Subgroup analyses based on the frequency of defecation for each patient during the pre-observation period for both trials revealed significant increases in bowel movements in patients with a low frequency of defecation and significant decreases in the bowel movements of patients with a high frequency of defecation during the intervention period in the BB536 groups. The combination of Trials 1 and 2 data revealed a modulatory effect of BB536 ingestion on the changes in bowel movements. Significantly increased bowel movements were observed in patients in the low frequency subgroup with significant intergroup differences ($P < 0.01$). Significantly decreased bowel movements were observed in patients in the high subgroup, but no significant intergroup differences were observed compared with the placebo group. BB536 ingestion increased the prevalence of normally formed stools. BB536 intake also significantly ($P < 0.01$) increased the cell numbers of bifidobacteria in fecal microbiota, and significant intergroup differences were observed at week 16. No adverse events were reported in any group.

CONCLUSION: Our results suggest that BB536 ingestion modulated the intestinal environment and may have improved the health care of elderly patients receiving enteral feeding.

Key words: Probiotics; *Bifidobacterium longum* BB536; Elderly; Defecation

Kondo J, Xiao JZ, Shirahata A, Baba M, Abe A, Ogawa K, Shimoda T. Modulatory effects of *Bifidobacterium longum* BB536 on defecation in elderly patients receiving enteral feeding. *World J Gastroenterol* 2013; 19(14): 2162-2170 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i14/2162.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i14.2162

INTRODUCTION

Enteral feeding is a common method of nutritional support for patients who are unable to achieve their nutritional requirements through an oral diet alone. No accurate data exist on the number of patients who receive enteral feeding. However, the number of new patients requiring enteral feeding in 2007 was approximately 130 000 in Japan[1], and this number is expected to increase in the future. Elderly people, particularly those who are hospitalized and receiving enteral feeding, exhibit significant problems with defecation, and the consequences of constipation or diarrhea may significantly impact their quality of life[2,3]. The prevalence of constipation is generally higher in elderly individuals who reside in nursing homes or hospitals compared with elderly individuals in the community[2]. Diarrhea, which is a potential consequence of enteral feeding, is observed in 2%-95% of patients who receive this therapy[3,4].

Intestinal microbiota are the largest source of microbial stimulation in the host, and these microbiota affect mucosal and systemic immunity[5]. The composition of the intestinal microbiota in elderly people is different from that in younger adults, and the number of bifidobacteria decreases with age[6,7]. Bifidobacteria in the intestinal microbiota may exert beneficial effects in the host, such as the promotion of gut maturation and integrity, antagonism against pathogens, and immune modulation[8].

Probiotics are currently used in the prevention and treatment of disease, specifically diseases of the intestinal environment. Several studies have investigated the beneficial effects of probiotics in the management of constipation and diarrhea in elderly patients[9-11]. However, these effects may be strain-dependent, and they are not consistently observed. Therefore, further investigation is required to clarify this relationship.

The probiotic strain *Bifidobacterium longum* (*B. longum*) BB536 was originally isolated from a healthy infant, and it is used in the dairy industry as a probiotic[12]. Several studies have evaluated the effects of BB536 on the intestinal environment in healthy adults with frequent constipation[12-14]. Seki *et al*[15] reported that the intake of BB536-supplemented milk improved constipation and increased the prevalence of intestinal *Bifidobacterium* in aged individuals in a preliminary study. Moreover, BB536 intake suppresses antibiotic-induced intestinal disorders[16].

The present study investigated the efficacy of BB536 in the health care of hospitalized elderly patients receiving enteral nutrition. We performed 2 double-blind, placebo-controlled trials using a 16-wk administration of BB536 to evaluate effects on health, defecation frequency, and the bifidobacterial composition of fecal microbiota in elderly patients receiving enteral nutrition.

MATERIALS AND METHODS

Subjects

Subject recruitment for this study was conducted in long-stay inpatients (age > 65 years) receiving enteral tube feeding at the Kitakyushu Hospital Group (Fukuoka, Japan). The subjects or their relatives provided written informed consent. The following exclusion criteria were used: presence of diabetes, renal dysfunction, severe infectious disease, autoimmune disease, immunodeficiency, pancreatic disease, or hepatic disease prior to the start of the study. The ethics committee of the Kitakyushu Hospital Group approved all study protocols, which followed the Declaration of Helsinki.

Test samples

Three types of study medications were used in the present study: placebo powder, BB536-L powder, and BB536-H powder. BB536-L and BB536-H powders contained lyophilized BB536 at doses of approximately 2.5×10^{10} and 5×10^{10} cfu, respectively, and the placebo powder contained only inactive ingredients (*i.e.*, primarily dextrin). Each dose was supplied in an aluminum sachet (2 g), and all sachets were identical in taste and appearance.

Clinical trials

Two trials were performed in this study, and both trials were performed using a double-blind, placebo-controlled, parallel-group design. Randomization for each group of participants was conducted using a minimization procedure to balance for gender, age, and hospital ward. The trial flows and schedules are presented in Figures 1 and 2, respectively. Routine enteral nutrition was provided to all the subjects during the trial period to maintain nutritional status. Participants, physicians, and other research staff in the study were unaware of treatment assignment. The study powder was suspended in drinking water and administered immediately after enteral feeding. The daily intake of energy and nutrients of each patient group during the trial period are summarized in Table 1. No significant differences in nutrient intake between the groups were observed.

Trial 1: The first trial was performed during the winter from the end of November 2009 to the end of March 2010. This period included one week for pre-observation and 16 wk for the ingestion of study medications. A total of 83 patients were randomized into 2 groups, and each

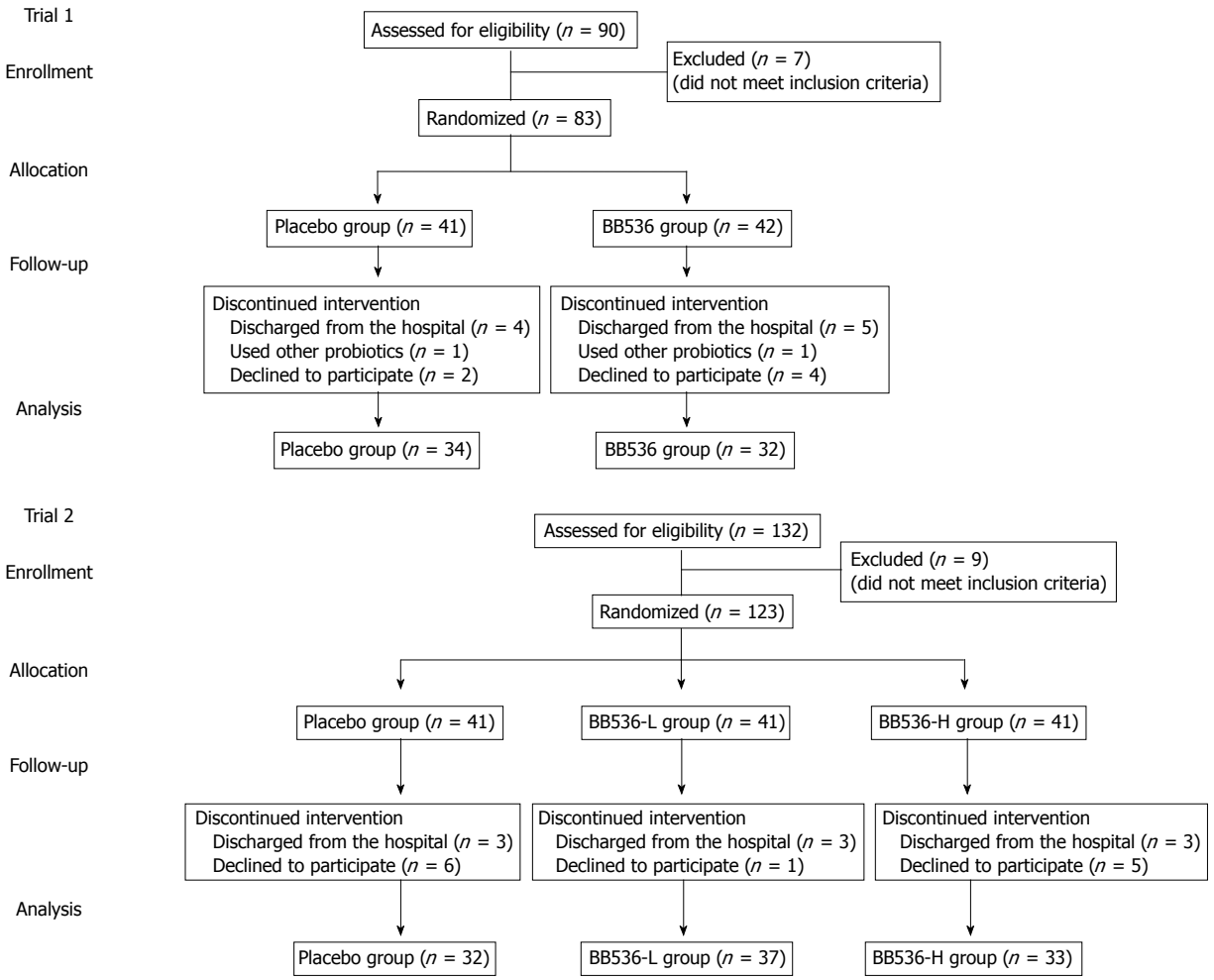

Figure 1 Trial protocol.

Table 1 Subject characteristics and daily intake of energy and nutrients

	Group	*n*	Gender (M/F)	Age (yr)	Total energy (kcal/d)	Protein (g/d)	Lipid (g/d)	Carbohydrates (g/d)	Dietary fiber (g/d)
Trial 1									
	Placebo	32	9/23	82.7 ± 9.5	884.7 ± 207.2	37.2 ± 11.6	28.8 ± 9.6	118.8 ± 30.6	10.8 ± 3.5
	BB536-H	34	8/26	85.8 ± 7.3	917.6 ± 162.6	37.5 ± 7.8	30.2 ± 10.3	124.2 ± 22.5	10.3 ± 3.7
Trial 2									
	Placebo	32	9/23	83.9 ± 7.5	798.1 ± 176.3	35.1 ± 11.9	24.5 ± 6.1	112.3 ± 31.4	9.6 ± 3.3
	BB536-L	37	9/28	84.4 ± 6.8	845.6 ± 186.9	37.0 ± 10.5	26.6 ± 9.9	118.0 ± 28.6	11.1 ± 4.6
	BB536-H	33	10/23	84.4 ± 10.1	854.8 ± 194.9	37.4 ± 10.7	26.0 ± 8.0	120.1 ± 29.9	10.4 ± 3.7

M: Male; F: Female.

group was assigned to receive placebo (placebo group) or BB536-H powder (BB536 group) once daily.

Trial 2: The second trial was performed to confirm the results of Trial 1, investigate the dose effect of BB536, and determine any possible influences of treatment on fecal microbiota. This trial was also conducted during the winter from the end of November 2010 to the end of March 2011. The trial period included one week for pre-observation and 16 wk for study medication ingestion. A total of 123 patients were randomized into 3 groups, and each group was assigned to receive the placebo (placebo group), BB536-L (BB536-L group), or BB536-H powder (BB536-H group) twice daily. Fecal samples were collected from each patient prior to (pre-observation week) and after the intervention (week 16). Fecal samples were collected in plastic tubes, cooled immediately after collection, and stored at -20 ℃ until analysis.

Clinical observations

Body temperature and the times of defecation were recorded daily. The occurrence of infection and fever and use of other medications, including antibiotics, were also recorded. A trained caregiver monitored stool characteristics during daily care, and stool form and consistency were evaluated using the Bristol Stool Form Scale. The

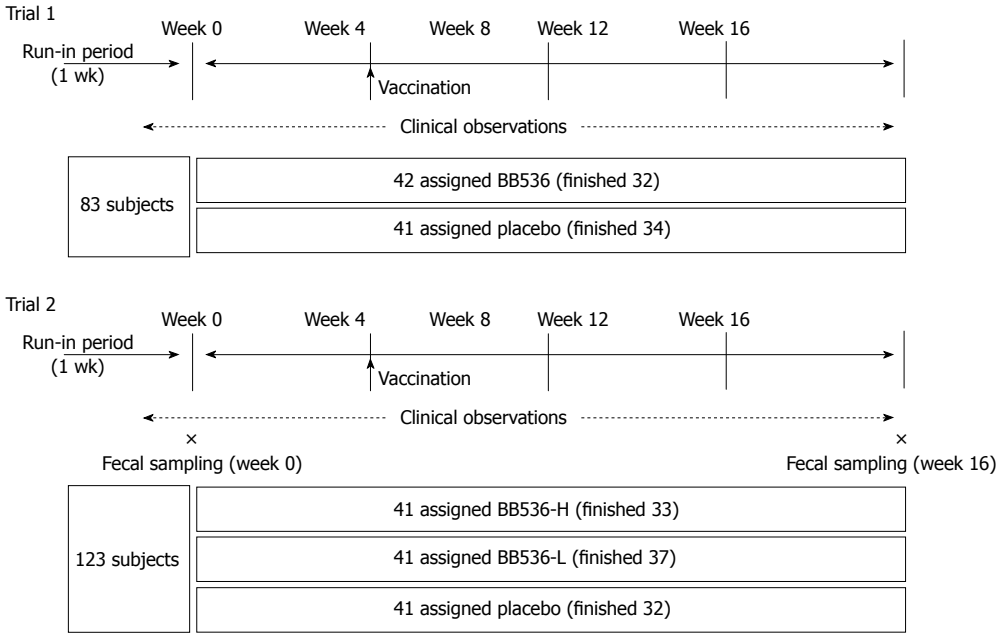

Figure 2 Intervention schedule.

Bristol Stool Form Scale scores range from 1 (separate hard lumps, like nuts and hard to pass) to 7 (watery, no solid pieces, entirely liquid); stools scored at 3 or 4 were considered normal stools[17].

Analysis of fecal microbiota

DNA was extracted from the fecal samples as described previously[18]. Briefly, each fecal sample (20 mg) was suspended in 1.0 mL phosphate-buffered saline (PBS) and centrifuged at 14 000 × *g*. The resulting pellet was washed twice with 1.0 mL PBS and resuspended in 450 μL of an extraction buffer [100 mmol/L Tris-HCl and 40 mmol/L ethylenediaminetetraacetic acid (EDTA) at pH 9.0] with 50 μL of 10% sodium dodecyl sulfate (SDS). Glass beads (300 mg, 0.1 mm diameter) and 500 μL of buffer-saturated phenol were added to the suspension, and the resulting mixture was vigorously vortexed for 30 s with a FastPrep™ FP 100A (Bio 101, Vista, CA, United States) device at a power level of 5.0. The mixture was centrifuged at 14 000 × *g* for 5 min, and 400 μL of the supernatant was extracted with phenol-chloroform; 250 μL of the supernatant was precipitated with isopropanol. Purified DNA was dissolved in 200 μL of a Tris-EDTA buffer at pH 8.0.

Real-time polymerase chain reaction (PCR) was performed using an ABI PRISM® 7500 Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA, United States), with SYBR® Premix Ex Taq (TaKaRa Shuzo, Japan) and ROX Reference Dye Ⅱ (TaKaRa Shuzo, Japan) as an internal standard. Primers for the bifidobacterial species and *B. longum* BB536 were used as described previously[19,20]. The amplification program consisted of 1 cycle at 94 ℃ for 10 s, followed by 40 cycles at 94 ℃ for 5 s and 60 ℃ for 30 s. Fluorescent products were detected at the last step of each cycle. Melting curves were obtained by heating from 60 ℃ to 95 ℃ in 0.2 ℃/s increments with continuous fluorescence data collection.

Statistical analysis

Data are expressed as means ± SD or SE. Daily recorded scores for body temperature and times of defecation were averaged weekly for each individual. Changes in values from baseline (week-1) were calculated based on the weekly scores. Weekly scores or changes were further averaged every 4 wk for analysis. The frequency of each stool type was summed for the total intervention period, and the prevalence of each stool type was calculated. Cell numbers for each bacterial target are expressed as means after logarithmic transformation for each group among individuals with cell numbers that exceeded the detection limit, which was 1×10^6 per gram wet weight of feces. However, statistical analyses were conducted on cell numbers after logarithmic transformation, in which cell numbers below the detection limit were substituted with 1×10^6. For analysis of sequence differences within a group, two-sequence differences were assessed using the paired Student *t*-test, and multi-sequence differences were analyzed using a repeated measures analysis of variance (ANOVA), followed by Dunnett's test for each time point against the baselines. For analysis of between group differences, two-group differences were evaluated using the Student *t*-test, and multi-group differences were evaluated using a non-repeated measures ANOVA, followed by the Student-Newman-Keuls test for comparisons of each group. Differences in changes from baseline between groups were evaluated using the Student *t*-test at each time point. *P* values less than 0.05 were considered statistically significant. Analyses were performed using SPSS software (Version 15.0J for Windows, Chicago, United States).

Table 2 Bowel movements during the intervention period

	Subgroups of subjects[1]	Intervention group	Subjects (*n*)	Bowel movements (times/wk)					*P* value[2]
				Week-1	Weeks 1-4	Weeks 5-8	Weeks 9-12	Weeks 13-16	
Trial 1									
	Whole	Placebo	34	4.88 ± 2.70	5.18 ± 2.57	5.20 ± 2.58	5.17 ± 2.74	4.8 ± 2.2	0.326
		BB536-H	32	5.53 ± 3.76	6.62 ± 3.83	6.37 ± 3.34	6.04 ± 3.25	6.0 ± 2.8	1.051
	Low	Placebo	19	3.00 ± 0.94	4.07 ± 1.37[a]	4.25 ± 1.94[a]	4.07 ± 1.34[a]	3.87 ± 1.13[a]	0.002
		BB536-H	14	2.93 ± 0.92	4.41 ± 1.89[b]	4.32 ± 1.85[b]	4.79 ± 1.92[b]	4.82 ± 1.92[b]	0.001
	Normal	Placebo	12	6.25 ± 1.36	5.46 ± 2.56	5.29 ± 2.07	5.23 ± 2.40	5.21 ± 2.69	0.564
		BB536-H	14	5.79 ± 1.19	7.02 ± 2.84	7.36 ± 3.16	6.27 ± 3.25	6.48 ± 2.7	0.340
	High	Placebo	3	10.50 ± 1.00	11.38 ± 1.77	10.38 ± 0.72	12.5 ± 3.22	8.50 ± 0.58	0.786
		BB536-H	4	13.75 ± 3.77	12.94 ± 4.94	10.06 ± 3.86	9.63 ± 4.75	8.75 ± 4.20[a]	0.044
Trial 2									
	Whole	Placebo	32	5.28 ± 3.34	5.02 ± 2.67	4.78 ± 2.54	4.73 ± 2.80	4.60 ± 2.20	0.563
		BB536-L	37	5.51 ± 4.12	6.10 ± 3.85	5.90 ± 3.43	5.11 ± 2.60	4.90 ± 3.00	0.044
		BB536-H	33	5.91 ± 4.30	6.12 ± 3.89	6.20 ± 3.51	6.30 ± 3.25	5.60 ± 3.80	1.075
	Low	Placebo	20	3.05 ± 0.76	3.73 ± 0.88	3.45 ± 1.05	3.41 ± 1.10	3.63 ± 1.09	0.387
		BB536-L	22	2.64 ± 1.05	3.69 ± 1.22[b]	3.74 ± 1.42[b]	3.70 ± 1.54[b]	3.44 ± 1.32[b]	0.001
		BB536-H	18	3.00 ± 1.03	3.96 ± 1.33[a]	4.24 ± 1.89[b]	4.35 ± 2.09[b]	3.83 ± 1.44[a]	0.015
	Normal	Placebo	6	6.83 ± 1.33	4.79 ± 1.16	5.04 ± 1.07	4.88 ± 1.61	4.79 ± 1.42	0.219
		BB536-L	7	7.00 ± 1.29	7.39 ± 3.15	7.86 ± 3.58	6.46 ± 1.81	6.61 ± 2.89	0.842
		BB536-H	8	6.50 ± 1.41	6.66 ± 3.70	7.47 ± 3.50	7.03 ± 3.11	5.66 ± 4.18	1.217
	High	Placebo	6	11.17 ± 0.98	9.54 ± 2.90	8.96 ± 2.54	9.00 ± 3.57	7.92 ± 2.59[a]	0.149
		BB536-L	8	12.13 ± 2.10	11.59 ± 3.01	10.13 ± 2.12	7.81 ± 2.89[b]	7.44 ± 4.03[b]	0.006
		BB536-H	7	12.71 ± 3.68	11.07 ± 4.08	9.79 ± 3.55[b]	10.46 ± 3.80[a]	10.25 ± 4.12[a]	0.040

Values are shown as mean ± SD. [1]Based on the results of bowel movements at week-1. Low, ≤ 4 times; Normal, 59 times; High, ≥ 10 times; [2]*P* values are results of repeated measures ANOVA for analyzing the significance of intragroup changes. [a]$P < 0.05$, [b]$P < 0.01$ *vs* week-1.

RESULTS

Baseline characteristics of participants and clinical observations

No significant differences in the baseline characteristics of patients were observed between the groups of either trial (Table 1). No significant changes in body temperature during the intervention period were observed between groups in either trial. A few patients experienced body temperatures > 38 ℃ and received antibiotics, but the incidence of fever was not significantly different between the groups in either trial (data not shown).

Changes in the frequency of defecation

No significant changes in the frequency of defecation were observed following treatments during Trial 1 (Table 2). However, significant changes were observed in the BB536-L group in Trial 2 but not in the placebo or BB536-H groups (Table 2).

The frequency of defecation varied for each patient during the pre-observation period. Therefore, subgroup analyses were performed for patients with infrequent (low) defecation (≤ 4 times a week), normal frequency of defecation (5-9 times a week), and a high frequency of defecation (≥ 10 times a week) at baseline (week-1). We observed significant changes in the frequency of defecation in the low frequency subgroup of the placebo and BB536 groups and the high frequency subgroup of the BB536 group in Trial 1. However, no significant changes were observed in the normal frequency subgroup of either the placebo or BB536 group or the high frequency subgroup of the placebo group during treatment (Table 2). Defecation frequency increased significantly after treatment in the low frequency subgroups of both the placebo and BB536 groups, and the frequency tended to be higher ($P < 0.1$) in the BB536 group compared with the placebo group at weeks 13-16. In contrast, defecation frequency decreased after treatment in the high frequency subgroup of the BB536 group but not in the placebo group, and significant differences were observed at weeks 9-12 and 13-16 in the BB536 group (Table 2).

Significant changes were observed in the frequency of defecation in the low and high frequency subgroups of the BB536 group but not the placebo group in Trial 2 (Table 2). No significant changes in the normal frequency subgroups of any of the three treatment groups were observed (Table 2). Defecation frequency increased significantly after treatment in the low frequency subgroups of both the BB536-L and BB536-H groups, and a trend for a difference was noted in the BB536-H group compared with the placebo group at weeks 9-12 ($P < 0.1$). In contrast, defecation frequency decreased after treatment in the high frequency subgroups of both the BB536-L and BB536-H groups. Significant differences were observed at weeks 9-12 and 13-16 in the BB536-L group and weeks 5-8, 9-12, and 13-16 in the BB536-H group (Table 2).

Combined analyses of Trials 1 and 2 for changes in the frequency of defecation

Figure 3 summarizes the changes in defecation frequency for the three subgroups in the two trials. Defecation frequency increased significantly in the low frequency subgroup of both placebo ($n = 39$) and BB536 ($n = 54$) groups. However, the frequency was significantly higher

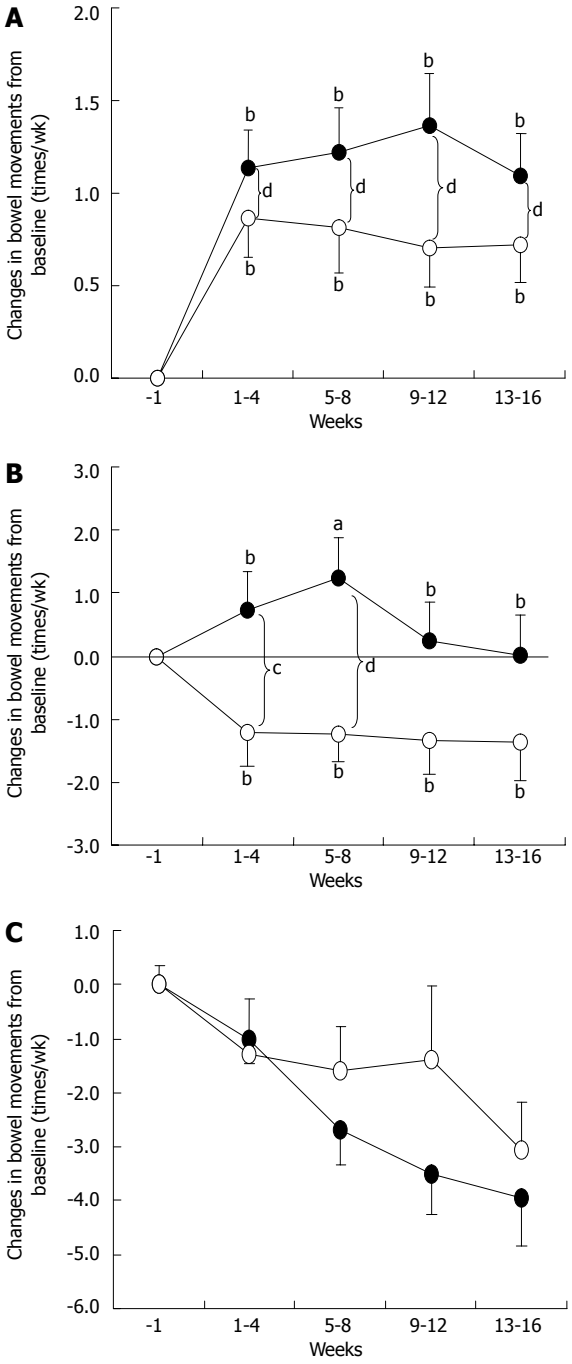

Figure 3 Effects of BB536 intake on changes in defecation frequency. A: Subgroup of patients with low infrequent defecation (≤ 4 times a week); B: Subgroup of patients with normal frequency of defecation (5-9 times a week); C: Subgroup of patients with high frequency of defecation (≥ 10 times a week) at baseline (week-1). Results present the summary of Trials 1 and 2 for the placebo (○) and BB536 groups (●) composed of the BB536 group in Trial 1 and BB536-H and BB536-L groups in Trial 2). Times of defecation were averaged weekly for each individual, and changes from baseline (week-1) were calculated. The weekly scores for changes were further averaged every 4 wk. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* week-1 group; $^{c}P < 0.05$, $^{d}P < 0.01$ between groups.

in the BB536 group compared with the placebo group. Defecation frequency increased significantly at weeks 5-8 in the normal frequency subgroup of the BB536 group (n = 29). However, defecation frequency decreased significantly during the intervention period in the placebo group (n = 18), and significant intergroup differences were observed at weeks 1-4 and 5-8. In contrast, defecation frequency decreased during the intervention period in the high frequency subgroup at weeks 5-8, 9-12, and 13-16 for the BB536 group (n = 19) but only at weeks 13-16 for the placebo group (n = 9). However, no significant intergroup differences were observed due to the small number of patients.

Changes in stool characteristics

Figure 4 presents the incidence of each stool type during the intervention. A significantly higher incidence of stool type 3 (*i.e.*, like a sausage but with cracks on its surface) and type 5 (soft blobs with clear-cut edges that could be passed easily) was observed in the BB536 group than in the placebo group in Trial 1. A significantly higher incidence of stool types 3 and 4 (like a sausage or snake, smooth and soft) was observed in the BB536-L group compared with the placebo group in Trial 2.

Effects on fecal microbiota

Real-time polymerase chain reaction analyses revealed that the cell numbers of total bifidobacteria, *B. longum* subsp. *longum*, and BB536 increased significantly after treatment in all 3 groups, and the cell numbers of these bacterial groups were significantly higher in the BB536 groups than in the placebo group (Table 3). The cell numbers of (*Bifidobacterium breve*) *B. breve* and *B. longum* subsp. *infantis* were significantly higher in the BB536-H group after treatment than before treatment. In addition, the cell numbers of (*Bifidobacterium adolescentis*) *B. adolescentis* were significantly higher in the BB536-H group than the placebo group at week 16. No differences in the cell numbers of the other dominant species of *Bifidobacterium* were observed after treatment.

DISCUSSION

The present results revealed obvious effects of BB536 therapy *vs* placebo in the normalization of defecation frequency in patients who exhibited low and high frequencies of defection. BB536 administration increased the incidence of close-to-normal stools (types 3-5, Figure 3), which is consistent with the results for defecation frequency. BB536 administration also increased the cell population of bifidobacteria in the microbiota of elderly patients.

The pathogenesis of constipation and diarrhea are multifactorial, and the definition of constipation and diarrhea requires the presence of clinical symptoms and changes in the frequency of defecation[21]. A careful diagnosis was not possible in the present study because the stools were monitored during daily care. Therefore, we could not classify low or high defecation frequencies as constipation or diarrhea, respectively. However, a defecation frequency ≤ 4 times per week may be considered mild constipation[22]. The present results suggested a modulatory effect of BB536 in the improving of bowel movements in individuals with a low and high frequency of defecation, which normalized the frequency of def-

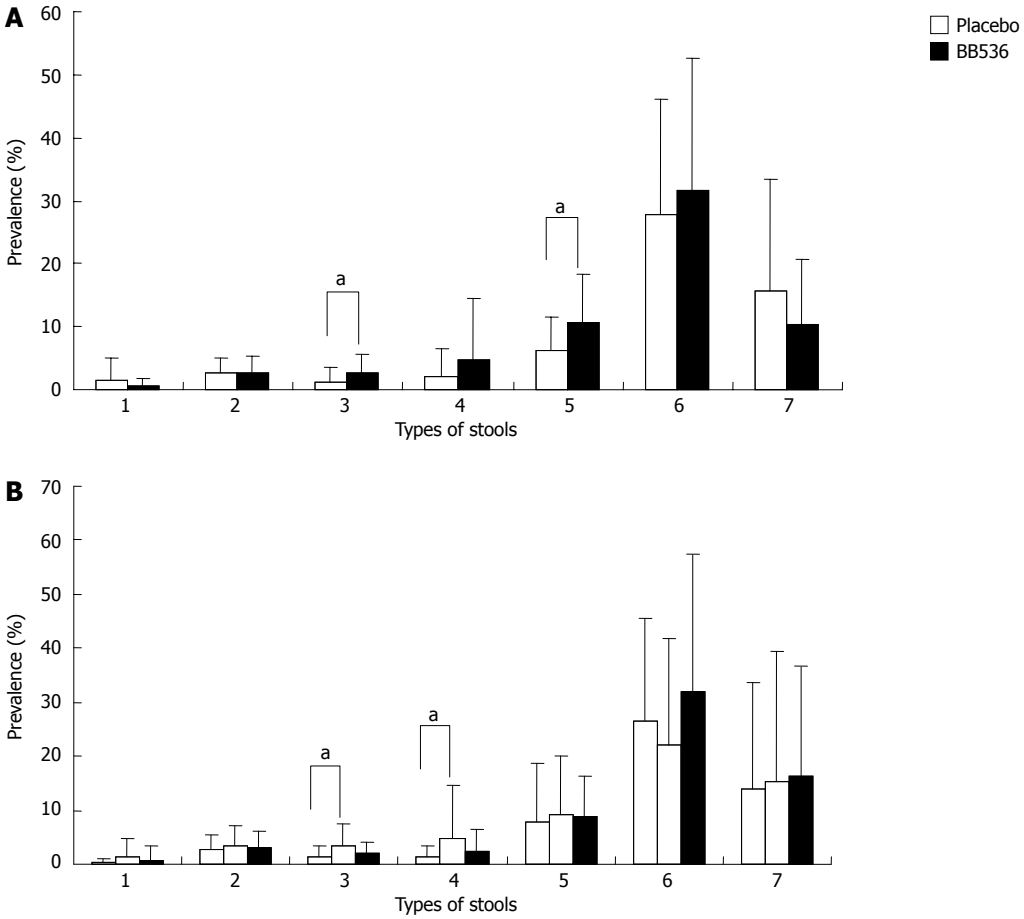

Figure 4 Effects of BB536 intake on stool form during treatment. A: Trail 1; B: Trail 2. Stool types for each bowel movement were recorded using the Bristol Stool Form Scale score, which ranges from 1 to 7. The frequency of each stool type was summed for the total treatment period, and the prevalence of each stool type was calculated. [a]$P < 0.05$ between groups.

ecation.

The effects of BB536 on fecal microbiota were investigated in Trial 2. We focused on the types of *Bifidobacteria* that are the major components of intestinal microbiota in humans and provide beneficial effects to human health[23]. The number of intestinal bifidobacteria decreases with age[6,7,24]. The populations of bifidobacteria in feces increased significantly after probiotic ingestion in the present study. These results confirmed previous findings that the ingestion of yogurt containing BB536 increased the population of bifidobacteria in healthy adults with a tendency toward constipation[13,14]. The administered strain was the primary contributor to this increase in the bifidobacterial microbiota population. However, increases in the cell numbers of *B. breve* and *B. adolescentis* were also observed in the BB536-H group. These results suggest the potential of BB536 administration in the modulation of the intestinal environment, which enhanced the proliferation of endogenous bifidobacterial species.

Trial 2 was performed to confirm the results of Trial 1 (*i.e.*, the beneficial effects on defecation frequency) and investigate the dose effect of BB536. We confirmed the effect of BB536 ingestion on defecation frequency in both trials. However, no significant differences in defecation frequency, stool types, or fecal microbiota were observed between the BB536-L and BB536-H groups, likely because the dose of the probiotic was only doubled. Further studies are required to investigate the dose response of BB536 using a broader dose range.

The present study had several strengths, including randomized treatment allocation, use of placebo controls, assessment of dose effect, evaluation using two successive studies, and evaluation of fecal microbiota during the study. This study also had several limitations, as previously discussed for other probiotic strains[25]. The results presented herein are applicable only to *B. longum* BB536 and cannot be generalized to other probiotic strains or products. Caution should be exercised in extrapolating these study outcomes to individuals with chronic and/or severe gastrointestinal complications. Another limitation may be the mild effect of the treatment compared with other therapies, such as prokinetics and laxatives, particularly when cost-effectiveness is considered. However, the clinical implications of prokinetic agents are controversial[26]. In contrast, probiotics are considered to be generally safe. Furthermore, as shown in the present study, *B. longum* BB536 showed a modulatory effect in improving the bowel movements of patients receiving enteral feeding whose bowel movements and frequency were not normal, *i.e.*, patients having either constipation or diar-

Table 3 Cell numbers of the dominant *Bifidobacterium*

Species of *Bifidobacterium*	Period	mean (log/g) ± SD (prevalence, %)		
		Placebo	BB536-L	BB536-H
All *Bifidobacterium*	Week-1	8.27 ± 1.32 (57.6)	8.68 ± 1.26 (62.2)	8.58 ± 0.94 (36.1)
	Week 16	8.41 ± 1.29 (78.8)[a]	9.05 ± 0.91 (94.6)[b,c]	8.94 ± 0.75 (94.4)[b,c]
B. longum subsp. longum	Week-1	6.91 ± 0.41 (15.2)	7.40 ± 0.88 (27)	7.29 ± 0.9 (30.6)
	Week 16	7.56 ± 0.94 (39.4)[b]	8.13 ± 0.74 (94.6)[b,d]	8.26 ± 0.65 (91.7)[b,d]
B. adolescentis	Week-1	ND (0)	ND (0)	10.06 (2.8)
	Week 16	6.64 ± 0.15 (36.4)	6.57 ± 0.21 (24.3)	6.97 ± 1.14 (25)
B. catenulatum	Week-1	11.12 ± 2.24 (9.1)	12.27 ± 3.01 (8.1)	12.88 ± 1.2 (5.6)
	Week 16	9.08 ± 0.36 (6.1)	8.98 ± 0.28 (8.1)	8.74 ± 0.05 (5.6)
B. breve	Week-1	7.91 ± 0.96 (39.4)	8.04 ± 0.87 (48.6)	7.84 ± 0.49 (22.2)
	Week 16	7.77 ± 1.00 (57.6)	8.34 ± 0.84 (48.6)	7.82 ± 0.82 (50.0)[b]
B. bifidum	Week-1	ND (0)	8.63 (2.7)	ND (0)
	Week 16	9.2 ± 0.66 (6.1)	7.81 ± 1.30 (5.4)	7.44 ± 1.24 (8.3)
B. longum subsp. *infantis*	Week-1	8.8 ± 0.46 (9.1)	8.00 ± 1.12 (13.5)	8.94 ± 0.05 (8.3)
	Week 16	7.71 ± 1.56 (18.2)	7.91 ± 1.04 (16.2)	8.16 ± 0.97 (25)[a]
BB536	Week-1	6.42 (3)	6.71 (2.7)	6.92 ± 0.26 (5.6)
	Week 16	6.98 ± 0.77 (24.2)[a]	7.97 ± 0.70 (89.2)[b,d]	8.13 ± 0.63 (91.7)[b,d]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* week-1; [c]$P < 0.05$, [d]$P < 0.01$ *vs* placebo group. *B. longum*: *Bifidobacterium longum*; *B. adolescentis*: *Bifidobacterium adolescentis*; *B. catenulatum*: *Bifidobacterium catenulatum*; *B. breve*: *Bifidobacterium breve*; *B. bifidum*: *Bifidobacterium bifidum*; ND: Not detected (< log 10^6 cells/g).

rhea. Such effects would contribute to an improved quality of life in the patients and a decreased burden of care for nurses or caregivers. In addition, although an immunoprotective effect was not observed in the present study because no patient experienced influenza infection during the study period, other studies have suggested immune-modulating and anti-infectious effects of BB536[28,29]. In the present study, we found that probiotic ingestion increased bifidobacteria in the microbiota. In addition, several studies have demonstrated the effects of administration of BB536 in eliminating harmful bacteria[13,14,30]. Based on these findings, we consider that this probiotic may represent an alternative strategy in the treatment of gastrointestinal disorders and health management in the elderly.

In conclusion, the present findings revealed that the 16-wk long-term ingestion of the probiotic BB536 strain modulated bowel movements and normalized defecation frequency in elderly patients receiving enteral feeding. BB536 administration also significantly increased the population of bifidobacteria in the intestinal microbiota. No adverse effects were associated with the ingestion of BB536. Overall, these results suggest that BB536 ingestion may improve health care in the elderly.

ACKNOWLEDGMENTS

We thank the patients and their guardians for their cooperation and the medical staff and attending physicians for their participation.

COMMENTS

Background

Elderly individuals, particularly patients who are hospitalized and receiving enteral nutrition, exhibit significant problems in defecation, which may impact on quality of life due to constipation or diarrhea. The development of novel therapeutic strategies is necessary to treat these patients more effectively, and probiotics are increasingly used as one alternative in the management of constipation.

Research frontiers

Several studies have investigated the beneficial effects of probiotics in the management of constipation and diarrhea in elderly patients. However, these effects may be strain-dependent, and they are not consistently observed. Therefore, further investigation is required to clarify this relationship. The present study investigated the efficacy of a probiotic *Bifidobacterium* strain in the health management of hospitalized elderly patients receiving enteral nutrition in two double-blind, placebo-controlled trials following a 16-wk administration of BB536.

Innovations and breakthroughs

Authors demonstrated effects of *Bifidobacterium longum* BB536 therapy *vs* placebo in the normalization of defecation frequency in patients who exhibited low and high frequencies of defection and increased the cell population of bifidobacteria in fecal microbiota.

Applications

The results of the present clinical trials suggest that the ingestion of the probiotic *Bifidobacterium* BB536 is an alternative strategy for the treatment of gastrointestinal disorders in the elderly.

Peer review

This is a formal good study of double-blind, placebo-controlled trials. The authors should discuss if probiotics should become part of regular EN in the elderly. In conclusion, I think that this had a good study design with interesting results for therapy.

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ORIGINAL ARTICLE

Correlation of human epidermal growth factor receptor 2 expression with clinicopathological characteristics and prognosis in gastric cancer

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Abstract

AIM: To investigate human epidermal growth factor receptor 2 (*HER2*) gene amplification and protein expression in Chinese patients with resectable gastric cancer and the association with clinicopathological characteristics and survival.

METHODS: One hundred and ninety-seven gastric cancer patients who underwent curative surgery procedures were enrolled into this study. *HER2* gene amplification and protein expression were examined using fluorescence *in-situ* hybridization (FISH) and immunohistochemistry (IHC) analysis on formalin-fixed paraffin-embedded gastric cancer samples from all patients. For scoring, Hofmann's HER2 gastric cancer scoring system was adopted. All cases showing IHC3+ or FISH positivity were defined as HER2 positive. Patient clinicopathological data and survival information were collected. Finally, χ^2 statistical analysis was performed to analyze the HER2 positivity rate amongst the subgroups with different clinicopathological characteristics including; gender, age, tumor location, Lauren classification, differentiation, TNM staging, depth of invasion, lymph node metastases and distant metastasis. The probability of survival for different subgroups with different clinicopathological characteristics was calculated using the Kaplan-Meier method and survival curves plotted using log rank inspection.

RESULTS: According to Hofmann's HER2 gastric cancer scoring criteria, 31 cases (15.74%) were identified as *HER2* gene amplified and 19 cases (9.64%) were scored as strongly positive for HER2 membrane staining (3+), 25 cases (12.69%) were moderately positive (2+) and 153 cases (77.66%) were HER2 negative (0/1+). The concordance rate between IHC and FISH analyses was 88.83% (175/197). Thirty-six cases were defined as positive for *HER2* gene amplification and/or protein expression, with 24 of these cases being eligible for Herceptin treatment according to United States recommendations, and 29 of these cases eligible according to EU recommendations. Highly consistent results were detected between IHC3+, IHC0/1 and FISH (73.68% and 95.42%), but low consistency was observed between IHC2+ and FISH (40.00%). The positivity rates in intestinal type and well-differentiated gastric cancer were higher than those in diffuse/mixed type and poorly-differentiated gastric cancer respectively (28.57% *vs* 13.43%, $P = 0.0103$; 37.25% *vs* 11.64%, $P < 0.0001$), but were not correlated with gender, age, tumor location or TNM stage, depth of invasion, lymph node metastases and distant metastasis. In poorly-differentiated gastric cancer patients, those without lymph node metastasis showed a higher HER2 positivity rate than those with lymph node metastasis (26.47% *vs* 7.14%, $P = 0.0021$). This association was not present in those

patients with well-differentiated gastric cancer (28.57% *vs* 43.33%, P = 0.2832). Within our patient cohort, 26 cases were lost to follow-up. The median survival time for the remaining 171 patients was 18 mo. The median survival times of the HER2 positive and negative groups were 17 and 18.5 mo respectively. Overall survival was not significantly different between HER2-positive and negative groups (χ^2 = 0.9157, P = 0.3386), but in patients presenting well-differentiated tumors, the overall survival of the HER2-positive group was significantly worse than that of the HER2-negative group (P = 0.0123). In contrast, patients with poorly differentiated and diffuse/mixed subtype gastric cancers showed no significant differences in overall survival associated with HER2. Furthermore, the median survival time of the HER2 positive group did not show any statistically significant differences when compared to the subgroups of gender, age, tumor location, TNM classification, lymph node metastases and distant metastasis.

CONCLUSION: Patients with intestinal type gastric cancer (GC), well-differentiated GC and poorly-differentiated GC without lymph node metastasis, may all represent suitable candidates for targeted therapy using Herceptin.

Key words: Gastric cancer; Human epidermal growth factor receptor 2; Gene amplification; Protein expression; Clinicopathological characteristics

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INTRODUCTION

Human epidermal growth factor receptor 2 (HER2) is a 185-kDa transmembrane tyrosine kinase receptor[1] and its gene amplification and protein overexpression play an important role in the proliferation, apoptosis, adhesion, angiogenesis and aggressiveness of many solid tumors[2], including; breast[3], colon[4], bladder[4], ovarian[5], uterine cervix[6], esophageal[7] and gastric cancer.

Herceptin (trastuzumab) has been approved[8] in the European Union and the United States for use in combination with 5-fluorouracil (5-FU) or capecitabine plus cisplatin for the first-line treatment of patients with HER2-positive metastatic adenocarcinoma of the stomach or gastro-esophageal junction according to the results of the 2010 trastuzumab for gastric cancer (ToGA) trial. However, precise patient inclusion criteria for Herceptin treatment is still not fully defined due to the lack of a standardized HER2 scoring system for gastric cancer[9,10]. For a clinician, defining the relationships between HER2 and clinicopathological characteristics can help to select suitable candidates.

Our study aimed to investigate the relationship between *HER2* gene amplification and protein overexpression in resectable gastric cancer patients and determine any correlations with relevant clinicopathological characteristics. Furthermore, we explored the influence of HER2 on disease prognosis in gastric cancer patients. Our study was conducted with a view towards the future introduction of Herceptin targeted therapy for the treatment of gastric cancer patients.

MATERIALS AND METHODS

Patients and tissue specimens

From July 2009 to January 2012, 197 gastric cancer patients who underwent curative surgery at Renji hospital, Shanghai Jiaotong University were enrolled into our study. Formalin-fixed, paraffin-embedded samples of tumors and corresponding normal stomach tissues from 197 gastric cancer patients were evaluated for HER2 protein and gene amplification using immunohistochemistry (IHC) and fluorescence *in-situ* hybridization (FISH) analysis. None of the patients had undergone prior preoperative radiation, chemotherapy or targeted therapy.

The study included 65 women and 132 men, with ages ranging from 22 to 88 years. The median age was 62 years. The tumor sample characteristics of all 197 cases are shown in Table 1. Of all the tumors examined, 31 (15.74%) were located in the cardiac region, 42 (21.32%) in the body, and 122 (61.93%) in the pylorus. The majority (98.98%) of the samples were primary tumors with only 2 recurrent tumors identified. According to Lauren classification, 63 (31.98%) tumors were intestinal-type and 134 (68.02%) were diffuse-type or mixed-type carcinomas. Poorly differentiated tumors (grades Ⅰ and Ⅱ) comprised 25.89%, whilst 74.11% of tumors were moderately differentiated (grades Ⅲ and Ⅳ). TNM classification revealed that 13 cases were stage Ⅰ (6.60%), 46 were stage Ⅱ (23.35%), 98 were stage Ⅲ (49.75%) and 40 were stage Ⅳ (20.30%). Postoperative follow-up ended in April, 2012.

FISH detection for HER2 gene amplification

FISH was conducted with the HER2 DNA Probe Kit (Invitrogen™ by Life Technologies) according to the manufacturer's instructions. Four-μm-thick sections were baked overnight at 56 ℃, deparaffinized in three 10 min changes of xylene and then rehydrated through two 5-min changes of 100% ethanol. The slides were then reduced for 18 min in SPOT-Light tissue pretreatment solution at > 98 ℃, and briefly washed in 3 × PBS at room temperature. The slides were then incubated for 16 min in enzyme reagent solution at 37 ℃ and washed in 3 × PBS at room temperature, dehydrated through 70%, 85%, and 100% ethanol, and allowed to air dry. After open air drying, the HER2 DNA probe kit (PathVysion HER2 DNA Probe Kit, Abbott Laboratories) which was denatured at

Table 1 Correlation of human epidermal growth factor receptor 2 expression with clinicopathological characteristics *n* (%)

Clinicopathological characteristics	*n*	HER2		χ^2	*P* value
		Positive	Negative		
Sex				1.2736	0.2591
Male	132	27 (20.45)	105 (79.55)		
Female	65	9 (13.85)	56 (86.15)		
Age (yr)				1.3056	0.2532
< 60	88	13 (14.77)	75 (85.23)		
⩾ 60	109	23 (21.10)	86 (78.90)		
Tumor site[1]				0.0409	0.9798
Cardiac	31	6 (19.35)	25 (80.65)		
Body	42	8 (19.05)	34 (80.96)		
Pylorus	122	22 (18.03)	100 (81.97)		
Lauren classification				6.5759	0.0103
Intestinal	63	18 (28.57)	45 (71.43)		
Diffuse/mixed	134	18 (13.43)	116 (86.57)		
Tumor differentiation				16.6003	< 0.0001
Well-differentiated	51	19 (37.25)	32 (62.75)		
Poorly-differentiated	146	17 (11.64)	129 (88.36)		
TNM classification				0.6754	0.879
Ⅰ	13	2 (15.38)	11 (84.62)		
Ⅱ	46	7 (15.22)	39 (84.78)		
Ⅲ	98	20 (20.41)	78 (79.59)		
Ⅳ	40	7 (17.50)	33 (82.50)		

[1]Two remnant samples were not included. HER2: Human epidermal growth factor receptor 2.

79 ℃ for 6 min, was applied onto each slide, a cover slip was added and then sealed with rubber cement. After 16 to 18 h of hybridization at 37 ℃, the slides were washed with 73 ℃ preheated post hybridization buffer for 5 min and dehydrated through 70%, 85% and finally 100% ethanol. After air drying, the slides were counter-stained with 14 μL diamidino-phenyl-indole, cover slips applied and then slides chilled for 30 min at 4 ℃. Finally, the slides were observed through a fluorescence microscope (OLYMPUS BX61).

Immunohistochemical staining

HER2 IHC analysis was performed on 4 μm thick tissue sections. Briefly, after deparaffinization and rehydration steps, the tissue samples were incubated in antigen retrieval solution at 99 ℃ for 40 min. Endogenous peroxidase activity was quenched by 5 min incubation with hydrogen peroxide. Sections were then incubated with HER2 antibody (Herceptest™, DAKO) for 30 min. Both the primary and secondary antibodies against human HER2 protein were applied for 30 min at room temperature and then the immunocomplexes were visualized with diaminobenzidine for 10 min and placed under a cover slip. Finally, the slides were viewed using light microscopy (LEICA DM2500).

Results scoring

An absolute *HER2* gene copy number lower than 6 or a HER2/Chr17 ratio of less than 2 was considered HER2 negative, whilst cases showing average gene copy numbers of HER2 ⩾ 6 or a gene/CEN17 fluorescence ratio ⩾ 2 were considered positive for gene amplification.

Table 2 Immunochemistry-fluorescence *in situ* hybridization concordance *n* (%)

FISH	IHC				Total
	3+	2+	1+	0	
Positive	14	10	7	0	31 (15.74)
Negative	5	15	21	125	166 (84.26)
Total	19 (9.64)	25 (12.69)	28 (14.21)	125 (63.45)	197

IHC: Immunochemistry; FISH: Fluorescence *in-situ* hybridization.

Additionally, tight gene clustering of HER2 signals was also defined as gene amplification. The above criteria are based on Hofmann's criteria in gastric cancer[9].

In the present study, the IHC score criteria on human gastric cancer also followed Hofmann's criteria[9]: no staining or < 10% tumor cell positive staining as 0/negative; faintly or barely perceptible staining on > 10% tumor cell membrane as 1+/negative; weak to moderate positive staining on > 10% tumor cells as 2+/(equivocal) positive; cohesive moderate to strong staining on the membrane will be scored as 3+/positive. All cases with IHC3+ or FISH positivity were defined as HER2 positive.

Statistical analysis

χ^2 statistical analysis was performed to assess the HER2 positivity rate amongst the subgroups with different clinicopathological characteristics. The probability of survival for different subgroups was calculated using the Kaplan-Meier method and the survival curves plotted using log rank inspection. All statistics were performed using 2-sided analysis, with a significance level of $P < 0.05$, using the "SAS9.13" statistical software package.

RESULTS

HER2 gene amplification and protein expression

The FISH and IHC analysis results for all 197 gastric cancer tissues are shown in Table 2. According to Hofmann's HER2 FISH scoring criteria, 31 cases (15.74%) were identified as *HER2* gene amplified and the other 166 cases (84.26%) were *HER2* gene amplification negative (Figure 1). Of the 197 samples examined by IHC (following Hofmann's criteria), 19 cases (9.64%) were scored as strongly positive for HER2 membrane staining (3+), 25 cases (12.69%) were moderately positive (2+), and 153 cases (77.66%) were HER2 negative (0/1+) (Figure 2).

The concordance rate between IHC and FISH analyses was 88.83% (175/197). Thirty-six cases were defined as HER2 positive and 24 cases were suitable for Herceptin treatment according to the recommendations of the United States[11]. However, when applying European Union[11] recomnmendations for Herceptin usage, 29 cases were identified as eligible for Herceptin treatment. This difference underscores the requirement for standardized and more precise eligibility criteria for correct identification of patients who are eligible for HER2 targeted therapy.

Of the 31 FISH-positive cases, 14 cases (45.16%)

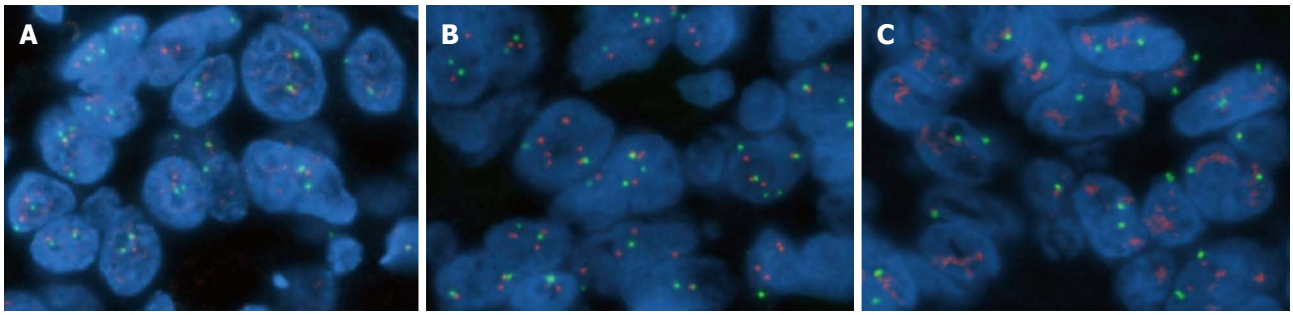

Figure 1 Fluorescent *in-situ* hybridization analysis of human epidermal growth factor receptor 2 gene amplification (× 600). A: Normal human epidermal growth factor receptor 2 (*HER2*) gene expression: Red signals (*HER2* gene), green signals [chromosome enumeration probe 17 (CEP17)], blue signals (nuclei lining dye); B: Positive *HER2* gene amplification: HER2:CEP17 > 2; C: Positive *HER2* gene amplification: HER2:CEP17 > 2 with clear red cluster signals observed.

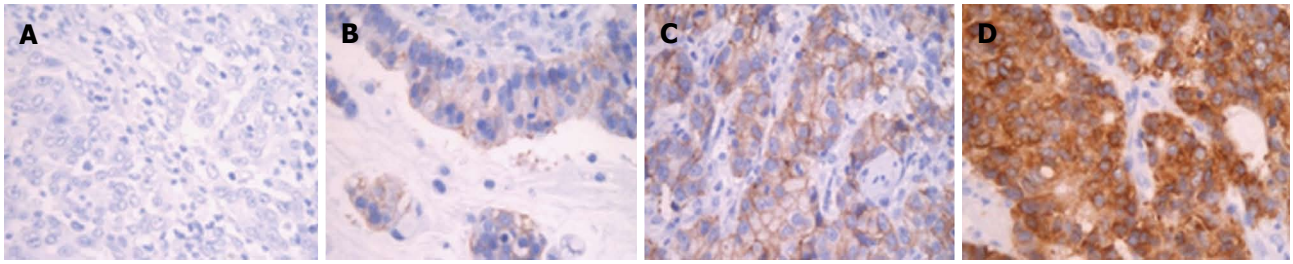

Figure 2 Immunohistochemical analysis of human epidermal growth factor receptor 2 protein expression (× 200). A: Immunohistochemical (IHC) 0: No staining on tumor cell membrane; B: IHC1+: Faintly perceptible staining on > 10% tumor cell membrane; C: IHC2+: Moderate staining on > 10% tumor cell membrane; IHC3+: Strong staining on > 10% tumor cell membrane.

Table 3 Correlation of human epidermal growth factor receptor 2 expression with tumor node metastasis staging *n* (%)

Clinicopathological characteristics	*n*	HER2		χ^2	*P* value
		Positive	Negative		
T				0.5782	0.4470
T1-T2	26	6 (23.08)	20 (76.92)		
T3-T4	171	29 (16.96)	142 (84.04)		
N				4.6274	0.2012
N0	55	8 (14.55)	47 (85.45)		
N1	83	20 (24.10)	63 (75.90)		
N2	33	5 (15.15)	28 (84.85)		
N3	26	2 (7.69)	24 (92.31)		
M				0.0000	1.0000
M0	185	33 (17.84)	152 (82.16)		
M1	12	2 (16.67)	10 (83.33)		

HER2: Human epidermal growth factor receptor 2.

were IHC3+ with a 100% concordance between IHC3+ and FISH, and 10 (32.26%) cases were IHC2+. None of the IHC 0 tumors demonstrated FISH amplification, and only 7 tumors in the IHC1+ group were found to be FISH positive with a ratio of 22.58%. High consistency results was detected between IHC3+, IHC0/1, and FISH scores (73.68% and 95.42%), but low consistency was observed between IHC2+ and FISH (40.00%).

Correlation of HER2 with clinicopathological characteristics

Significantly different HER2 positivity rates were observed when comparing intestinal-type gastric cancers with diffuse/mixed-type cancers (28.57% *vs* 13.43%, $P = 0.0103$), and well-differentiated cases with poorly-differentiated cases (37.25% *vs* 11.64%, $P < 0.0001$). No relationship was observed between the HER2 positivity rate and sex, age, tumor site and TNM GC classification ($P > 0.05$; Table 1). Furthermore, within the subgroups, no relationship was observed between HER2 positivity and depth of invasion, lymph node metastasis or distant metastasis (Table 3).

Within the poorly-differentiated gastric cancer patient group, those without lymph node metastasis showed a higher HER2 positivity rate than those with lymph node metastasis (26.47% *vs* 7.14%, $P = 0.0021$). This association was not observed in the well-differentiated gastric cancer patient group (28.57% *vs* 43.33%, $P = 0.2832$).

Survival analysis

Of our 197 gastric cancer patients, 26 cases were lost in follow-up. The median survival time for the remaining 171 patients was 18 mo (range: 0-33 mo). During the follow-up time, 60 deaths occurred (35.09%), 57 of which were disease-related. One patient died of perioperative pulmonary infection, and two cases died of heart disease and multiple organ failure, respectively.

The median survival time of the HER2 positive (29 cases) and negative groups (142 cases) was 17 mo and 18.5 mo, respectively. Nevertheless, the HER2 positive gastric cancer patients did not show statistically significant reductions in mean survival times, nor lower 1-year or 2-year survival rates. Furthermore, no statistically significant differences were observed in overall survival

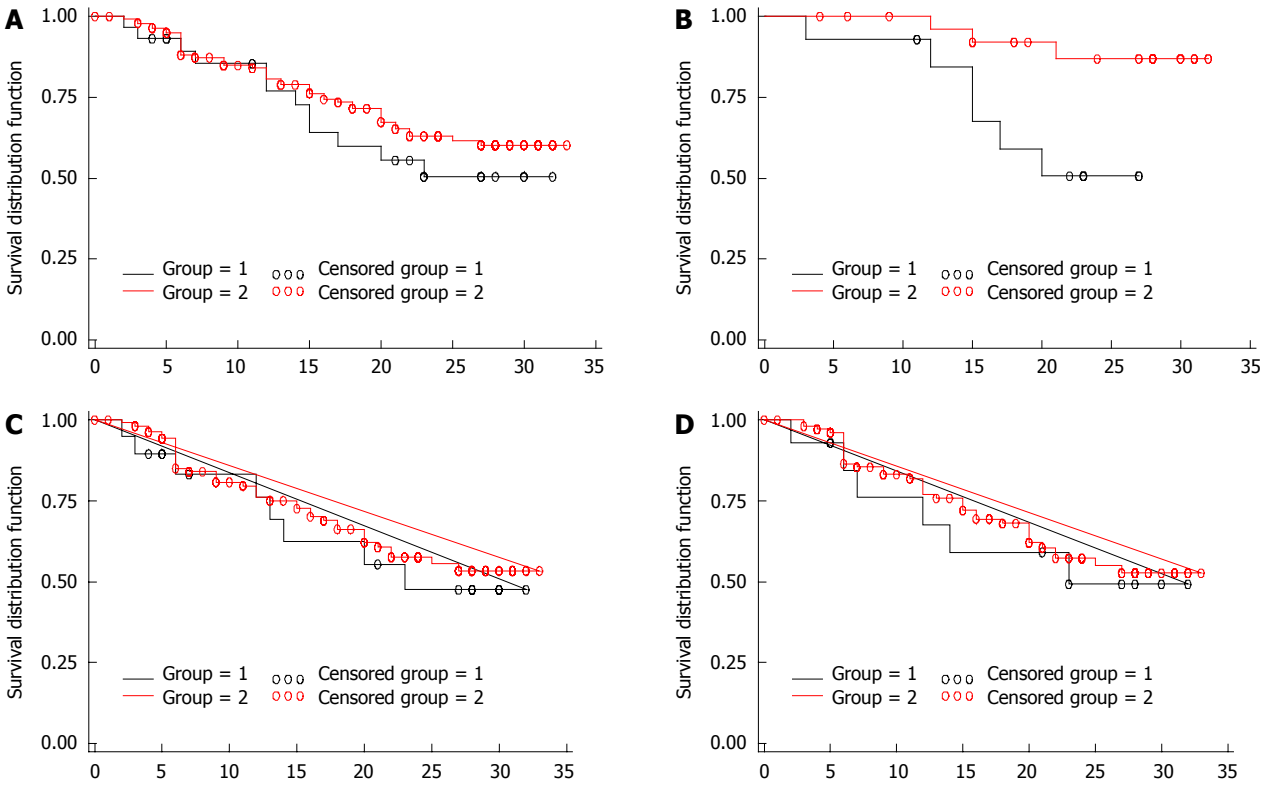

Figure 3 Kaplan-Meier survival analysis. A: Overall survival curves of 171 gastric cancer patients according to human epidermal growth factor receptor 2 (HER2) detection (P = 0.3386); B: Survival curve of patients with well differentiated gastric cancer according to HER2 expression (P = 0.0123); C: Survival curve of patients with poorly differentiated gastric cancer according to HER2 expression (P = 0.0988); D: Survival curve of patients with the diffuse/mixed type gastric cancer according to HER2 expression (P = 0.6623).

times between the HER2 positive and negative groups (χ^2 = 0.9157, P = 0.3386; Figure 3A).

Within the well differentiated gastric cancer patient group, patients with HER2 tumor positivity had poorer outcomes than those with HER2 negative tumors. The well differentiated HER2 positive patient group exhibited shorter mean survival time (18.5 mo *vs* 27.5 mo) and lower 1-year and 2-year survival rates compared to the HER2 negative group (84.42% *vs* 96.00%; 50.65% *vs* 86.89%; P = 0.0123; Figure 3B). The median survival time of the HER2 positive group did not show any statistical associations when compared to the subgroups of sex, age, tumor site, TNM classification, depth of invasion, lymph node metastases and distant metastasis in gastric cancer (Table 4). Within the poorly differentiated and diffuse/mixed type gastric cancer patient groups, no statistically significant differences were observed between the HER2 positive and HER2 negative groups (Figure 3C and D).

DISCUSSION

HER2 gene amplification and protein overexpression in gastric cancer were first reported in 1986[12,13]and have since been confirmed by numerous studies, highlighting ranges in both *HER2* gene amplification rates from 16%-27.1% by FISH analysis and HER2 protein overexpression from 8.2%-53.4% by IHC analysis. The variability within these results is likely due to several factors including sample size, study design and differences in geographic location[14]. However, the most important variability factor is likely a consequence of having no standardized HER2 test and scoring criteria[15]. In the present study, both FISH and IHC scoring criteria followed that of Hofmann[9] which is considered to be the most appropriate HER2 scoring system in human gastric cancer. Furthermore, to ensure the reliability of our results, we followed the guidelines on HER2 detection in breast cancer, recommended by the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP)[16] and used the test kit certified by the United States Food and Drug Administration.

Herceptin (trastuzumab) is a recombinant human monoclonal antibody designed to target and block the function of HER2 by directly binding to the extracellular domain of the receptor[1,17]. It has been used for the treatment of HER2 overexpressing breast cancer for more than 10 years and was approved by the European Medicines Agency[18] in 2010 for use in combination with capecitabine or 5-FU and cisplatin for metastatic gastric or GE junction cancers, based on data from the 'ToGA" clinical trial. The exact anti-tumor mechanism of Herceptin is not fully understood, however some mechanisms have been postulated[17,19-23] including interruption of HER2 mediated cell signaling pathways and cell cycle progression; induction of antibody-dependent cell-mediated cytotoxicity and apoptosis; induction of

Table 4 Relationship of different clinicopathological characteristics and prognosis

Clinicopathological characteristics	HER2 positive			HER2 negative			χ^2	*P* value
	Median survival time (mo)	1-year survival rate	2-year survival rate	Median survival time (mo)	1-year survival rate	2-year survival rate		
Sex								
Male	20	74.34%	50.18%	20	83.96%	69.00%	2.2591	0.1328
Female	10	100.00%	50.00%	16.5	74.50%	51.79%	0.0182	0.8927
Age (yr)								
≤ 60	23	100.00%	57.14%	20	80.54%	61.81%	0.0104	0.9186
> 60	15	67.55%	49.13%	18	80.62%	64.35%	1.6356	0.2009
Tumor site								
Cardiac	19	66.67%	50.00%	15	69.51%	49.15%	0.0494	0.8242
Body	16.5	62.50%	62.50%	14	67.80%	44.07%	0.1561	0.6927
Pylorus	17	85.56%	46.67%	20	87.00%	73.25%	2.3295	0.1269
Lauren classification								
Intestinal	17	84.85%	50.91%	27	89.17%	76.53%	2.3604	0.1244
Diffues/mixed	14	67.53%	49.24%	16.5	76.99%	57.24%	0.1907	0.6623
Tumor differentiation								
Well-differentiated	18.5	84.42%	50.65%	27.5	96.00%	86.89%	6.2701	0.0123
Poorly-differentiated	14	67.88%	49.49	17	76.56%	56.71%	0.0988	0.7532
TNM classification								
Ⅰ and Ⅱ stages	18.5	68.57%	57.14%	21.5	93.60%	79.20%	2.9813	0.0842
Ⅲ and Ⅳ stages	17	82.59%	45.88%	16.5	73.32%	54.12%	0.0263	0.8711
T								
T1-T2	17	66.67%	66.67%	28	100.00%	92.31%	3.4587	0.0629
T3-T4	15	91.30%	46.99%	17	77.47%	58.26%	0.2953	0.5869
N								
N0	14	68.57%	51.43%	21	90.46%	74.98%	2.0667	0.1505
N1-N3	18.5	79.19%	49.49%	17	75.73%	57.27%	0.0531	0.8177
M								
M0	17	78.67%	54.69%	20	84.41%	66.01%	0.7842	0.3757
M1	11.5	50.00%	0.00%	5	0.00%	0.00%	0.5900	0.4424

HER2: Human epidermal growth factor receptor 2.

anti-angiogenesis effects and increasing receptor turnover by endocytosis. As clinical surgeons, we should be readily and accurately able to identify which patients are suitable for Herceptin treatment. An accurate and reliable HER2 scoring system, together with clinical information, may help us to better determine whether a gastric cancer patient is a potential candidate for targeted therapy using Herceptin.

The relationship between *HER2* gene amplification and protein expression in gastric cancer patients is controversial[24,25]. Nevertheless, more recent studies have reported a high concordance between gene amplification and protein overexpression using FISH and IHC approaches[11,26,27]. Indeed, the ToGA trial[28] (which recruited the largest population of gastric cancer patients to date-3807) reported a HER2 FISH and IHC concordance rate of 87.5%, and further reported that HER2 IHC3+ cases were almost all entirely *HER2* gene amplified (97.5% of cases). However, 22.5% of HER2 FISH positive cases in the ToGA trial were HER2 IHC negative, a finding which differs from the situation observed in breast cancer, where almost all HER2 IHC 0/1+ samples are HER2 FISH negative[14]. In our study, the overall HER2 positive rate (FISH and IHC combined) was 18.27% while 15.74% of cases showed *HER2* gene amplification by FISH and 9.64% of patients showed HER2 protein overexpression by IHC analysis. The concordance between the two detection methods was 88.83%. Of the 31 FISH-positive cases, 14 cases (45.16%) were IHC3+, with a 100% concordance between IHC3+ and FISH, and 10 (32.26%) cases were IHC2+. None of the IHC0 tumors showed FISH amplification, and only 7 tumors in the IHC1+ group were found to be FISH positive with a ratio of 22.58%. A high degree of data consistency was observed between IHC3+ and IHC0/1 with FISH (73.68% and 95.42%); however, low scoring consistency was observed between IHC2+ and FISH (40.00%). Thus, our data highlights the need and importance of further clarifying the relationship between *HER2* gene amplification and protein overexpression in gastric cancer.

In our study, no relationship was observed between HER2 positivity and sex, age and TNM classification ($P > 0.05$). However, intestinal-type and well-differentiated gastric cancer cases showed a higher HER2 positive rate than diffuse/mixed-type and poorly-differentiated cancer cases. This finding is in keeping with similar data from the ToGA trial and other published studies[29,30]. Of interest, the ToGA trial reported a higher HER2 positivity rate in GE junction cancers compared to other gastric cancers (33.2% *vs* 20.9%, $P < 0.001$)[17]. Our study, as well as that of another group[31], showed no statistically significant difference between HER2 positivity and the gastric tumor site. Within the poorly-differentiated gastric cancer patient group, those patients without lymph node

metastasis showed a higher HER2 positivity rate when compared to those with lymph node metastasis (26.47% *vs* 7.14%, $P = 0.0021$). No difference in HER2 positivity was observed, however, when comparing lymph node metastasis status in the well-differentiated gastric cancer patient group (28.57% *vs* 43.33%, $P = 0.2832$). The underlying molecular mechanisms behind the varying HER2 positivity rates in the different histological GC subtypes are clearly complex and require further investigation.

The role of HER2 as a prognostic factor in gastric cancer has been controversial due to significant differences in historical study results. More recent studies, however, indicate that HER2 is a poor prognostic factor in gastric cancer patients[32-35], especially those with liver metastases[36]. Whilst our study did not show any correlation between HER2 status and overall survival, patients with well-differentiated HER2 positive tumors showed poorer survival times compared to patients with HER2 negative tumors. We speculate that HER2 status has a mild impact on gastric cancer patient survival and may not constitute an independent prognostic factor in gastric cancer patients. Clearly, further research is required to explain the impact of HER2 on development and prognosis of gastric cancer.

In conclusion, an accurate and standardized scoring system for HER2 expression in gastric cancer patients is of clear importance and utility in the optimal selection of patients for Herceptin therapy. Our studies highlight intestinal-type, well-differentiated and poorly-differentiated gastric cancer patients without lymph node metastasis as the three main candidate patient groups for targeted therapy using Herceptin. Finally, we advocate further detailed research on the mechanism(s) through which HER2 expression drives progression of gastric cancer and consideration of additional studies to explore the role of HER2 as an independent prognostic factor.

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COMMENTS

Background

Gastric cancer (GC) is one of the most prevalent cancers worldwide, with poor prognosis. Herceptin (trastuzumab) can improve overall survival without compromising safety in patients with human epidermal growth factor receptor 2 (HER2)-positive metastatic gastric cancer. However, a standardized HER2 scoring system is still required. Studies on the correlation of HER2 and clinicopathological characteristics could help clinicians to optimally select suitable candidates for targeted therapy using Herceptin.

Research frontiers

HER2 inhibition is playing a significant role as a new treatment option for gastric cancer. Numerous countries have approved the use of Herceptin for the treatment of gastric cancer and increasingly, HER2 has become a "hot" research topic. An accurate and reliable HER2 scoring system is necessary to select suitable candidates for Herceptin targeted therapy.

Innovations and breakthroughs

To date, there have been limited studies to determine any correlations of HER2 expression with clinicopathological characteristics and prognosis in Chinese patients with resectable gastric cancer. Intestinal type gastric cancer patients, well-differentiated gastric cancer patients and poorly-differentiated gastric cancer patients without lymph node metastasis showed a higher HER2 positivity rate and thus could represent ideal candidates for targeted-therapy using Herceptin.

Applications

The study results suggest that an accurate HER2 scoring system plays an important role with clinical significance. Patients with intestinal-type gastric cancer, well-differentiated gastric cancer and poorly-differentiated gastric cancer without lymph node metastasis are ideal candidates for targeted therapy using Herceptin.

Peer review

The paper makes sense to search for the gastric cancer patients in Jiangsu province. The study design is valid and the data is sufficient.

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ORIGINAL ARTICLE

Involvement of interstitial cells of Cajal in experimental severe acute pancreatitis in rats

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Abstract

AIM: To observe the changes in interstitial cells of Cajal (ICC) in rats with experimental severe acute pancreatitis (SAP).

METHODS: A total of twenty-four SD rats were randomly divided into two groups (n = 12), namely the sham (S) group and the SAP group; the SAP rat model was established by retrograde injection of 5% sodium taurocholate (1.0 mL/kg) into the pancreatic duct. Twenty-four hours later intestinal motility was assessed by testing small intestinal propulsion rate, and then the rats were sacrificed. The pancreas and jejunum were resected and underwent routine pathologic examination. Immunohistochemical staining was used to detect c-kit-positive cells in the jejunum. Expression of c-kit mRNA was detected by real-time polymerase chain reaction, and the expression of c-kit protein was evaluated by Western blotting. Ultrastructure of ICC was evaluated by transmission electron microscopy.

RESULTS: There was bleeding, necrosis and a large amount of inflammatory cell infiltration in pancreatic tissue in the SAP group, while in jejunal tissue we observed a markedly denuded mucosal layer, loss of villous tissue and a slightly dilated muscular layer. The small intestinal propulsion rate was 68.66% ± 2.66% in the S group and 41.55% ± 3.85% in the SAP group. Compared with the S group, the rate of the SAP group decreased sharply. The density of c-kit-positive cells in the SAP group was significantly lower than in the S group; the respective mean densities were 88.47 ± 10.49 in the S group and 56.11 ± 7.09 in the SAP group. The levels of c-kit protein and mRNA were 0.36 ± 0.04 and 1.29 ± 0.91 in the SAP group, respectively, which were significantly lower than those in the S group (0.53 ± 0.06, 0.64 ± 0.33, respectively). In the SAP group, ICC profiles showed the same change tendency, such as vacuolation of mitochondria, irregular vacuoles and loosened desmosome-like junctions.

CONCLUSION: Decreased c-kit-positive cells and ultrastructural changes in ICC resulting from blockade of the c-kit signaling pathway are involved in the intestinal dysmotility associated with SAP.

Key words: Severe acute pancreatitis; c-kit; Interstitial cells of Cajal; Real-time polymerase chain reaction; Ultrastructure

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INTRODUCTION

In 1893, the Spanish neuro-histologist, Cajal, discovered

interstitial cells of Cajal (ICC) within the gastrointestinal wall. Since then, especially in the most recent two decades, a number of studies have established the roles of ICC in normal functions of the gastrointestinal wall primarily in 4 major groups: ICC in the submuscular plexus; ICC within the circular and longitudinal layers of muscle; ICC in the myenteric plexus (ICC-MY, also called ICC-MP); ICC in the deep muscular plexus. These cells function as pacemaker cells in the gastrointestinal wall to generate slow waves that spread from ICC to smooth muscle cells for triggering calcium entry, as a result of depolarization, and contraction as a basis for peristalsis and segmentation. They maintain normal neurotransmission and regulate mechanical activities in the gastrointestinal tract[1-5]. More recently, the discovery of c-kit along with its endogenous ligand, stem cell factor (SCF), have dramatically advanced ICC investigations in this field[6,7]. Presentation of SCF increases expression of c-kit immunoreactive ICC in culture while loss-of-function mutations of the c-kit gene cause deficiency of ICC; these have shown that the SCF/c-kit signal pathway is essential for the maintenance of ICC[8-11]. Imatinib, a novel and potent inhibitor of c-kit, abolished the spontaneous movements in circular muscles of the mouse small intestine[12], and this result suggests that the c-kit signaling of ICC plays an essential role in the spontaneous mechanical activity of intestine. Disorders of ICC may result in gastrointestinal motility dysfunctions, which lead to a number of gastrointestinal diseases, including severe acute pancreatitis (SAP). Furthermore, investigators have found that damage of ICC occurred in the muscular layer of the small intestine in experimental acute pancreatitis[13].

Despite the association of SAP with gastrointestinal motility disturbances on the basis of evidence acquired through both observational clinical[14] and experimental investigations[15,16], the detailed mechanisms of the changes in gastrointestinal motility in SAP have not been clearly elucidated. Thus, we hypothesized that ICC might play an important role in the pathogenesis of gastrointestinal dysmotility in SAP. In the present study we tested our hypothesis in a rat model of SAP.

MATERIALS AND METHODS

Animal model establishment

Twenty-four adult male Sprague-Dawley (SD) rats with body weight between 200 g and 250 g were purchased from the animal research center of the affiliated Drum Tower Hospital of Nanjing University Medical School and randomly divided into two groups of equal number (n = 12 each): the sham (S) group and the severe acute pancreatitis (SAP) group. To establish the SAP rat model, freshly prepared 5% sodium taurocholate solution was injected at a volume of 1.0 mL/kg from the duodenal papilla into the pancreatic duct. In the S group, the duodenum and pancreas of animals were manually manipulated a few times after laparotomy. All procedures took place under sterile conditions and all animals were housed under pathogen-free conditions in the animal facility with a 12-h light/dark cycle and free access to food and water. The study protocol was approved by the Medical Ethics Committee of the Hospital.

Assessment of small intestinal propulsion rate

Twenty-three hours after the operative procedure for the establishment of the SAP animal model, gastric gavage with 1 mL of methylene blue solution was performed in both groups. One hour later, the rats were euthanized *via* CO_2 asphyxiation and the small intestine in each rat was removed from the abdominal cavity. All the mesentery tissues were stripped and the total length of the small intestine from the pyloric sphincter of the distal stomach to the distal end of the ileum was measured. The movement of the methylene blue solution in the small intestine was observed and recorded. The small intestinal propulsion rate was calculated as the product of the distance of the methylene blue traveled within 30 min immediate after the removal of the small intestine divided by the total length of the small intestine.

Histopathologic examination of the pancreas and the jejunum

Both the pancreas and the jejunum were removed at the time of harvest of the small intestine described above. Four segments of the jejunum 15 cm distal to the ligament of Truiz, approximately 10 mm each in length, were collected for the following study. One segment of the jejunum was opened, cleaned, and inspected macroscopically along with the pancreas that was transversely sectioned, for visible pathologic changes. After gross examination, both organs were fixed with 10% buffered neutral formalin solution for 24 h. The tissue from both organs was sectioned at 4 μm in thickness. Histology sections were stained with hematoxylin and eosin and evaluated microscopically by experienced pathologists.

Real-time polymerase chain reaction

In the jejunal tissue freshly harvested previously, total RNA was isolated from the jejunum segments with mucosa stripped using TRIzol® reagent following the manufacturer's instructions. The reverse transcription (RT) was performed in a 20 μL reaction mixture containing 1 μg total RNA by using a PrimeScript RT Reagent Kit (Perfect Real Time, Takara Bio Inc., Otsu, Japan) according to the manufacturer's protocol. The RT reaction product was amplified by using the SYBR Premix Ex Taq (Takara Bio Inc., Otsu, Japan) and ABI PRISM 7500 Real-time PCR system according to the manufacturer's protocol. Primers of c-kit were as follows: 5'-TGGATCAGCAAATGTCACAACAAC-3' (forward) and 5'-TAGGCCTCGAACTCAACAACCA-3' (reverse). The predicted size of the c-kit-PCR product was 132 bp. The primers of β-actin were: 5'-TCGTGCGTGACATTAAAGAG-3' (forward) and 5'-ATTGCCGATAGTGATGACCT-3' (reverse). The predicted size of the β actin-PCR product was 134 bp. Mean fold changes for each sample were calculated

by using the $2^{-\Delta\Delta Ct}$ method as previously described[17].

Immunohistochemical staining

The segments of jejunum harvested previously were immersed in a fixative containing 4% paraformaldehyde for 6 h at 4 ℃. Then the segments were embedded with the optimum cutting temperature compound and sectioned at 10 μm in thickness. The tissue section was mounted on glass slides. For the c-kit staining, tissue sections were incubated with 0.3% Triton X in 10% normal rabbit serum for 60 min and then incubated with the goat anti-c-kit polyclonal antibody (clone sc-1494; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, United States) at 4 ℃ overnight. Next, we applied a biotin-free polymeric horseradish peroxidase (HRP)-linked antibody conjugate system for 20 min followed by DAB condensed chromogen for 5 min. Tissue sections were counterstained with hematoxylin and eosin (HE). For negative control experiments, the primary antibody was omitted. Images of c-kit-positive cells were taken in 4 randomly chosen fields (× 200 magnification) per tissue section. The positive cell density was assessed with the Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, United States).

Western blotting

A segment of the jejunum was cut along the mesenteric axis and stripped of the mucosa. The remaining jejunal tissue was immediately snap-frozen in liquid nitrogen and stored at -80 ℃. After homogenization in extraction buffer (50 mmol/L Tris-Cl (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, and 1 mmol/L PMSF), the lysate was collected and centrifuged at 4 ℃ for 15 min at 10 000 r/min to remove the insoluble material. The protein concentration of the supernatant was measured by spectrophotometry using the BCA protein assay method (Pierce, Rockford, IL, United States). The samples were electrophoresed on a 10% SDS-polyacrylamide gel, and transferred to a PVDF transfer membrane (Millipore, Bedford, MA, United States). The membrane was then incubated with 5% skimmed cow's milk overnight at 4 ℃ to block nonspecific binding sites and then incubated with the primary c-kit antibody (clone sc-1494; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, United States) applied for 1 h at room temperature. After washing, the secondary matching peroxidase-conjugated antibody was applied to the membrane and incubated for 1 h at room temperature. Specific protein bands were visualized with an X-ray film using the chemiluminescence detection kit (ECL Western blotting detection; Millipore Corp). Optical density of the bands was analyzed with software Quantity One.

Electron microscopy

Immediately after resection, blocks of jejunal tissue were cut and immersed into a fixative containing 5% glutaraldehyde and stored at 20 ℃ for at least 2 h. Following fixation, tissues were cut into small pieces (1 mm × 2 mm) and further fixed in 5% glutaraldehyde overnight, and then rinsed for 60 min in 0.1 mol/L phosphate buffer, pH 7.3, and postfixed in 2% OsO_4 in 0.1 mol/L phosphate buffer for 2 h. The tissue specimens were subsequently dehydrated and embedded. Thin sections were cut at 1 μm in thickness and stained with toluidine blue for light microscopy to select suitable areas for ultrathin sectioning. Ultrathin sections were cut at 70-80 nm, mounted onto copper grids, and stained with lead citrate for electron microscopy with a Philips Morgagni 261 EM microscope.

Statistical analysis

The data obtained were expressed as mean ± SD. Comparison between the two groups was performed by using the Student *t*-test, and the differences with $P < 0.05$ were considered as statistically significant. All data were analyzed with SPSS 13.0 software (SPSS Inc., Chicago, IL, United States).

RESULTS

Small intestinal propulsion rate

The small intestinal propulsion rate was significantly lower in the SAP group than in the S group. The respective rate was 68.66% ± 2.66% in the S group and 41.55% ± 3.85% in the SAP group.

Pathological changes

Under gross examination, the pancreas and jejunum in the SAP group appeared edematous at 24 h. The jejunum was full of yellow intestinal juice and ascites, and adhesions of organs were observed in 2 rats of the SAP group. Under light microscope examination, the pancreas from the S group exhibited no signs of pancreatitis (Figure 1A). Histological evaluation of the pancreas in rats with SAP revealed widespread acinar cell necrosis accompanied by edema, visible hemorrhage and inflammatory cell infiltrate (Figure 1B). In the S group, the structure of jejunum was normal (Figure 1C). In the SAP group, the mucosa was markedly denuded and partial loss of villous tissue with crypt layer infarction was also seen. Muscular layers showed slight alteration characterized by dilated thickness (Figure 1D).

Immunohistochemical staining

c-kit-positive cells could be divided into two cell populations. One population was mast cells situated within the mucosa layer (Figure 2A). The second population was ICC with large oval nuclei, sparse cytoplasm and branching processes; these cells were situated in the submuscular plexus (Figure 2B) and the intermuscular septa (Figure 2C). The density of c-kit-positive cells in the SAP group was significantly lower than in the S group; the respective mean densities were 88.47 ± 10.49 in the S group and 56.11 ± 7.09 in the SAP group.

Western blotting

Western blotting analysis using an antibody to c-kit on

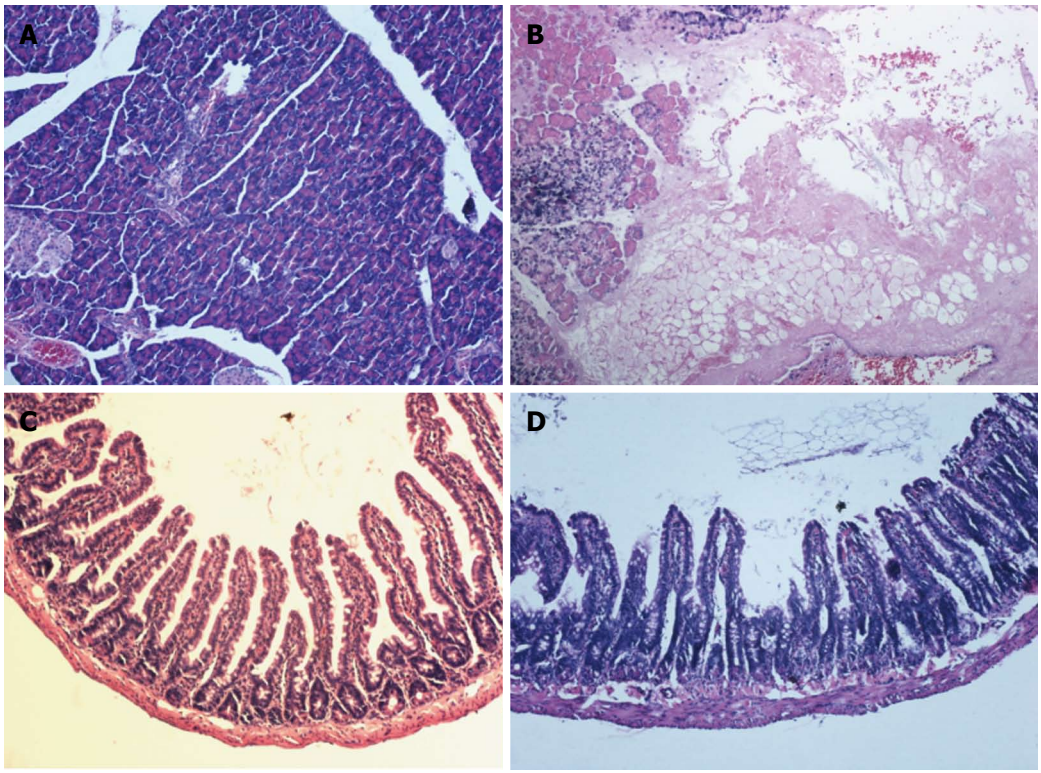

Figure 1 Histological sections from pancreas and jejunum. A: The pancreas of the sham (S) group shows a normal exocrine and endocrine pancreatic architecture; B: The pancreas of severe acute pancreatitis (SAP) rats shows necrosis of the acinar cells accompanied by edema and hemorrhage; C: The structure of jejunum in the S group is normal; D: The mucosa was markedly denuded and the muscular layer was edematous in the SAP group. Magnification × 200.

tissue from the jejunum detected a protein band at approximately 145 kDa that corresponded to the molecular weight of c-kit protein (Figure 3A). The c-kit band density was clearly observed in the S group rats, but significantly reduced in comparison to the SAP group (relative protein expression: the S group, 0.53 ± 0.06; the SAP group, 0.36 ± 0.04, $P < 0.05$; Figure 3B). Consistent with immunohistochemical staining, lower levels of c-kit protein were demonstrated in the SAP group.

c-kit mRNA expression

Decreased expression of c-kit mRNA was demonstrated compared with the S group (Figure 4).

Ultrastructure of ICC

As previously described, ICC in control tissue are present in triangular or fusiform shapes. The nucleus of ICC is very voluminous surrounded by a small perinuclear cytoplasm that expands with long prolongations which are called cytoplasmic processes. The cytoplasm of these cells presents a higher electron density than the cytoplasm of the surrounding muscle cells. ICC contain mitochondria, rough and smooth endoplasmic reticulum, thin and intermediate filaments, caveolae, Golgi apparatus, free ribosomes and cytoplasmic vesicles. They are closely associated with smooth muscle and often network with other ICC. Some of them are intercalated between nerves and smooth muscle cells (Figure 5A-C).

In contrast with control tissues, confluent vacuoles were frequently present in ICC in tissue from the SAP group (Figure 5D). Mitochondria appeared damaged in some vacuolated processes (Figure 5E). Ultrastructural preservation of other cellular elements and organelles was mostly unaffected. Damage of the desmosome-like junctions between ICC and smooth muscle was also seen (Figure 5F).

DISCUSSION

SAP is a very common clinical disease and its mortality rate ranges from 10% in the case of sterile necrosis to 25% in the case of infected pancreatic necrosis[18,19]. Many studies have indicated that gastrointestinal dysmotility in rats with SAP could lead to the translocation of bacteria from the gut, thus resulting in pancreatic infections which have been suggested to be a major cause of death in SAP[20,21]. So it is very important to investigate the possible mechanisms of gastrointestinal dysmotility in SAP in order to reduce the mortality rate of SAP.

We used retrograde injection of 5% sodium taurocholate from the duodenal papilla to establish an SAP rat model. Pancreatic pathological changes, such as pancreatic hemorrhage, necrosis and infiltration of inflammatory cells, could be observed at 24 h after modeling. All these changes were consistent with patients with SAP. This demonstrated that the animal model of SAP was successfully established. In addition, a significantly decreased small intestine propulsion rate in rats with SAP was observed. Our results confirmed that experimental SAP induced intestinal motility disturbances as previously shown[22].

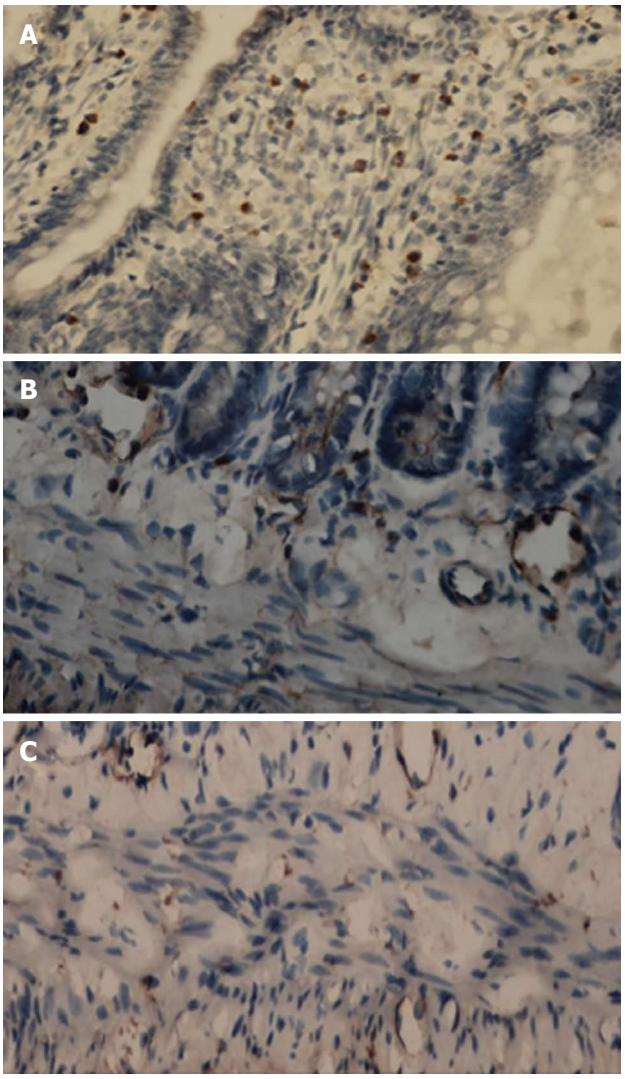

Figure 2 Immunohistochemistry for c-kit. A: Mucosal mast cells in rats retain c-kit positivity (internal control); B: c-kit-positive interstitial cells of Cajal (ICC) in the submuscular plexus in the sham group; C: c-kit-positive ICC in the intermuscular septa in the severe acute pancreatitis group. Magnification × 200.

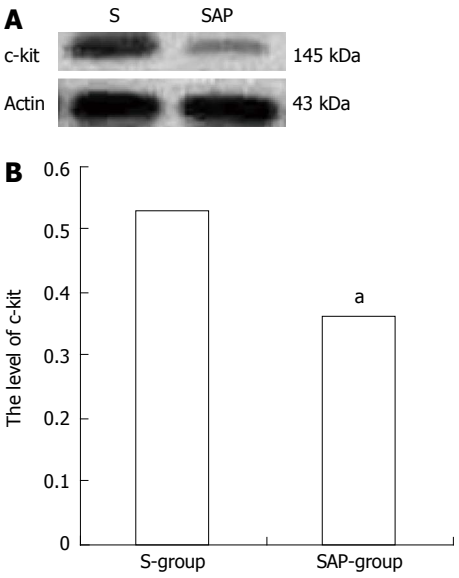

Figure 3 Expression of c-kit protein. A: Bands of Western blotting of c-kit (145 kDa). β-actin is a loading control; B: Statistical analysis of relative density of Western blotting between two groups. Data are represented as mean ± SD. [a]*P* < 0.05 *vs* sham (S) group.

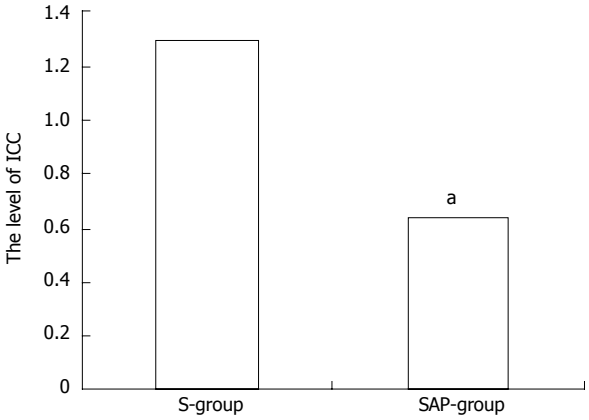

Figure 4 Mean optical density of c-kit mRNA. Each bar represents the mean ± SD (vertical line). [a]*P* < 0.05 *vs* sham (S) group. ICC: Interstitial cells of Cajal.

So far, the pathogenic mechanisms of pancreatitis-induced intestinal motility disturbances are largely unknown. It is well documented that ICC are implicated in the control of gastrointestinal motility. For example, decreased numbers or disrupted networks of ICC are associated with a number of human gastrointestinal motility disorders, including slow transit constipation[23,24], pseudo-obstruction[25-27] and diabetic enteropathy[28]. The potential role of ICC in the pathogenesis of gastrointestinal dysmotility in SAP has attracted attention. ICC can be classified into several subtypes according to their location in the gut wall; ICC at the level of the MY generates slow waves, and studies have confirmed that damage in the network of ICC-MY resulted in change of spontaneous mechanical contractions of the gut in a variety of human disease processes[29-31]. All these studies were focused on ICC-MY and spontaneous mechanical contractions. In addition to generating slow waves, other subsets of ICC are engaged in mediating enteric neural signals to the smooth muscles and acting as mechanosensors. In the present study, total ICC were observed by immunohistochemical staining. Around the submuscular plexus and in the intermuscular septa, we have demonstrated a decrease of c-kit-positive cells in these regions in the SAP group. Consistent with immunohistochemical staining, lower levels of c-kit protein were demonstrated in the SAP group.

Investigators have examined the ultrastructure of ICC by transmission electron microscopy in their intestinal obstruction model[30] and surgical resection model[31]. These findings all suggested that an actual change in ICC phenotype occurred from the ultrastructural appearance. Moreover, functionally mature ICC redifferentiated toward a smooth muscle cell phenotype when kit receptors were blocked[32]. Similarly, in our study, morphological changes such as vacuolation of mitochondria, irregular vacuoles and loosened desmosome-like junctions were

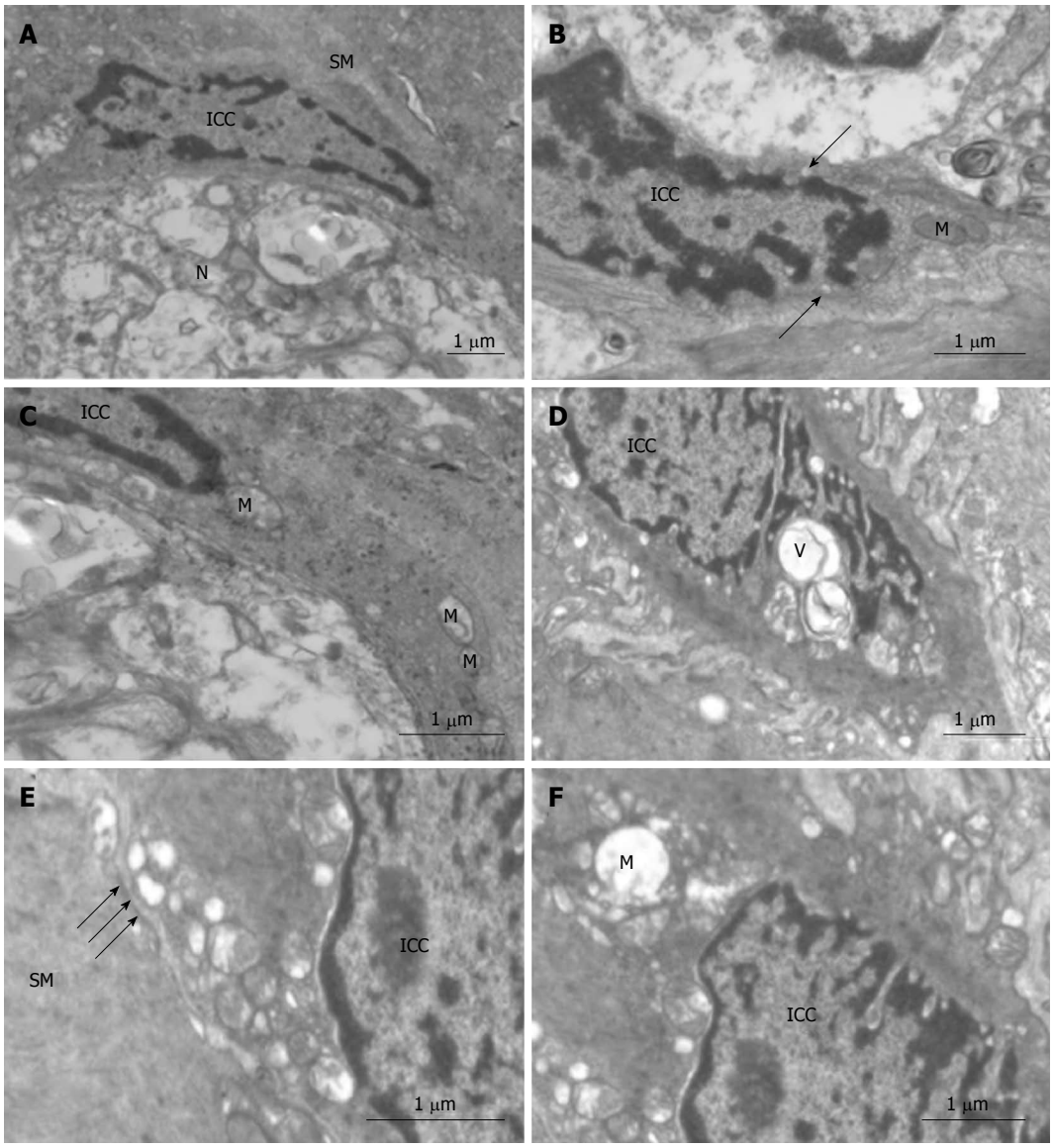

Figure 5 Ultrastructure of interstitial cells of Cajal. A-C: Control. A: Interstitial cells of Cajal (ICC) with fusiform nuclear morphology show an elongated nucleus with scarce perinuclear cytoplasm, and are situated between the smooth muscle (SM) and the enteric nerve (N). The cytoplasmic processes surround the external contours of the enteric nerve; B: Caveolae are lining cytoplasmic membrane (arrows); C: The processes of some ICC have characteristically numerous mitochondria (M); D-F: Severe acute pancreatitis. D: Vacuoles (V) are present in the ICC; E: The density of desmosome-like junction between ICC and smooth muscle is lower (arrows); F: Vacuolated mitochondria are present in the processes of ICC.

present in ICC in the SAP group, while the ultrastructure of ICC is normal in the S group. However, there is not sufficient evidence to support the theory that ICC transdifferentiate towards a smooth muscle cell phenotype. Although we did not investigate the amplitudes and frequencies of slow waves of the jejunum generated by ICC, it could be speculated that loss of ICC and changes of the ultrastructure influenced the function of ICC and eventually resulted in gastrointestinal dysmotility. The precise cellular changes that occur in response to the blockade of the c-kit signaling pathway are an extremely interesting direction for future investigation.

The present study further demonstrated that the expression of c-kit mRNA was significantly down-regulated in the SAP group. These data are consistent with previous reports that c-kit is down-regulated in the sigmoid colon of patients with slow transit constipation[33] and in the gallbladders of guinea pigs on a high cholesterol diet[34]. In the gastrointestinal tract, development and maintenance of the ICC phenotype have been linked to intracellular signaling *via* c-kit. Beckett *et al*[35] have shown that blocking c-kit signaling during late gestation results in failure of ICC networks and pacemaker function to develop in the murine small intestine. Other investigators have shown that blockade of c-kit signaling caused redifferentiation of functionally mature ICC toward a smooth muscle cell phenotype[32]. In the present study, we provide additional evidence that the c-kit signaling pathway may be responsible for development and maintenance of the ICC. However, further studies are needed to demonstrate whether and when these changes could be restored to normal.

In conclusion, this study has disclosed that decreased

c-kit-positive cells and degenerative ultrastructural changes of ICC were present in the jejunum of rats with SAP, and that all these changes resulted from blockade of the c-kit signaling pathway. This study may provide new insights into pathological mechanisms of gastrointestinal motility disturbances in SAP. Since loss and proliferation of c-kit-positive cells lead to a variety of human gastrointestinal motility disorders[36-38] and gastrointestinal stromal tumors[39,40], thus developing the means to manipulate the ICC phenotype may have profound therapeutic benefits for these patients.

COMMENTS

Background

The incidence of intestinal dysmotility increases the mortality of patients with severe acute pancreatitis (SAP), but until now, the mechanism of this dysmotility is largely unknown. Many studies have reported that interstitial cells of Cajal (ICC), which are known as pacemaker cells, are associated with gastrointestinal dysmotility diseases.

Research frontiers

Loss and proliferation of ICC lead to a variety of human gastrointestinal motility disorders and gastrointestinal stromal tumors. However, the detailed changes of ICC in SAP are not clearly elucidated. In this study, the authors demonstrate that the loss and ultrastructural changes of ICC could be a potential mechanism for intestinal dysmotility in SAP.

Innovations and breakthroughs

Recent reports have highlighted the importance of ICC in gastrointestinal motility disorders and gastrointestinal stromal tumors. In gastrointestinal motility disorders, loss of ICC was present. This is the first study to report that loss of ICC was also present in SAP. Furthermore, the studies would suggest that the loss of ICC may result from blockade of the c-kit signaling pathway.

Applications

This study provided new insights into pathological mechanisms of gastrointestinal motility disturbances in SAP. Developing the means to manipulate the ICC may have profound therapeutic benefits.

Terminology

ICC were firstly described by the Spanish neuro-histologist Cajal. ICC are involved in processes such as generation of slow waves, neurotransmission and regulation of mechanical activities; all these processes are thought to be crucial in intestinal motility. SAP is a special type of acute pancreatitis accounting for 10% to 20% of all acute pancreatitis episodes; it is a dangerous condition with more complications and higher mortality.

Peer review

The authors examined the expression of c-kit and ultrastructural changes of ICC in jejunum in rats with experimental severe acute pancreatitis. The study revealed that decreased c-kit positive cells and degenerative ultrastructural changes of ICC were present; these changes were correlated to blockade of c-kit signaling pathway. The results are interesting and may provide new insights into pathological mechanisms of gastrointestinal dysmotility in SAP.

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ORIGINAL ARTICLE

Comparative evaluation of intragastric bile acids and hepatobiliary scintigraphy in the diagnosis of duodenogastric reflux

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Abstract

AIM: To assess the diagnostic value of a combination of intragastric bile acids and hepatobiliary scintigraphy in the detection of duodenogastric reflux (DGR).

METHODS: The study contained 99 patients with DGR and 70 healthy volunteers who made up the control group. The diagnosis was based on the combination of several objective arguments: a long history of gastric symptoms (*i.e.*, nausea, epigastric pain, and/or bilious vomiting) poorly responsive to medical treatment, gastroesophageal reflux symptoms unresponsive to proton-pump inhibitors, gastritis on upper gastrointestinal (GI) endoscopy and/or at histology, presence of a bilious gastric lake at > 1 upper GI endoscopy, pathologic 24-h intragastric bile monitoring with the Bilitec device. Gastric juice was aspirated in the GI endoscopy and total bile acid (TBA), total bilirubin (TBIL) and direct bilirubin (DBIL) were tested in the clinical laboratory. Continuous data of gastric juice were compared between each group using the independent-samples Mann-Whitney *U*-test and their relationship was analysed by Spearman's rank correlation test and Fisher's linear discriminant analysis. Histopathology of DGR patients and 23 patients with chronic atrophic gastritis was compared by clinical pathologists. Using the Independent-samples Mann-Whitney *U*-test, DGR index (DGRi) was calculated in 28 patients of DGR group and 19 persons of control group who were subjected to hepatobiliary scintigraphy. Receiver operating characteristic curve was made to determine the sensitivity and specificity of these two methods in the diagnosis of DGR.

RESULTS: The group of patients with DGR showed a statistically higher prevalence of epigastric pain in comparison with control group. There was no significant difference between the histology of gastric mucosa with atrophic gastritis and duodenogastric reflux. The bile acid levels of DGR patients were significantly higher than the control values (Z: TBA: -8.916, DBIL: -3.914, TBIL: -6.197, all $P < 0.001$). Two of three in the DGR group have a significantly associated with each other (r: TBA/DBIL: 0.362, TBA/TBIL: 0.470, DBIL/TBIL: 0.737, all $P < 0.001$). The Fisher's discriminant function is followed: Con: Y = 0.002TBA + 0.048DBIL + 0.032TBIL - 0.986; Reflux: Y = 0.012TBA + 0.076DBIL + 0.089TBIL - 2.614. Eighty-four point zero five percent of original grouped cases were correctly classified by this method. With respect to the DGR group, DGRi were higher than those in the control group with statistically significant differences (Z = -5.224, $P < 0.001$). Twenty eight patients (59.6%) were deemed to be duodenogastric reflux positive by endoscopy, as compared to 37 patients (78.7%) by hepatobiliary scintigraphy.

CONCLUSION: The integrated use of intragastric bile acid examination and scintigraphy can greatly improve the sensitivity and specificity of the diagnosis of DGR.

Key words: Duodenogastric reflux; Diagnosis; Intragastric bile acids; Hepatobiliary scintigraphy

Core tip: The study results suggest that total bile acid is the most important factor of the bile acids to determine duodenogastric reflux (DGR) by using a variety of statistical methods. Using the receiver operator curve, we found the hepatobiliary scintigraphy is better than the examination of gastric juice in the diagnosis of DGR. From this study, the biggest revelation is that we can research other medical problems particularly using many statistical methods.

Chen TF, Yadav PK, Wu RJ, Yu WH, Liu CQ, Lin H, Liu ZJ. Comparative evaluation of intragastric bile acids and hepatobiliary scintigraphy in the diagnosis of duodenogastric reflux. *World J Gastroenterol* 2013; 19(14): 2187-2196 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i14/2187.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i14.2187

INTRODUCTION

Duodenogastric reflux (DGR) is a natural physiological phenomenon which is commonly defined as the transport of duodenal contents from the duodenum to the stomach[1]. Chernov *et al*[2] concluded that DGR was involved in the formation of the internal gastric environment, which played a significant role in gastric digestion and that its regulation was affected by the coordinated motor and evacuated performance of the gastroduodenal junction and duodenum. Duodenal fluid causes an increase in inflammatory cells in the gastric mucosa, decrease in parietal cells, hyperplasia of mucous cells and changes in glandular morphology. Patients with DGR will feel heartburn, nocturnal cough and chest pain, nausea, epigastric pain, gassy or bloating feelings, vomiting and so on. DGR has been implicated in the pathogenesis of a variety of upper gastrointestinal disorders including esophagitis, gastritis, duodenal and gastric ulcers[3].

With the increasing number of research in this field, reliable, repeatable and simple methods of assessment of DGR are required, especially in the early stage. Earlier used techniques included radiology, endoscopy and intubation methods such as nasogastric aspiration of bile marker or the measurement of bile acids in fasting gastric aspirates. At present, several methods are available to detect duodenogastric reflux in general hospital. For example, intubation of the upper gastrointestinal tract is the essential method to assess the extent and severity of tissue damage of duodenogastric reflux disease in our daily work. This intubation should be gentle because it may causes disturbances in gastric and duodenal motility. The conventional and most widely accepted method of diagnosing DGR is the measurement of intragastric bile acid in the gastric juice aspirated through nasogastric tube and hepatobiliary scintigraphy. In the last few years, scintigraphic radiological techniques, such as imaging with hepatobiliary scintigraphy, has become available to study dynamic duodenogastric reflux[4-6], but they also have limitations[7,8].

The aim of this study was to represent the visualization of endoscopy, to measure intragastric bile acids aspirated at endoscopy and to compare them with DGR index (DGRi) assessed by hepatobiliary scintigraphy to assess the sensitivity and specificity of these two techniques in the diagnosis of DGR.

MATERIALS AND METHODS

Patients and methods

A total of 99 patients (41 male and 58 female) with DGR were undergoing esophagogastroduodenoscopy (EGD) from September 2011 to March 2012 at Shanghai Tenth People's Hospital, Tongji University. The diagnosis of DGR was based on the combination of the following arguments: a long history of gastric symptoms poorly responsive to prokinetics, mucosa-protective medicines, H_2-blockers and/or proton-pump inhibitors (PPI), gastroesophageal reflux symptoms unresponsive to PPI, gastritis on upper GI endoscopy, and/or at histology, presence of a large amount of bile in the gastric cavity at > 1 endoscopic examination, pathologic at 24-h intragastric bile monitoring with the Bilitec device. The gastric juice was often lucidity or light yellow-green and/or associated mucosal change in these patients' endoscopic images. Before investigation, all patients were interviewed by the senior author for the presence of both upper abdominal symptoms (heartburn, regurgitation, nocturnal cough and chest pain) and dyspeptic symptoms (nausea, epigastric pain, gassy or bloating feelings, vomiting). None of the patients had diabetes mellitus, neurological disorders, vascular diseases, collagen diseases, neoplastic diseases or inflammatory bowel disease. Acute cases and patients who had previously undergone gastrectomy or esophagotomy were excluded.

As a control group, 70 consecutive patients (35 male and 35 female) who needed EGD for an annual medical check-up were enrolled. None had undergone earlier esophageal, gastric or biliary surgery; and none had earlier gastrointestinal diseases or was on medication which would influence gastric acidity or motility. After this, all patients underwent upper gastrointestinal endoscopy and found gastric juice was normal and the gastric mucosa was not damaged obviously under the macroscopic observation.

The protocol of this study was approved by the ethics committee of the Shanghai Tenth People's Hospital. Written informed consent was obtained from all participants.

Endoscopic study

Endoscopic examination was performed to find the evidence of DGR in all patients using fiber optic gastroduodenoscopy (The GIF-H260 and Q260 endoscopes, Olympus Medical Systems Co., Tokyo, Japan). To ensure the most accurate results possibly, every patient was not taken any food or drink for 8-10 h before examination to allow a valid examination of the upper gastric intestinal (GI) tract and to lower the risk of vomiting.

The doctor explained the test to everyone, including the possibility of biopsy and risks such as the need to remove polyps or other surgical procedures and asked to sign a consent form agreeing to the procedure. At the same time, all the participants informed the endoscopy team about any medications he/she was taking and any allergy or bad reactions in previous tests. People who have had cardiac valve replacement or blood vessel graft suggested to continue medications to prevent infection. All dentures and eyeglasses prior to begin an upper endoscopy were removed. Each of the subjects was given a topical anesthetic before the test to numb his/her throat to prevent gagging. The patient was placed on his/her left side and had a plastic mouthpiece placed between his teeth to keep his mouth opening that makes easier to pass the tube. The doctor lubricated the endoscope, passed it through the mouthpiece, and then asked him to swallow it. The doctor guided the endoscope under direct visualization through his esophagus to the first part of small intestine (duodenum). Any saliva was cleared using a small suction tube that was removed quickly and easily after the test.

The doctor inspected portions of the linings of everyone's esophagus, stomach, and the first part of small intestine and then re-inspects them as the instrument is withdrawn. To determine the presence and severity of DGR, biopsies of gastric inflammation was necessary to be performed in the antrum of the stomach. All endoscopic examinations were done by well-trained endoscopists, and three expert endoscopists examined the endoscopy photographs to determine whether the attending endoscopists had diagnosed accurately. The endoscopic diagnosis was established by consensus of two or three expert endoscopists and the attending endoscopist.

Histopathology

Biopsy samples, no less than four sequential sections, were taken from the inflammatory mucosa for each enrolled patient. Mucosal erythema, erosion or ulcerations of the gastric wall were usually considered signs of gastric inflammation. Biopsy specimens were immediately placed in a 10% buffered formalin solution, routinely processed, and embedded in paraffin in the department of Pathology. Two sections were stained with hematoxylin and eosin (HE). At the same time, 23 patients with chronic atrophic gastritis were reviewed for comparison. The estimation of inflammatory was made only when the biopsy specimen consisted of intestinal columnar epithelial cells with goblet cells. All biopsy examinations were done by well-trained clinical pathologists and the pathological diagnosis was established by consensus of two or three expert pathologists.

Determination of bile acids in gastric juice

For all patients, resting gastric juice was aspirated through a sterile wash tube inserted down the biopsy channel of the gastroscope. The gastric aspirate was stored at -20°C until batch analysis. The concentration of free and total bile acid was made by the steroid dehydrogenase method (Modular P800, Hoffmann-La Roche Ltd, Basel, Switzerland), performed in duplicate with a mean coefficient of variation of 5% for each patient. The mean overall percentage recovery was 89 percent and the variance was less than 10 percent in duplicate analyses. In the present study, three bile acids were analyzed in accordance with the clinical processes: total bile acid (TBA), total bilirubin (TBIL) and direct bilirubin (DBIL).

Duodenogastric reflux imaging

Twenty eight patients of DGR group and 19 persons of control group were subjected to hepatobiliary scintigraphy for the diagnosis of DGR. ^{99m}Tc-ethyl hepatic iminodiacetic acid (EHIDA) imaging was performed using single-photon emission-computed tomography (SPE-CT)/CT (PHILIPS Precedence 16 SPE-CT/CT, Koninklijke Philips Electronics NV, The Netherlands) in accordance with our institution's standard protocol. Stress and rest images were acquired 1 h after injecting 111-185 MBq (3-5mCi) of technetium 99m ethyl hepatic iminodiacetic acid, [^{99m}Tc(CO)$_3$(EHIDA)]$^-$.

Patients were in fasting, non-smoking for 4-12 h and oral potassium perchlorate 400 mg was taken to close the thyroid function before examination. DGR was studied scintigraphically using a modified and extended version of the conventional hepatobiliary scintigraphy. The study was conducted with the patient in the supine position and the gamma camera detector placed above the patients' abdomen. About 111-185 MBq [^{99m}Tc(CO)$_3$(EHIDA)]$^-$ (^{99m}Tc-EHIDA) was injected intravenously. Gallbladder contraction was then stimulated by a fatty meal and/or intravenous cholecystokinin (1-5 units/kg). SPECT was performed by acquiring 32 projections over 180° (from 45°RAO to 45°LPO) on a circular, 400-mm field of view gamma camera. Serial images of the liver and hepatobiliary system were obtained at every 5 min up to one hour, followed by imaging at every 10 min for the next two hours. At the end of the study, 20-40 MBq ^{99m}Tc-EHIDA was given orally to confirm the location of the stomach if necessary.

In this research, the films of all participants, showing both SPECT and planar projection image, were evaluated retrospectively by two nuclear consultant radiologists working together. Scans were scored as positive for DGR only if the two physicians agreed on the presence of DGR. Retrograde movement of radioactivity from the duodenum into the stomach was considered abnormal and diagnostic of DGR. DGRi was calculated to estimate

Table 1 Comparison of demographic and clinical characeristics of duodenogastric reflux group and control group

	DGR group (n = 99)	Control group (n = 70)
Age (mean ± SD)	48.6 ± 16.2	50.1 ± 13.2
Gender (male/female)	41/58	35/35
Epigastric pain (yes)	72.7%[1]	17.10%
Nausea/vomit (yes)	20.2%[1]	7.10%
Bitter taste (yes)	31.3%[1]	4.30%
Sour regurgitation (yes)	23.2%[1]	8.60%
Retrosternal pain (yes)	18.2%[1]	1.40%
Anorexia (yes)	26.30%	10.00%

[1]Statistically significant differences ($P < 0.05$). DGR: Duodenogastric reflux.

the severity of DGR, following the formula:

$$\text{DGRi}(\%) = \frac{\text{Supreme count rate in the stomach}}{\text{Intrahepatic supreme count rate}} \times 100\%$$

Statistical analysis

All statistical analyses were performed using Statistical Analysis Software IBM SPSS Statistics 20 (Chicago, IL, United States). The significance level was set at 0.05 for all statistical tests. Values are expressed as mean ± SD or stand error of mean. Continuous data of gastric juice and DGRi were using the Independent-samples Mann-Whitney *U*-test between DGR and control group. The relationship among the TBA, DBIL and TBIL of DGR group was analysed by Spearman's rank correlation test and Fisher's linear discriminant analysis. The comparison between intragastric bile acids and hepatobiliary scintigraphy in the diagnosis of DGR was demonstrated by receiver operating characteristic (ROC) curve.

RESULTS

Characteristics of enrolled patients

Characteristics of the enrolled patients are shown in Table 1. The group of patients with DGR was 41 males and 58 females, with a mean ± SD age of 48.62 ± 16.20 years (95%CI: 45.39-51.85). The group of patients without DGR was 35 males and 35 females, with a mean ± SD age of 50.16 ± 13.23 years (95%CI: 47.00-53.31). The group of patients with DGR showed a statistically higher prevalence of epigastric pain in comparison with that without DGR.

Endoscopic study and histopathology

The images of patients which were got in the endoscopic examination were revealed in Figure 1. The gastric juice of DGR patients was lucidity or light yellow-green and/or associated mucosal changes. Pathologically the reflux was associated with infiltration of mononuclear leukocytes, neutrophilic granulocytes, and eosinophilic granulocytes and with foveolar hyperplasia in the gastric mucosa. Our results suggest that postprandial duodenogastric bile reflux is characterized by superficial inflammatory changes in the gastric mucosa. Reviewed with past recording, there is no significant difference between atrophic gastritis and duodenogastric reflux (Figure 2).

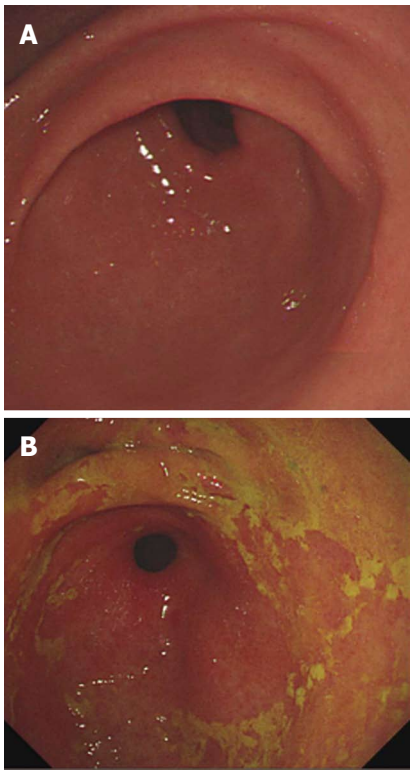

Figure 1 Comparison between the review of endoscopic evaluation control group (A) and duodenogastric reflux group (B). Compared with control group, the gastric mucous paste of duodenogastric reflux patient is usually yellow or green and has bile dyeing like islands.

Determination of bile acids in gastric juice

Gastric juice was successfully collected from all enrolled patients, and the concentration of bile acids in gastric juice was measured in the clinical laboratory. Analysis of the gastric aspirates was described in the Table 2, Figure 3. The bile acids levels of DGR patients were significantly higher than the control values (Z: TBA: -8.916, DBIL: -3.914, TBIL: -6.197, all $P < 0.001$). Using Nonparametric correlations, two of three in the DGR group have a significantly associated with each other (r: TBA/DBIL: 0.362, TBA/TBIL: 0.470, DBIL/TBIL: 0.737, all $P < 0.001$). Using the Fisher's linear discriminant analysis, we found the canonical correlation is 0.631 ($P < 0.001$). The standardized canonical discriminant function coefficient of TBA, DBIL and TBIL is individually 0.899, 0.084 and 0.152, from which we found TBA is the most important factor in the diagnosis of DGR in the examination of gastric juice. The Fisher's discriminant function is followed: Con: Y = 0.002TBA + 0.048DBIL + 0.032TBIL - 0.986; Reflux: Y = 0.012TBA + 0.076DBIL + 0.089TBIL - 2.614. Eighty-four point zero five percent of original grouped cases were correctly classified by this method. In other words, the result of endoscopy and gastric juice biochemistry detection were consistent more than 80%

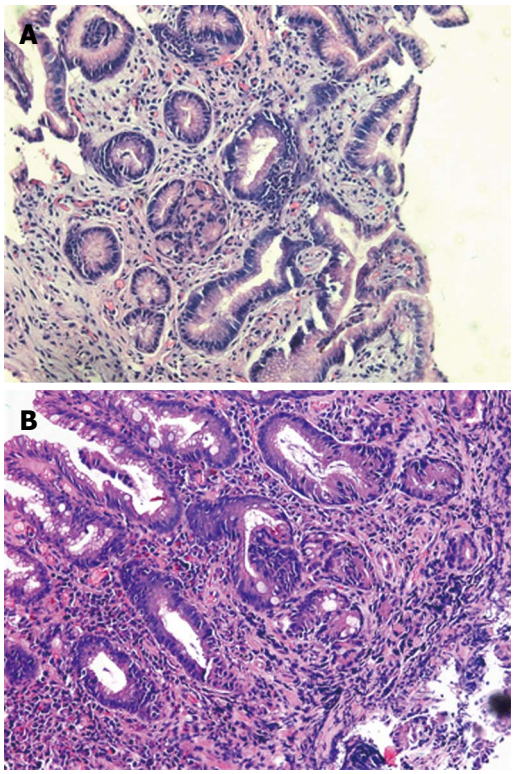

Figure 2 Representative hematoxylin and eosin staining of gastric tissue from chronic atrophic gastritis (A) and duodenogastric reflux (B). Isolated metaplasia of glandular epithelium and mild inflammation of the lamina propria was found in the tissue of duodenogastric reflux patients (original magnification, × 200).

Table 2 Results of gastric juice analyses between duodenogastric reflux group and control group

	Type	mean ± SD	Range	95%CI	Z	Sig.
TBA	Con	44.51 ± 56.53	0.80-235.80	31.05-58.01	-8.916	0.000
	Reflux	263.64 ± 171.61	0.70-660.50	229.41-297.87		
DBIL	Con	1.87 ± 1.85	0.00-8.90	1.43-2.31	-3.914	0.000
	Reflux	5.43 ± 6.12	0.00-23.70	4.20-6.65		
TBIL	Con	1.63 ± 1.34	0.10-7.40	1.31-1.94	-6.197	0.000
	Reflux	5.49 ± 5.51	0.30-21.90	4.39-6.59		

Sig.: Asymp. Sig. (2-tailed) or exact Sig.; TBA: Total bile acid; TBIL: Total bilirubin; DBIL: Direct bilirubin.

by this method. The sensitivity and the specificity is separately 83.8% and 84.3%.

Duodenogastric reflux imaging

When hepatobiliary scintigraphy was administered by constant intravenous infusion it resulted in an increased elimination in bile for the first 80-100 min, and the concentration in bile then remained relatively constant for the rest of the test. Normally no increase in radioactivity in the stomach can be recorded, while the local radioactivity of the stomach increased during the investigation in DGR patients (Figure 4). The DGRi of DGR group were higher than those of the control group significantly (Z = -5.224, P < 0.001) (Figure 5). Twenty eight patients (59.6%) were deemed to be duodenogastric reflux positive by endoscopy, as compared to 37 patients (78.7%) by hepatobiliary scintigraphy. In this study, we also found some patients who were not determined with DGR by endoscopy were found the clue of duodenogastric reflux in the hepatobiliary scintigraphy. Furthermore, 11 patients were evaluated twice by the hepatobiliary scintigraphy at intervals ranging from 3-14 d. The result was identical in 8 patients, from which it indicates the good reproducibility of the test.

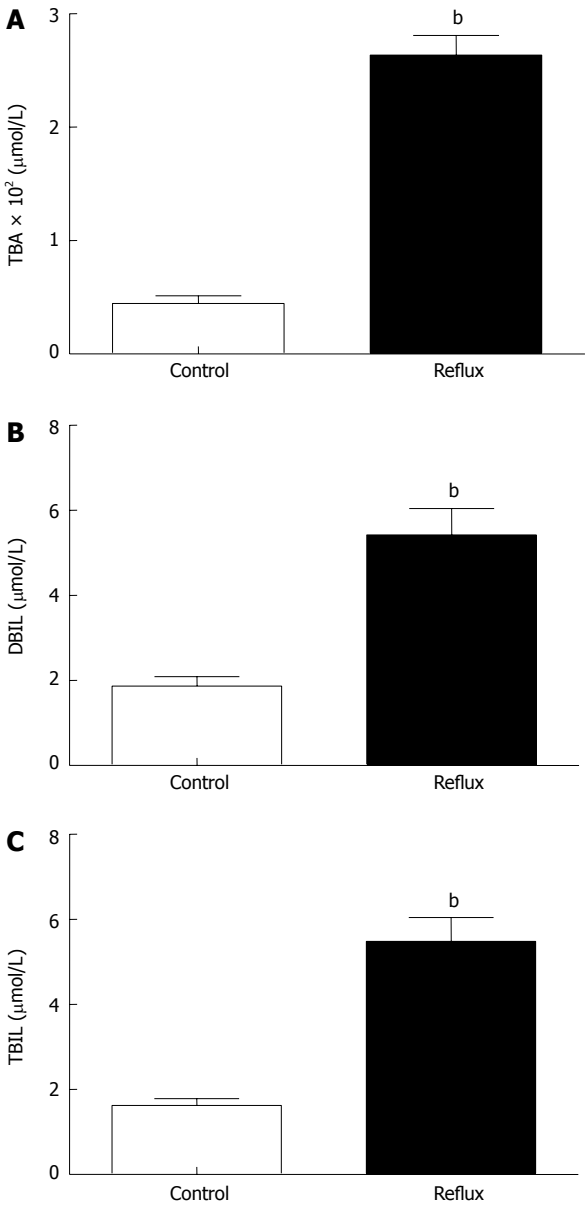

Figure 3 Intragastric concentrations of total bile acid (A), direct bilirubin (B) and total bilirubin (C) aspirated in endoscopy examination in duodenogastric reflux group and control group. Patients with duodenogastric reflux had a significantly higher total bile acid (TBA), total bilirubin (TBIL) and direct bilirubin (DBIL) compared to controls (^bP < 0.001 *vs* control group). Data are expressed as mean ± SE and difference was calculated using the independent-samples Mann-Whitney *U*-test.

DISCUSSION

DGR is a natural physiological phenomenon often oc-

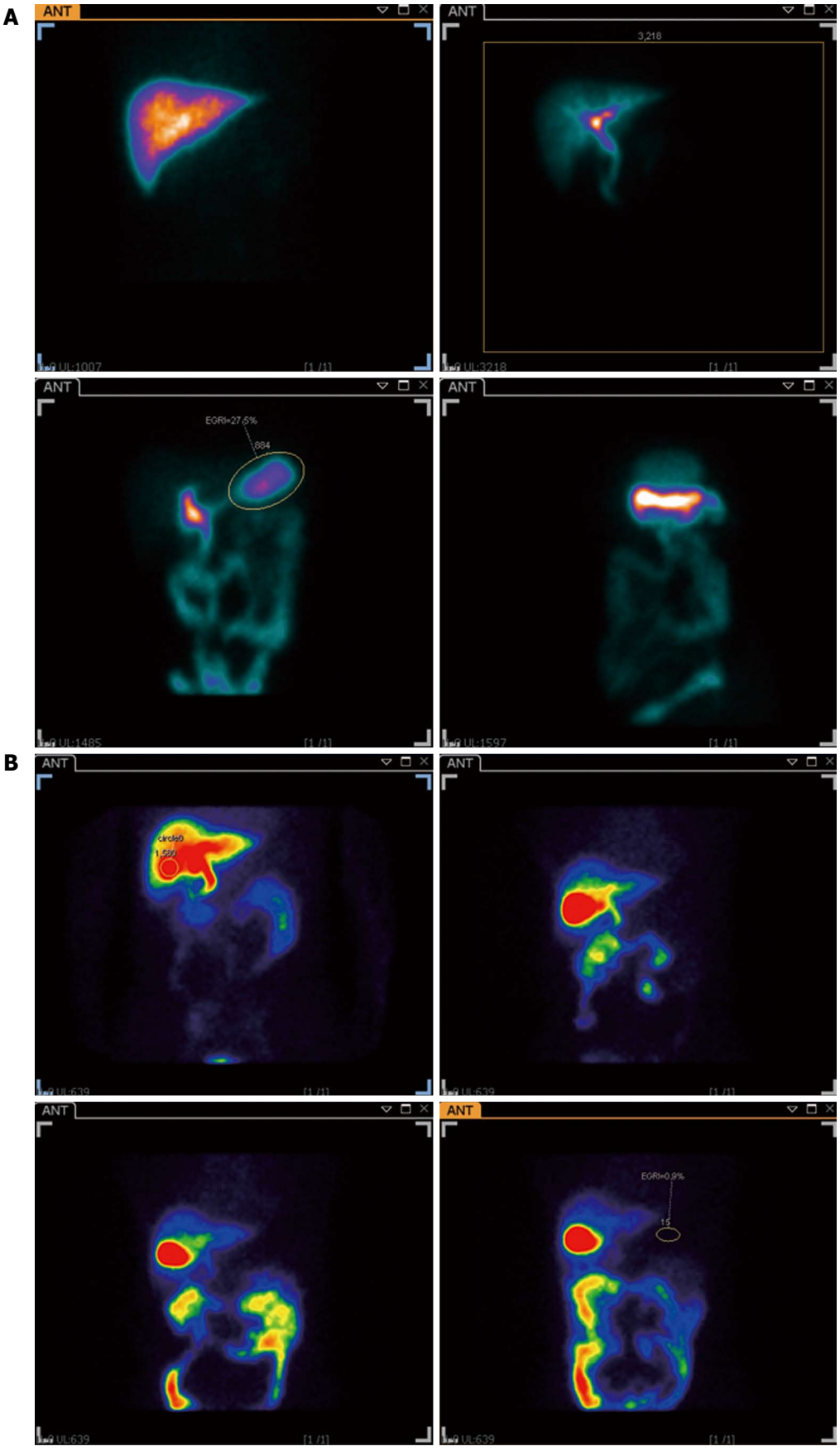

Figure 4 Examination of duodenogastric reflux by ^{99m}Tc-ethyl hepatic iminodiacetic acid test. A: One episode of duodenogastric reflux, of which the duodenogastric reflux index is 27.5%, is shown in the gastric localization (yellow circle) in the third image; B: A normal study in which no reflux is seen in the gastric region (yellow circle) outlined in the last picture.

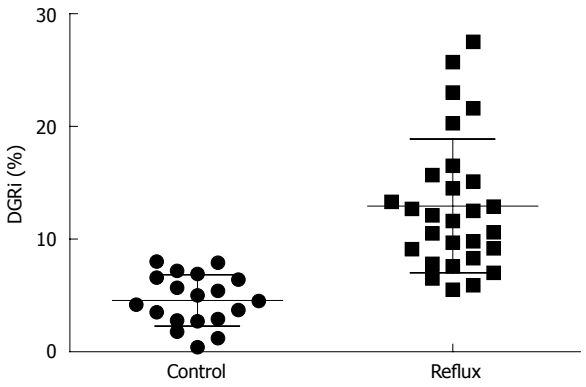

Figure 5 Comparison of duodenogastric reflux group and control group in the scintigraphy. The reflux rates of duodenogastric reflux group in the patients were higher than those in the control group with statistically significant differences (Z = -5.224, P < 0.001). Data was calculated using the independent-samples Mann-Whitney U-test. DGRi: Duodenogastric reflux index.

curring during the early hours of the morning and postprandial period[9]. It is commonly understood to mean the passing into the stomach of duodenal fluid containing secretions from the intestinal mucosa, bile and pancreatic fluid[10]. The prevalence of upper gastrointestinal symptoms and frequency of established diagnosis of upper gastrointestinal disease is greatest for the patients with marked DGR, being approximately twice that of patients without evidence of DGR[11]. For over a century DGR has been considered the main cause of the primary or secondary alkaline gastritis and plays the basic role in the pathogenesis of gastritis and other GI tract diseases (reflux oesophagitis, gastric ulcer, progressing metaplasia or oesophageal and gastric cancer). In the previous researches, DGR occurred in 30% to 40% of adult patients presenting with acid reflux esophagitis or gastroesophageal reflux disease[12,13]. It is common even in asymptomatic subjects, especially in gastric and duodenal ulcer patients, gastric surgery, gallstone patients, patients undergoing gallbladder operations and cases of chronic pulmonary disease. DGR is a physiologic event, but also that the pathologic presence of duodenal juice in the foregut lumen may account for the development of Barrett's metaplasia and dysplasia[14,15], and for that of gastric polyps[16], as well. Excessive DGR has been associated with the development of antral gastritis, gastric ulcers, alkaline esophagitis, esophageal or gastric adenocarcinoma, and intestinal metaplasia of the gastric mucosa[17-20]. Gastric mucosal damage induces mast cell degranulation and a release of vasoactive mediators, such as histamine, leading to vascular congestion and lamina propria edema[21]. Accurate detection of DGR has been a major problem for many years. The exact pathogenic features of bile reflux in unoperated stomach as well as its contributions to gastric mucosal lesions in chronic gastritis are still remaining unrevealed[22]. The clinical diagnosis of excessive DGR is usually based on endoscopic observation of bile reflux found in the stomach, antral gastritis or ulceration, or the histologic documentation of foveolar hyperplasia, vascular congestion, lamina propria edema or chemical gastritis[23-25].

The various techniques employed to detect DGR are endoscopy gastroduodenal intubation and direct sampling, gastric pH monitoring, ambulatory gastric bilirubin monitoring and hepatobiliary scintigraphy. Among them, the use of the intubation technique is considered non-physiologic since it is invasive and thereby may spuriously provoke reflux. Gastric pH monitoring is cumbersome, entails the use of sophisticated instruments and is uncomfortable for the patients. Scintigraphic documentation of DGR is technically easy, simple and physiologic as it is noninvasive[3]. Bilitec method reliably identified the presence of bilirubin and it has made feasible to quantitatively detect duodenogastroesophageal reflux of bile[26]. Just *et al*[27] showed that there was no correlation between an alkaline pH and the presence of bilirubin. Due to methodological discrepancies, research into the significance of duodenogastric reflux in the diagnosis of DGR has yielded varying results. Combined with past researches and practice, we think the diagnosis of DGR is still based on the systematic analysis of endoscopy, gastric fluid samples obtained by intubation and hepatobiliary scintigraphy, a more physiological, non-invasive method.

Endoscopy is one of the principal means of studying upper abdominal complaints for routine clinical purposes and is considered as a minimally invasive procedure, since it does not require an incision into one of the major body cavities and does not require any significant recovery after the procedure (unless sedation or anesthesia has been used). Stein *et al*[28] reported that upper gastrointestinal endoscopy had lower accuracy and predictive value than scintigraphy or gastric pH monitoring in the assessment of DGR. We can find duodenogastric reflux under direct visualization in our daily clinical work. But this is only a temporary phenomenon for the most part, and not on behalf of the patient's disease status. The chief source of error in this technique is the possible effect of the intubation in either promoting or hindering reflux. Therefore, endoscopic findings only give us an intuitive, subjectivity evidence of the bile reflux and the test is largely a qualitative one.

In addition to the observation of DGR situation, we did pathological examinations during the routine gastroscopy examination. It has been demonstrated in animal experiments that duodenal fluid caused an increase in inflammatory cells in the gastric mucosa, a decrease in parietal cells, a hyperplasia of mucous cells and changes in glandular morphology. The important factor is the antrum which serves to protect the mucosa of the gastric body from the toxic effects of DGR. In our research, we found the atrophic gastritis was very common in patients with severe reflux in endoscopy, and we didn't found the histopathology significantly different between DGR and atrophic gastritis, which was consistent with the previously research[29].

The assessment of gastric fluid, an important work in the endoscopic progress, is another important impact in the diagnosis of DGR. The surfactant effect of bile acids

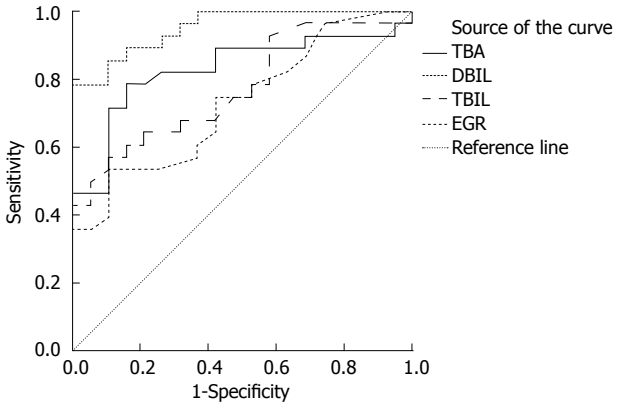

Figure 6 Receiver operator curve for ^{99m}Tc-ethyl hepatic iminodiacetic acid test and gastric juice analyses in the diagnosis of duodenogastric reflux. Area under the curve was of 0.953 for ethyl hepatic iminodiacetic acid scintigraphy (P < 0.001, 95%CI: 0.901-1.000), 0.830 for total bile acid (TBA) (P < 0.001, 95%CI: 0.709-0.950), 0.722 for direct bilirubin (DBIL) (P = 0.008, 95%CI: 0.587-0.872) and 0.773 for total bilirubin (TBIL) (P = 0.002, 95%CI: 0.642-0.905).

is closely related to their hydrophobic-hydrophilic balance. Bile acids have a surfactant effect for lipid absorption[30], and they may have a cytotoxic action if the surfactant effect is too strong[31,32]. Indeed, Heuman reported that the hydrophilic-hydrophobic balance of bile acids correlates with their toxicity, and increasing hydrophobicity was associated with increasing cytotoxicity towards the gastrointestinal epithelium[33]. Therefore, the bile acids may also have some roles in the formation of duodenogastric gastritis and in the diagnosis of DGR. In our study, we found there was a good correlation between TBA and DBIL, TBA and TBIL, DBIL and TBIL in DGR group. When we used Fisher's linear discriminant analysis to analyze the three indexes in the determination of DGR, we found TBA was the most important factor in the diagnosis and created two formats to discriminant the diagnosis of DGR. The consistency between the direct vision of endoscopy and gastric juice examination was nearly 84%. By this method, the sensitivity and the specificity was separately 83.8% and 84.3% and this is the first time that we used this method to determine DGR.

Hepatobiliary scintigraphy, using ^{99m}Tc-EHIDA derivatives, is superior to upper gastrointestinal endoscopy in the detection of DGR and also has the advantage of being non-invasive and physiological. A hepatobiliary tracer is injected intravenously and ^{99m}Tc-EHIDA excreted through the liver into the biliary tract and further into the duodenum in cholescintigraphy. When DGR happened, ^{99m}Tc-EHIDA passes into the duodenum and via reflux into the stomach. About 60% (28/47) of the isotope dose was secreted into the bile in 1.5 h. In the past researches, a good correlation was shown between the severity of mucosal changes on histology and the presence of DGR on scintigraphy[34,35]. The present study not only confirms this sensitive method for the diagnosis of DGR, but also proves its superiority over intragastric bile acids estimation (Figure 6). When we used ROC curve, we found the hepatobiliary scintigraphy was better than the examination of gastric juice (Figure 6). This means the hepatobiliary scintigraphy has better sensitivity and specificity. From the statistical analysis, we also found TBA was the most important factor of bile acids to determinate the diagnosis of DGR, which was in accordance with the result of the standardized canonical discriminant function coefficient of TBA. But the method gave no information on the nature of the reflux fluid, *i.e.*, the substances neither contained in bile, nor did it measure anything more than bile reflux. It is well accepted that hepatobiliary scintigraphy recorded only a relatively short period. Being noninvasive, physiological and good repeatability, hepatobiliary scintigraphy appears suitable for routine clinical use in the diagnosis of DGR[36,37].

All in all, the results of endoscopy, discriminant function of intragastric bile acid examination and scintigraphy were correlated with the final diagnosis of DGR. Integrated use of these three methods will help improve the accuracy of diagnosis of DGR.

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COMMENTS

Background

Duodenogastric reflux (DGR) is a natural physiological phenomenon often occurring during the early hours of the morning and postprandial period, which is commonly understood to mean the passing into the stomach of duodenal fluid containing secretions from the intestinal mucosa, bile and pancreatic fluid. Earlier used techniques employed to detect DGR included gastroduodenal intubation and direct sampling, gastric pH monitoring, endoscopy, gastric mucosal biopsy and hepatobiliary scintigraphy, but every method has its limit in the diagnosis of DGR.

Research frontiers

Gastric pH monitoring is cumbersome, entails the use of sophisticated instruments and is uncomfortable for the patients. Bilitec method reliably identified the presence of bilirubin and it has made feasible to quantitatively detect duodenogastroesophageal reflux of bile. Due to methodological discrepancies, research into the significance of duodenogastric reflux in the diagnosis of DGR has yielded varying results.

Innovations and breakthroughs

This is the first time that we used the Fisher's linear discriminant analysis to determine the bile acids in gastric juice and found total bile acid is the most important factor in the diagnosis of DGR. Using the Receiver operator curve, authors found the hepatobiliary scintigraphy is better than the examination of gastric juice.

Applications

By understanding the advantages and disadvantages of intragastric bile acids and scintigraphy, this study demonstrates the hepatobiliary scintigraphy have better sensitivity and specificity than intragastric bile acids in the diagnosis of DGR and the integrated use of these two methods can greatly improve the accuracy and sensitivity of the diagnosis of DGR.

Terminology

Hepatobiliary scintigraphy is a radionuclide diagnostic imaging study that evaluates hepatocellular function and patency of the biliary system by tracing

the production and flow of bile from the liver through the biliary system into the small intestine. Sequential images of the liver, biliary tree and gut are obtained. Computer acquisition and analysis as well as pharmacological interventions are frequently employed.

Peer review

Many reports evaluate duodenogastric reflux with endoscopic examination or gastric juice examination. Hepatobiliary scintigraphy can check objectively dynamic duodenogastric reflux and is no invasive method. This report results hepatobiliary scintigraphy is a useful method for evaluating duodenogastric reflux and help improve the accuracy of diagnosis of duodenogastric reflux with integrated use of endoscopy and intragastric bile examination.

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ORIGINAL ARTICLE

miRNA-338-3p suppresses cell growth of human colorectal carcinoma by targeting smoothened

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Abstract

AIM: To investigate the regulative effect of miRNA-338-3p (miR-338-3p) on cell growth in colorectal carcinoma (CRC).

METHODS: The lentiviral vector pLV-THM-miR-338-3p and pLV-THM-miR-338-3p-inhibitor were constructed. The recombinant viral vector encoding the pre-miR-338-3p or miR-338-3p-inhibitor and the two packaging plasmids psPAX2 and pMD2.G were cotransfected into human embryonic kidney 293T cells to package lentivirus. The supernatant containing the lentivirus particles was harvested to determine the viral titer, and this supernatant was then used to transduce CRC-derived cell line, SW-620. Flow cytometry was utilized for sorting the green fluorescent protein $(GFP)^+$ cells to establish the SW-620 cell line stably expressing pre-miR-338-3p or miR-338-3p-inhibitor. Moreover, the expression of miR-338-3p was determined by real-time reverse transcriptase polymerase chain reaction, and Western blotting was used to detect the expression of the smoothened (SMO, the possible target of miR-338-3p) protein in SW-620 cells. Furthermore, the status of CRC cell proliferation and apoptosis were detected by 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide assay and flow cytometry, respectively.

RESULTS: Restriction enzyme digestion and DNA sequencing demonstrated that the lentiviral vector pLV-THM-miR-338-3p and pLV-THM-miR-338-3p-inhibitor were constructed successfully. GFP was expressed after the SW-620 cells were transduced by the lentivirus. Expression of miR-338-3p in SW-620 cells transduced with the lentivirus pLV-THM-miR-338-3p was significantly increased (relative expression 3.91 ± 0.51 *vs* 2.36 ± 0.44, $P < 0.01$). Furthermore, overexpression of miR-338-3p inhibited the expression of SMO protein in SW-620 cells, which showed obviously suppressed proliferation ability [cellular proliferation inhibition rate (CPIR) 61.9% ± 5.2% *vs* 41.6% ± 4.8%, $P < 0.01$]. Expression of miR-338-3p in SW-620 cells transduced with the lentivirus pLV-THM-miR-338-3p-inhibitor was significantly decreased (relative expression 0.92 ± 0.29 *vs* 2.36 ± 0.44, $P < 0.01$). Moreover, the downregulated expression of miR-338-3p caused upregulated expression of the SMO protein in SW-620 cells, which showed significantly enhanced proliferation ability (CPIR 19.2% ± 3.8% *vs* 41.6% ± 4.8%, $P < 0.01$). However, anti-SMO-siRNA largely, but not completely, reversed the effects induced by blockage of miR-338-3p, suggesting that the regulative effect of miR-338-3p on CRC cell growth was indeed mediated by SMO.

CONCLUSION: miR-338-3p could suppress CRC growth by inhibiting SMO protein expression.

Key words: Colorectal carcinoma; Hsa-miRNA-338-3p; Smoothened; Lentivirus

Core tip: The previous study has shown that loss of miR-338-3p expression is associated with clinical aggressiveness of colorectal carcinoma (CRC). In this study, the authors demonstrated that forced expression of miR-338-3p in CRC cells suppressed cell proliferation and induced apoptosis, whereas inhibition of miR-338-3p in CRC cells promoted growth. We described miR-338-3p as a direct regulator of smoothened (SMO) expression in CRC, showing a new mechanism responsible for SMO upregulation in CRC. This study provides evidence for antiangiogenic activity of miR-338-3p in the development of CRC and it may develop as a useful biomarker or therapeutic target in CRC.

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INTRODUCTION

Colorectal carcinoma (CRC) is one of the leading causes of cancer-related death worldwide with an estimated one million new cases and 500 000 deaths annually. The CRC incidence and mortality in China have increased rapidly in the past few decades[1]. Screening for CRC allows early-stage diagnosis of the malignancy and potentially reduces mortality. New targeted therapies directed against molecules involved in the pathogenesis of CRC have recently been reported to be safe and effective[2,3]. With the advent of new chemotherapeutic agents, such as angiogenesis inhibitor and transforming growth factor-α inhibitors, there is growing interest to identify new prognostic biomarkers and therapeutic targets for this disease[4].

miRNAs are a new class of small noncoding RNAs that regulate the expression of target genes through translational repression or mRNA cleavage/decay[5,6]. Genome-wide studies have demonstrated that miRNA genes are frequently located at cancer-associated genomic regions or in fragile sites, and in minimal regions of loss of heterozygosity or of amplification, or in common breakpoint regions, indicating the potential roles of miRNAs in tumorigenesis[7,8]. miRNAs have been demonstrated to play an important role in the multistep processes of carcinogenesis, either by oncogenic or tumor suppressor function[9]. Studies of miRNAs have been extended to many types of tumors, including CRC. These studies have revealed that miRNAs may be potential diagnostic or prognostic tools for cancer, and the identification of target mRNAs is a key step for assessing the role of aberrantly expressed miRNAs in human cancer[10].

miR-338-3p has recently been discovered and is involved in cell growth. Although miR-338-3p is known to be specifically expressed in neuronal tissue, little is known about its abundance and function during carcinogenesis[11,12]. We have found that miR-338-3p is downregulated in several CRC samples compared with adjacent non-tumorous tissues, suggesting that miR-338-3p might act as tumor suppressor in CRC, however, the targets that it regulates in CRC have not been established. Smoothened (SMO) protein is related to G-protein-coupled receptors, and is the key activator of the Hedgehog (Hh) signaling pathway[13,14]. Upregulation of SMO in CRC is correlated with higher biological aggressiveness, advanced stage, poor differentiation, larger tumor size, and high proliferative activity[15]. Furthermore, it is also well known that SMO regulation, both in physiological and pathological conditions, is mostly at a post-transcriptional level[16]. Moreover, with the application of bioinformatics predictions, we have found that miR-338-3p and SMO mRNA 3'-untranslated region (UTR) have complementary binding sites. Thus, we inferred that the noncoding RNA, miR-338-3p, acts as a local regulator of SMO by binding to the 3'-UTR of its mRNA, thereby modulating CRC development. In order to verify this hypothesis, we investigated the regulative effect of miR-338-3p on cell proliferation and apoptosis in CRC. We aimed to reveal a new regulatory mechanism of miR-338-3p in the development of CRC, and provide a new miRNA and target gene for clinical application.

MATERIALS AND METHODS

Construction of transfer vector pLV-THM-miR-338-3p and pLV-THM-miR-338-3p-inhibitor

The lentiviral vectors used in this study were pLV-THM, psPAX2, and pMD2.G, which were a transfer vector, packaging plasmid, and envelope plasmid, respectively. The sequences of interest were inserted into the transfer vector between the *Mlu*I and *Cla*I restriction sites according to the Addgene protocol. The third generation of self-inactivating, lentivirus plasmid, pLV-THM (HIV-1-based vector; Addgene, Cambridge, MA, United States), which contains a CMV-driven enhanced green fluorescence protein (GFP) reporter and an H_1 promoter upstream of the restriction sites (*Mlu*I and *Cla*I), was used as the transfer plasmid and was linearized by digesting the vector with the restriction enzymes. The sequence of the mature miR-338-3p (5'-UCCAGCAUCAGUGAUUUUGUUG-3') was obtained from miRBase (http://www.mirbase.org/). The pre-miR-338-3p and miR-338-3p-inhibitor oligonucleotides were chemically synthesized by Sangon Biotech Co. Ltd. (Shanghai, China) and were inserted between the *Mlu*I and *Cla*I sites of the pLV-THM plasmid. After the pre-miR-338-3p and miR-338-3p-inhibitor lentiviral-based vector were transformed into competent *Escherichia coli* DH5α cells

using the calcium chloride method, antibiotic-resistant colonies were selected on LB-ampicillin agar plates. After colony selection and further propagation, the plasmid was extracted using the alkaline lysis method. The plasmid DNA was then analyzed by restriction enzyme digestion and sequence analysis. The plasmid containing the target gene was digested with the restriction enzymes and amplified by polymerase chain reaction (PCR). The clones with positive PCR results were subjected to DNA sequencing.

Cell lines and culture

Human embryonic kidney 293T (HEK-293T) cells (Invitrogen, Carlsbad, CA, United States) and the human CRC-derived cell line SW-620 (Shanghai Institutes for Biological Science, CAS, China) were cultured in Dulbecco's Modified Eagle's Medium high glucose supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT, United States) at 37 ℃ in a humidified incubator with 5% CO_2. The medium was changed every 3 d, and the cells were trypsinized with trypsin/ethylene diamine tetraacetic acid when 80%-90% confluence was reached. Cells at passages 4-8 were used for the experiments.

Lentiviral packaging and virus collection

Twenty-four hours prior to transfection, the HEK-293T cells in logarithmic growth phase were trypsinized, and the cell density was adjusted to 1.0×10^6 cells/mL with complete culture medium. The cells were reseeded into 15-cm cell culture dishes and cultured for 24 h prior to transfection. The cells were 90%-95% confluent on the day of transfection. The recombinant viral vector encoding the miR-338-3p or miR-338-3p-inhibitor and the two packaging plasmids psPAX2 and pMD2.G were extracted with a plasmid extraction kit (Invitrogen) and cotransfected into HEK-293T cells according to the manufacturer's instructions. After 8 h transfection, the cell culture medium was replaced with fresh complete medium. After 24 h transfection, the expression of GFP was determined. After 48 h transfection, the culture medium was collected and centrifuged at 4000 × *g* at 4 ℃ for 10 min to remove any cellular debris. The supernatant was filtered through a 0.45-μm filter into a Plus-20 centrifugal ultrafiltration unit and centrifuged at 4000 × *g* to obtain a high-titer lentivirus stock. The lentivirus without the transgene was used as the negative control and was produced in the same manner.

Virus transduction and fluorescent cell selection

SW-620 cells were seeded at 1.0×10^5 cells per well in 24-well plates in DMEM containing 10% FBS. After 24 h incubation, the cells were transduced with each lentivirus stock (3.0×10^5 Titer Units). The SW-620 cells were then incubated for an additional 48-72 h prior to identifying the GFP^+ cells by flow cytometry (Becton Dickinson, San Jose, CA, United States).

Detection of miR-338-3p expression by real-time reverse transcriptase RT-PCR

Total RNA from SW-620 cells was prepared using the TRIzol reagent (Invitrogen) after viral transduction. The precipitate was dissolved in diethylpyrocarbonate-treated water, and a nucleic acid protein analyzer (Beckman Coulter, Fullerton, CA, United States) was used to determine the RNA concentration. The purity and integrity of the RNA were identified as follows: the A_{260nm}/A_{280nm} was ⩾ 1.8, and the band ratio of 28 S RNA to 18 S RNA was ⩾ 1.5 in formaldehyde denaturing gel electrophoresis. Accurate quantitation of the mature miR-338-3p was obtained using the TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA, United States). The reverse transcription reaction was performed using 10 ng total RNA and the looped primers. Real-time PCR was performed using the standard TaqMan MicroRNA Assays protocol on the iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, United States). The PCR reaction (20 μL) included 1.33 μL reverse transcription product, 1 × TaqMan Universal PCR Master Mix, No AmpErase UNG, 0.2 μmol/L TaqMan probe, 1.5 μmol/L forward primer, and 0.7 μmol/L reverse primer. The reactions were incubated in a 96-well plate at 95 ℃ for 10 min followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ for 1 min. The miR-338-3p expression level was measured using the Ct (threshold cycle) method. Ct is the fractional cycle number at which the fluorescence of each sample passes the fixed threshold. The ΔΔCT method for relative quantitation of gene expression was used to determine the miR-338-3p expression levels. The ΔCT was calculated by subtracting the Ct of U6 from the Ct of the miR-338-3p. The ΔΔCT was calculated by subtracting the ΔCT of the reference sample from the ΔCT of each sample. The fold change was calculated using the equation $2^{-\Delta\Delta CT}$. The TaqMan MicroRNA Assays for U6 RNA was used to normalize the relative abundance of miR-338-3p.

miRNA target prediction

The analysis of miR-338-3p-predicted targets was performed using the algorithms TargetScan (http://targetscan.org/), PicTar (http://pictar.mdc-berlin.de/) and MiRanda (http://www.microrna.org/microrna/home.do).

Detection of SMO protein expression by Western blotting

SW-620 cells were rinsed twice with cold PBS and were then lysed in ice-cold lysis buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.6), 0.1% SDS, 1% Nonidet P-40, and protease inhibitor cocktail (Boehringer Mannheim, Lewes, United Kingdom). The samples were cleared by centrifugation at 13 000 × *g* for 10 min. The cellular protein (50 μg) was subjected to SDS-PAGE and electrotransferred to polyvinylidine fluoride membranes (Immobilon, Bedford, MA, United States). After blocking in 20 mmol/L Tris-HCl, (pH 7.6) containing 150

mmol/L NaCl, 0.1% Tween-20, and 5% nonfat dry milk, the membranes were incubated with primary antibodies against SMO or β-actin, which was used as a sample loading control, overnight at 4 ℃. The membranes were then incubated with horseradish-peroxidase-conjugated secondary antibody. The blot was developed using the ECL detection kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ, United States) according to the manufacturer's instructions.

Cell proliferation assay

The status of cell proliferation was determined by 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Amresco, Solon, OH, United States) assay. Exponentially growing SW-620 cells were adjusted to 2.5×10^4 cells/mL with DMEM, plated in 96-well plates (Corning, Corning, NY, United States) at 200 μL/well and then incubated for 12 h according to routine procedure. After being transduced with each lentivirus stock and incubated for 48 h (5 duplicate wells for each sample), 20 μL/well MTT (5 g/L) was added to each well. The medium was then removed after 4 h incubation and 100 μL/well dimethyl sulfoxide was added to dissolve the reduced formazan product. Finally, the plate was read in an enzyme-linked immunosorbent microplate reader (Bio-Rad 2550) at 490 nm. The cellular proliferation inhibition rate (CPIR) was calculated using the following formula: CPIR = (1 - average *A* value of experimental group/average *A* value of control group) × 100%.

Apoptosis assay

The effects of miR-338-3p on CRC cell cycle and apoptosis were examined by flow cytometry. Pretreated SW-620 cells were harvested and washed twice with PBS, fixed with 70% ethanol at -20 ℃ for 30 min, and stored at 4 ℃ overnight, then washed with PBS again, treated with 100 mL 100 mg/L RNase at 37 ℃ for 30 min, and stained with 100 mL 50 mg/L propidium iodide at 4 ℃ for 30 min in the dark. The multiplication cycle and apoptotic rate were assayed using flow cytometry, and the data were analyzed using CellQuest software. The percentages of cells in the G_0/G_1 phase and S phase, and the apoptotic rate were measured by calculating the ratio of the number of corresponding cells to the number of total cells. For each sample, 10 000 cells were measured.

Statistical analysis

The relative expression analysis of the target gene was performed using REST-XL (Relative Expression Software Tool, available at http://www.wzw.tum.de/genequantification). All data in the experiment were presented as the mean ± SD. Comparisons between the groups were analyzed with one-way ANOVA and Student-Newman-Keuls *Q* test, using SPSS version 13.0 software (SPSS Inc., Chicago, IL, United States). $P < 0.05$ was considered statistically significant.

RESULTS

Lentivirus package and transduction

HEK-293T cells were cotransfected with the transfer plasmid, pLV-THM-transgene, the packaging plasmid, psPAX2, and the envelope plasmid, pMD2.G. The high-titer lentivirus was harvested as the stock virus solution. GFP was expressed 48 h after the SW-620 cells were transduced by the lentivirus, and the cells were observed under a fluorescence microscope (Figure 1A, B). This suggests that the miR-338-3p or miR-338-3p-inhibitor vector was successfully transduced into the SW-620 cells, which provides the basis for further studies regarding the molecular function of miR-338-3p in CRC cells. The GFP^+ fluorescent cells were then identified and harvested using flow cytometry for the next experiment (Figure 1C-E).

Real-time reverse transcriptase-PCR detecting miR-338-3p expression in CRC cells after lentivirus transduction

To study the expression pattern of miR-338-3p in SW-620 cells after lentivirus transduction, we performed real-time reverse transcriptase (RT)-PCR to detect miR-338-3p expression in the SW-620 cells. Real-time RT-PCR indicated that the miR-338-3p cDNA increased exponentially and then reached a plateau. The miR-338-3p amplification curve was a typical reverse S pattern (Figure 2A) and showed higher amplification efficiency. The miR-338-3p PCR product was 72 bp long, the corresponding Tm was 84.09 ± 0.15 ℃, the melting temperature was even, and the shape of the peak was sharp (Figure 2B). As shown in Figure 2C, the expression level of miR-338-3p in the pLV-THM-miR-338-3p group was more than one-third of the expression in the control cells that were transduced with the blank pLV-THM vector, whereas the expression level of miR-338-3p in the pLV-THM-miR-338-3p-inhibitor group decreased significantly compared with the control group ($P < 0.01$). Thus, we established the SW-620-miR-338-3p and SW-620-miR-338-3p-inhibitor cell lines successfully to observe the corresponding biological effect.

SMO is a target of miR-338-3p in CRC

Most miRNAs are thought to control gene expression by base-pairing with the miR-recognizing elements found in their messenger target. We then used all three currently available major prediction programs, including TargetScan, Miranda and PicTar, to analyze the potential interaction between miR-338-3p and SMO. SMO mRNA was predicted by all of the algorithms and revealed potential miR-338-3p target sites in its 3'-UTR (Figure 3A).

To check if miR-338-3p actually affected SMO expression in CRC cells, we analyzed the consequence of the ectopic expression of miR-338-3p. We transfected the pre-miR-338-3p and miR-338-3p-inhibitor into SW-620 cells by lentivirus transduction as described above, and we searched for changes in SMO protein

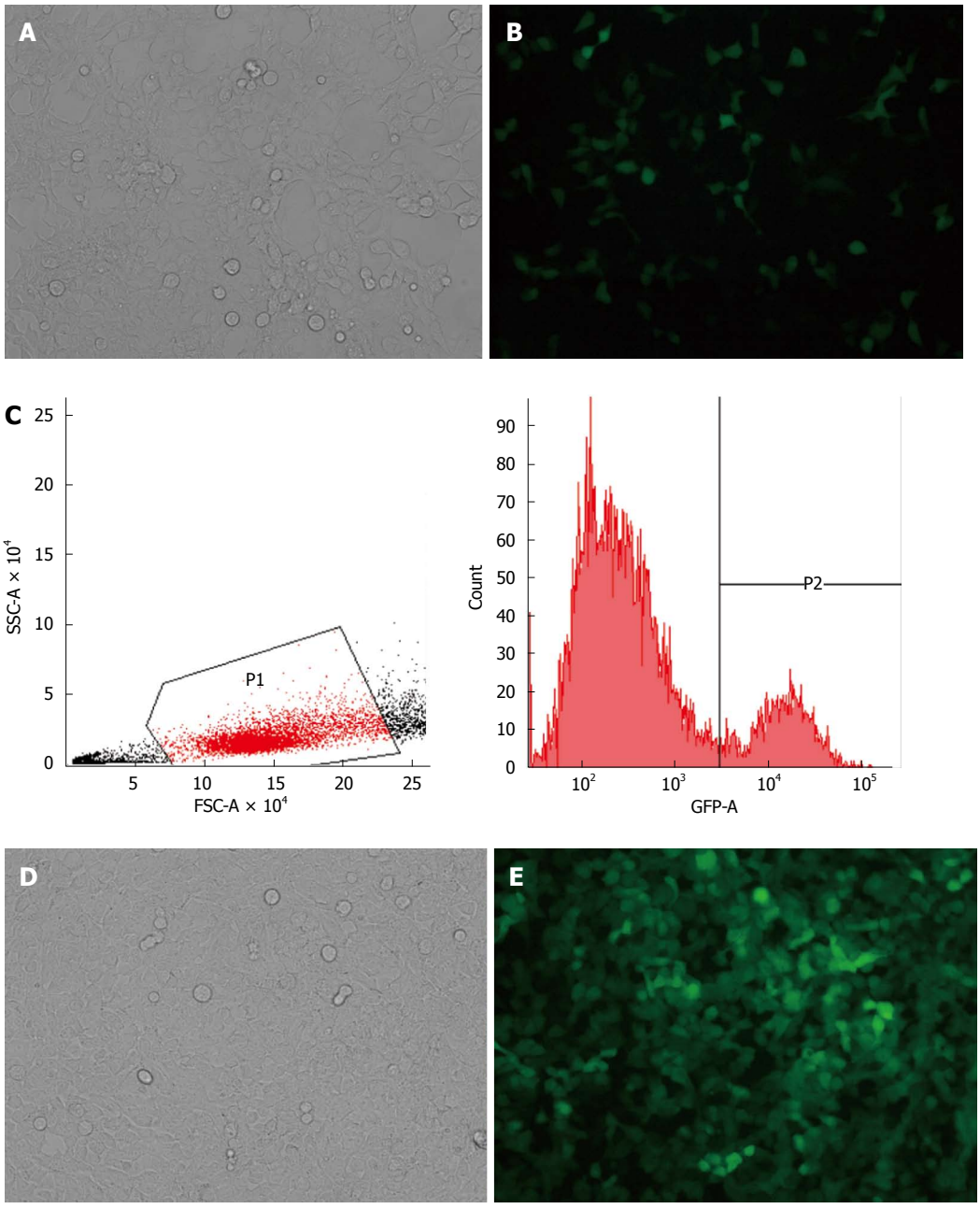

Figure 1 SW-620 cells transduced by lentivirus before and after flow cytometry selection. A, B: SW-620 cells transduced by lentivirus before flow cytometry selection (A: Light microscopy; B: Fluorescent microscopy × 40); C: SW-620 cells with green fluorescent protein$^+$ were distinguished by flow cytometry; D, E: SW-620 cells transduced by lentivirus after flow cytometry selection (D: Light microscopy; E: Fluorescent microscopy × 40).

levels by Western blotting analysis. Introduction of pre-miR-338-3p caused a significant increase of miR-338-3p value and decreased SMO protein levels in SW-620 cells. Conversely, miR-338-3p-inhibitor caused a significant decrease of miR-338-3p value and increased SMO protein level (Figure 3B). This result strongly validates a post-transcriptional regulation of SMO protein by miR-338-3p.

miR-338-3p suppresses proliferation and induces apoptosis in CRC cells

SMO has a key role in the cell cycle, particularly in the growth arrest at the G_1/S transition, therefore, we further tested if the cell growth potential of stably transduced CRC cells expressing miR-338-3p or miR-338-3p-inhibitor was modified as a consequence of the demonstrated SMO alteration. First, to evaluate the effect of miR-338-3p on CRC cell proliferation, growing SW-620 cells were transduced with lentivirus pLV-THM-miR-338-3p or pLV-THM-miR-338-3p-inhibitor for 48 h and the cell proliferation was determined by MTT assay. We observed a significant increase in proliferation after transduction of pLV-THM-miR-338-3p-inhibitor (Figure 4A, $P < 0.01$). In contrast, pre-miR-338-3p significantly inhibited cell proliferation (Figure 4A, $P < 0.01$). These data indicate that cell proliferation can

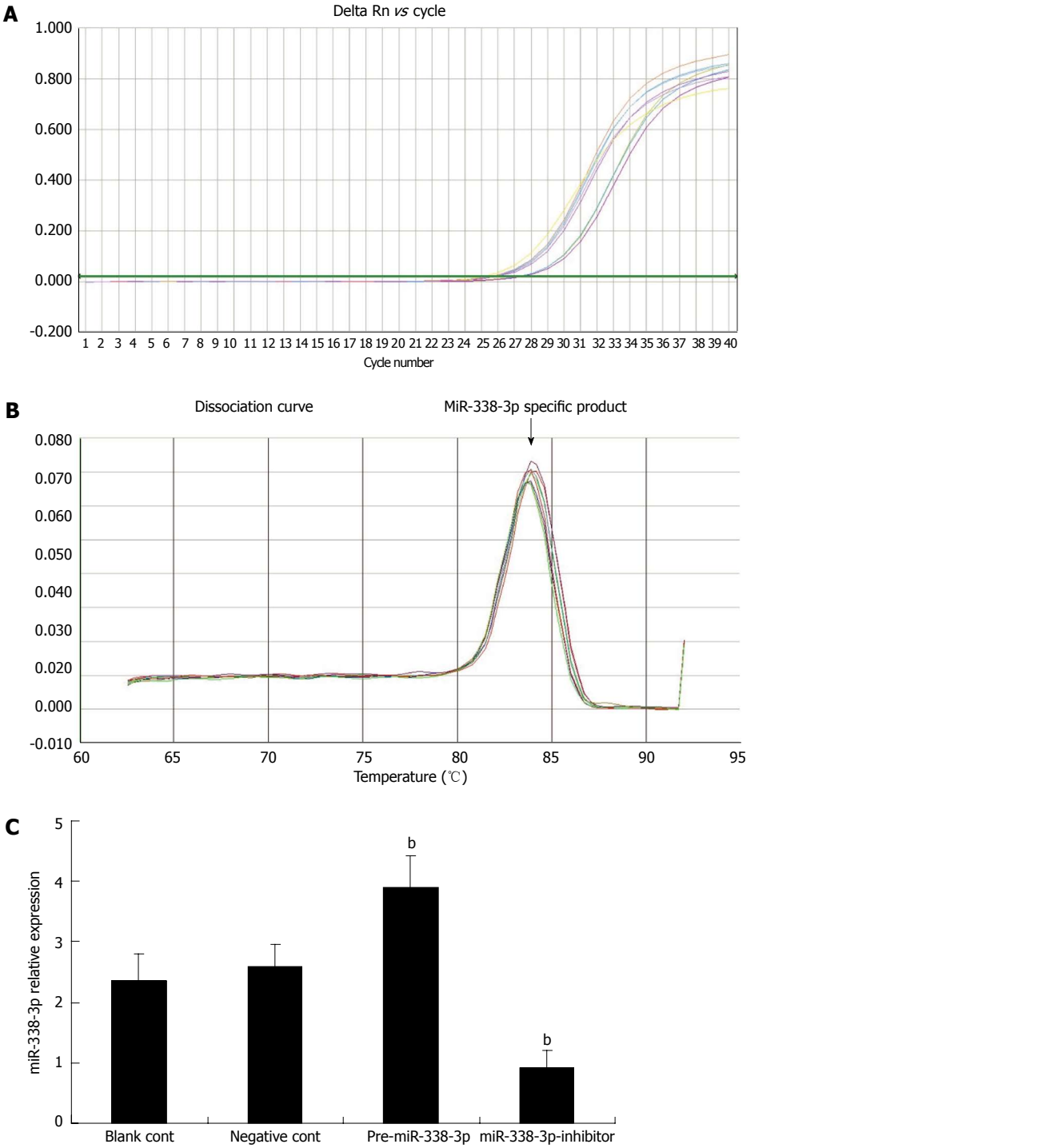

Figure 2 Real-time reverse transcriptase-polymerase chain reaction analysis detecting miRNA-338-3p expression in SW-620 cells. A: miRNA-338-3p (miR-338-3p) cDNA concentrations, Log value as ordinate, Ct value as abscissa; B: Tm of miR-338-3p was 84.09 ℃; C: Expression of miR-338-3p detected by real-time reverse transcriptase-polymerase chain reaction. Expression of U6 snRNA was used as an internal control. $^{b}P < 0.01$ *vs* control group.

be significantly suppressed by increased miR-338-3p expression. Second, we performed flow cytometry analysis after exposure to miR-338-3p or miR-338-3p-inhibitor to investigate CRC cell-cycle phase distribution. SW-620 cells overexpressing miR-338-3p had a significant decrease in the S-phase population and a increase in the G_0/G_1 population compared with cells transduced with negative control lentivirus (Figure 4B, $P < 0.01$). On the contrary, miR-338-3p-inhibitor significantly increased the S-phase and decreased the G_0/G_1 population (Figure 4B, $P < 0.01$). Third, we investigated the effect of miR-338-3p on apoptosis by flow cytometry and found that apoptosis increased dramatically in SW-620 cells after transduction with lentivirus pLV-THM-miR-338-3p, suggesting that miR-338-3p may function as a strong apoptotic inducer in human CRC cells (Figure 4C-F). These

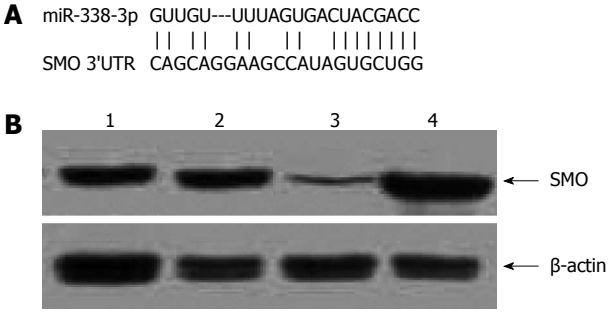

Figure 3 miRNA-338-3p regulates expression of smoothened in SW-620 cells. A: Smoothened (SMO) 3'-untranslated region potentially targeted by miRNA-338-3p (miR-338-3p) as predicted by TargetScan; B: Western blotting analysis showing SMO protein expression in SW-620 cells. β-actin was used as a housekeeping gene to normalize SMO protein expression. Lane 1: blank control; lane 2: SW-620 cells transduced with lentivirus pLV-THM-control; lane 3: SW-620 cells transduced with lentivirus pLV-THM-miR-338-3p; lane 4: SW-620 cells transduced with lentivirus pLV-THM-miR-338-3p-inhibitor. The results are representative of three independent experiments.

results confirm the potential tumor-suppressor activity of miR-338-3p in CRC.

CRC cell proliferation suppression by miR-338-3p is mediated by SMO

If miR-338-3p suppression of CRC cell proliferation was indeed mediated by SMO, we would expect that the SMO-specific and irreversible antagonist anti-SMO-siRNA would abolish this effect. To test this hypothesis, we measured the changes in proliferation induced by pre-miR-338-3p or miR-338-3p-inhibitor in CRC cells previously transfected with anti-SMO-siRNA. The aim was to study if and how the SMO-depleted cellular environment responded to pre-miR-338-3p or miR-338-3p-inhibitor. SW-620 cells were pretreated with or without anti-SMO-siRNA (50 nmol/L) for 24 h prior to transduction with lentivirus pLV-THM-miR-338-3p or pLV-THM-miR-338-3p-inhibitor, and cell proliferation was determined by MTT assay. A reduction in SMO level, by means different from miR-338-3p overexpression, led to analogous outcomes. When we transfected SW-620 cells with anti-SMO-siRNA, SMO protein was reduced by about 80% (Figure 5A), and we observed a sharp decrease in cell proliferation as compared with negative controls (Figure 5B, $P < 0.01$). Thus, reducing SMO protein levels in CRC cells, either by miR-338-3p overexpression or by anti-SMO-siRNA transfection, is sufficient to induce a comparable decrease in cell growth.

When lentivirus pLV-THM-miR-338-3p was transduced into SW-620 cells previously treated with anti-SMO-siRNA, we observed that anti-SMO-siRNA and pre-miR-338-3p seemed to co-operate to inhibit the growth rate (Figure 5B). However, when lentivirus pLV-THM-miR-338-3p-inhibitor was transduced into SW-620 cells previously treated with anti-SMO-siRNA, we observed that the enhancement of cell proliferation by miR-338-3p-inhibitor was largely abrogated by anti-SMO-siRNA (Figure 5B, $P < 0.01$). These results indicated that the promotive effect of miR-338-3p-inhibitor on CRC cell growth was largely, but not completely, mediated by SMO, suggesting that miR-338-3p-inhibitor could also activate some SMO-independent signaling pathway to promote CRC cell growth in addition to upregulation of SMO.

DISCUSSION

With the advent of new chemotherapeutic agents, clarification of the molecular pathogenesis of CRC is crucial for developing effective therapeutic strategies to improve patient outcome[17,18]. The miR-338 gene is located on chromosome 17 and produces two mature forms (miR-338-3p and miR-338-5p). Tsuchiya *et al*[19] have reported that miR-338-3p contributes to the formation of epithelial basolateral polarity by facilitating the translocalization of β1-integrin to the basolateral membrane, which highlights a potentially important role for miR-338-3p in the process of epithelial cell differentiation. Huang *et al*[20] have demonstrated that a decrease in miR-338-3p expression in hepatocellular carcinoma, which is another type of epithelial-cell-derived cancer, was significantly associated with TNM stage, vascular invasion, intrahepatic metastasis, tumor size, and tumor grade. Our previous study has also shown that loss of miR-338-3p expression is associated with clinical aggressiveness of CRC. Moreover, the miR-338-3p expression was not only related to TNM stage but also to tumor invasion and migration. The level of miR-338-3p expression at TNM stages Ⅲ and Ⅳ was lower than that at stages Ⅰ and Ⅱ, and the tumors which invaded adjacent tissues or organs had less miR-338-3p expression than those limited to the wall of the colon and rectum (data not shown). Thus, miR-338-3p may be an important tumor suppressor, which can cleave or inhibit the targeted mRNAs of tumor promoters, and play a role in the progression of CRC. However, lack of knowledge about the targets for miR-338-3p hampers a full understanding of the biological functions deregulated by miR-338-3p aberrant expression. To confirm the molecular mechanism of miR-338-3p in CRC, it is necessary to observe the biological effects of the up- and down-regulation of miR-338-3p. Thus, we constructed a CRC-derived cell line in which miR-338-3p was stably over- or under-expressed by transducing the lentivirus vector, pLV-THM-miR-338-3p or pLV-THM-miR-338-3p-inhibitor, into SW-620 cells[21]. Notably, we showed that our combined lentivirus specifically enhanced or inhibited endogenous miR-338-3p to induce the corresponding biological effect. The successful construction of the lentiviral vector provides the basis for further studies regarding the molecular function of miR-338-3p in CRC[22-24].

To extend our previous observation, we focused on the role of miR-338-3p in regulation of proliferation

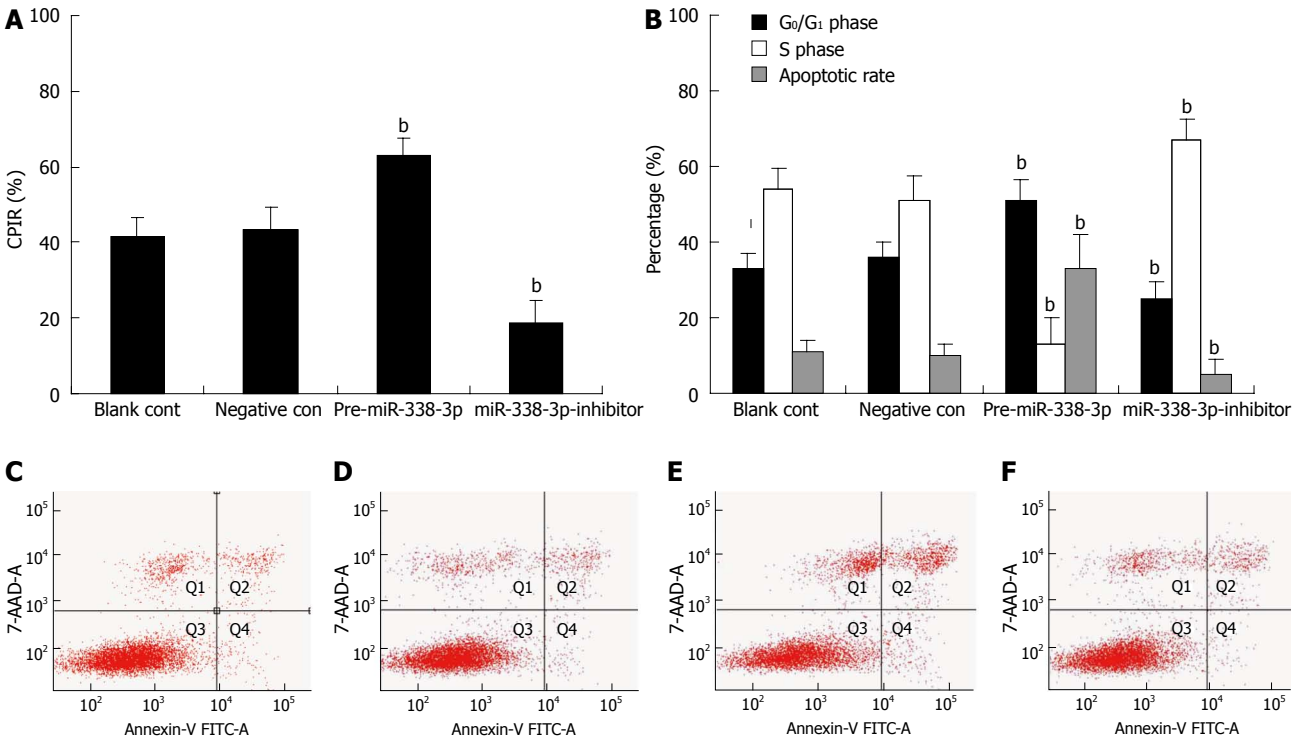

Figure 4 Effects of miRNA-338-3p on cell proliferation and apoptosis in colorectal carcinoma cells. A: Cell proliferation was determined by 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide assay. Cellular proliferation inhibition rate (CPIR) in the presence of pre-miRNA-338-3p (miR-338-3p) or miR-338-3p-inhibitor was compared with that of the controls; $n = 6$, mean ± SD. $^bP < 0.01$ *vs* control group; B: Effects of pre-miR-338-3p and miR-338-3p-inhibitor on cell-cycle in SW-620 cells. The percentages of cells in G_0/G_1 phase and S phase and apoptotic rate were measured by computing the ratio of the number of corresponding cells to total cells; $n = 3$, mean ± SD. $^bP < 0.01$ *vs* control group; C-F: Apoptosis analysis of transduced cells by flow cytometry. C: Blank control; D: SW-620 cells transduced with lentivirus pLV-THM-control; E: SW-620 cells transduced with lentivirus pLV-THM-miR-338-3p; F: SW-620 cells transduced with lentivirus pLV-THM-miR-338-3p-inhibitor. The right lower quadrant ($FITC^+/PI^-$) shown as apoptotic cells.

and apoptosis in CRC. We found that the proliferative potential was suppressed after restoration of miR-338-3p expression in CRC cells transduced by lentivirus vector, pLV-THM-miR-338-3p. However, the downregulation of miR-338-3p, due to transducing by lentivirus vector pLV-THM-miR-338-3p-inhibitor into SW-620 cells, induced CRC cell proliferation. Cell cycle status and apoptosis are usually closely associated. Cells failing to progress to mitosis are destined for apoptosis. Besides cell-cycle arrest, the inhibition of cell growth observed in CRC cells with pre-miR-338-3p may also be a result of increased apoptosis. In this study, treatment of lentivirus pLV-THM-miR-338-3p caused G_0/G_1 phase arrest and blocked cells from entering S phase. Interestingly, as seen in other tumor cells, we clearly demonstrated that pre-miR-338-3p induced significant apoptosis in CRC cells, as demonstrated by flow cytometry. These data demonstrate that miR-338-3p is a potential tumor suppressor for CRC. However, the exact mechanisms of miR-338-3p remain unknown.

With the application of bioinformatics prediction programs, such as TargetScan, PicTar and MiRanda, we found that miR-338-3p and the 3'-UTR of SMO mRNA had complementary binding sites. From this, we hypothesized that SMO may be a new target of miR-338-3p in CRC; however, this finding has not yet been reported. SMO, a seven-membrane-spanning receptor is a fundamental component of the Hh signaling pathway and an important anticancer drug target[25-27]. Once activated, SMO triggers a series of intracellular events with resultant activation of the zinc finger transcription effectors including Gli, which in turn regulates cell proliferation, differentiation, apoptosis and invasion[28-30]. It has been reported that 3-Keto-*N*-(aminoethyl-aminocaproyl-dihydrocinnamoyl) cyclopamine (KAAD-cyclopamine), a synthetic specific antagonist of SMO, markedly inhibits hepatocellular carcinoma cell growth and motility by binding to SMO[31]. Indeed, in our study, downregulation of SMO occurred in response to lentivirus vector pLV-THM-miR-338-3p transduction into CRC cells, and significant upregulation of SMO occurred in response to lentivirus vector pLV-THM-miR-338-3p-inhibitor transduction. Consistent with Huang *et al*[32], our results suggest that SMO is a direct target of miR-338-3p in CRC cells.

We deduced that miR-338-3p inhibited CRC cell proliferation, likely through downregulating SMO. To confirm this, we performed RNA interference to knock down SMO in CRC cells before transduction with miR-338-3p-inhibitor. We showed that anti-SMO-siRNA could significantly, but not completely, inhibit miR-338-3p-inhibitor-induced proliferation of CRC cells.

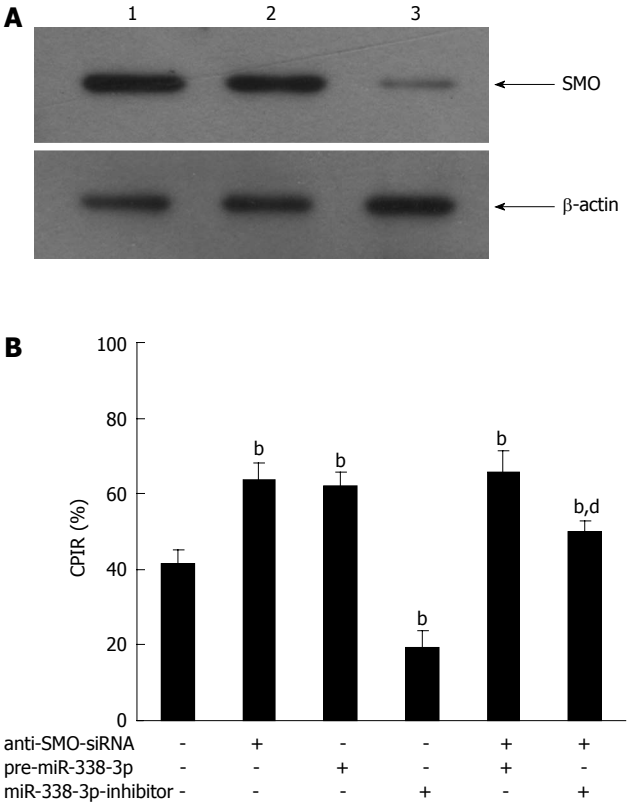

Figure 5 Ectopic expression of miRNA-338-3p affects proliferation of colorectal carcinoma cells by targeting smoothened. SW-620 cells were pretreated with or without anti-smoothened (SMO)-siRNA (50 nmol/L) for 24 h prior to transduction with lentivirus pLV-THM-miRNA-338-3p (miR-338-3p) or pLV-THM-miR-338-3p-inhibitor. A: Western blotting analysis showing that SMO protein reduced markedly after transfection with anti-SMO-siRNA. Equal loading was confirmed by using β-actin. Lane 1, blank control; lane 2, SW-620 cells transfected with control siRNA; lane 3, SW-620 cells transfected with anti-SMO-siRNA; B: Cell proliferation was determined by 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide assay. Enhancement of SW-620 cell proliferation by miR-338-3p-inhibitor was largely, but not completely, abrogated by anti-SMO-siRNA [cellular proliferation inhibition rate (CPIR) from 19.2% to 50.9%]; n = 3, mean ± SD. $^{b}P < 0.01$ *vs* negative control group. $^{d}P < 0.01$ *vs* sole miR-338-3p-inhibitor group.

These results confirmed that the inhibitory effect of miR-338-3p on CRC cell proliferation was largely, but not completely, mediated by SMO, suggesting that miR-338-3p could regulate other SMO-independent signaling pathways to promote CRC growth. We think that our results, which identify SMO as a target for miR-338-3p in the context of CRC cell line, fit well within a dynamic view of the miRNA-mediated regulation of gene expression. It is well known and widely predicted that the relationship between miRNAs and target mRNAs is not a "one to one" connection, because the same mRNA can be regulated by more than one miRNA, and that the choice of how many and which miRNAs target one 3'-UTR is strongly determined by the specific cellular environment[33-35]. An miRNA that regulates targets playing opposite roles in the control of cell proliferation may act as a tumor suppressor in some cancers and as an oncogene in others, depending on which targets are driving tumorigenesis in that specific cellular milieu[36].

In summary, we have described miR-338-3p as a direct regulator of SMO expression in CRC, showing a new mechanism responsible for SMO upregulation in CRC. These findings further outline the importance of miR-338-3p in CRC carcinogenesis. However, it should be emphasized that our results were generated from cultured CRC cells and that they might not necessarily and comprehensively reflect the situation *in vivo*[37]. Further experiments, beyond the scope of this study, are required to elucidate the antitumor mechanisms of miR-338-3p in athymic mice.

COMMENTS

Background

miRNAs regulate gene expression by mainly binding to the 3'-untranslated region (UTR) of the target mRNAs, leading to mRNA degradation or translation inhibition. miRNAs are aberrantly expressed in various cancers, suggesting that they play a vital role as a novel class of oncogenes or tumor suppressor genes, depending on the targets they regulate.

Research frontiers

Colorectal carcinoma (CRC) is one of the most serious malignancies in China. Our previous study has shown that loss of miRNA-338-3p (miR-338-3p) expression is associated with clinical aggressiveness of CRC. In this study, the authors report the regulatory effect of miR-338-3p on proliferation and apoptosis of CRC cells.

Innovations and breakthroughs

Some human miRNAs are consistently deregulated in human cancer, suggesting a role for these genes in tumorigenesis. Authors previous study has also shown that loss of miR-338-3p expression is associated with clinical aggressiveness of CRC. The authors demonstrated that forced expression of miR-338-3p in CRC cells suppressed cell growth, whereas inhibition of miR-338-3p promoted cell growth. Furthermore, smoothened (SMO) was identified as a direct target of miR-338-3p. The antiangiogenic role of miR-338-3p was determined as tumor suppressor.

Applications

This study indicates that miR-338-3p suppresses cell growth by targeting the *SMO* gene in CRC *in vitro* and miR-338-3p might be a novel potential strategy for CRC treatment.

Terminology

Most miRNAs are thought to control gene expression by base-pairing with the miR-recognizing elements, 3'-UTR, found in their messenger target. Not surprisingly, with the application of bioinformatics predictions, we find that miR-338-3p and SMO mRNA 3'-UTR has complementary binding sites.

Peer review

miR-338-3p could suppress CRC growth ability by inhibiting SMO protein expression. This study provides evidence for antiangiogenic activity of miR-338-3p in the development of CRC, and may be developed as a useful biomarker or therapeutic target in CRC.

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BRIEF ARTICLE

Non-invasive panel tests for gastrointestinal motility monitoring within the MARS-500 Project

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Abstract

AIM: To develop an integrated approach for monitoring gastrointestinal motility and inflammation state suitable for application in long-term spaceflights.

METHODS: Breath tests based on the oral administration of ^{13}C-labeled or hydrogen-producing substrates followed by the detection of their metabolites ($^{13}CO_2$ or H_2) in breath were used to measure gastrointestinal motility parameters during the 520-d spaceflight ground simulation within the MARS-500 Project. In particular, the gastric emptying rates of solid and liquid contents were evaluated by ^{13}C-octanoic acid and ^{13}C-acetate breath tests, respectively, whereas the orocecal transit time was assessed by an inulin H_2-breath test, which was performed simultaneously with the ^{13}C-octanoic acid breath test. A ready-to-eat, standardized pre-packaged muffin containing 100 mg of ^{13}C-octanoic acid was used in the ^{13}C-octanoic acid breath test to avoid the extemporaneous preparation of solid meals. In addition, a cassette-type lateral flow immunoassay was employed to detect fecal calprotectin, a biomarker of intestinal inflammation. Because no items could be introduced into the simulator during the experiment, all materials and instrumentation required for test performance during the entire mission simulation had to be provided at the beginning of the experiment.

RESULTS: The experiments planned during the simulation of a manned flight to Mars could be successfully performed by the crewmembers without any external assistance. No evident alterations (*i.e.*, increasing or decreasing trends) in the gastric emptying rates were detected using the ^{13}C-breath tests during the mission simulation, as the gastric emptying half-times were in the range of those reported for healthy subjects. In contrast to the ^{13}C-breath tests, the results of the inulin H_2-breath test were difficult to interpret because of the high variability of the H_2 concentration in the breath samples, even within the same subject. This variability suggested that the H_2-breath test was strongly affected by external factors, which may have been related to the diet of the crewmembers or to environmental conditions (*e.g.*, the accumulation of hydrogen in the simulator microenvironment). At least in closed microenvironments such as the MARS-500 simulator, ^{13}C-breath tests should therefore be preferred to H_2-breath tests. Finally, the fecal calprotectin test showed significant alterations during the mission simulation: all of the crewmembers were negative for the test at the beginning of the simulation but showed various degrees of positivity in at least one of the subsequent tests, thus indicating the onset of an intestinal inflammation.

CONCLUSION: Breath tests, especially those ^{13}C-based, proved suitable for monitoring gastrointestinal motility in the 520-d isolation experiment within

MARS-500 project and can be applied in long-term spaceflights.

Key words: Breath test; Gastrointestinal inflammation; Gastrointestinal motility; Spaceflight; Stress

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INTRODUCTION

A manned mission to Mars is currently starting to garner a consistent level of support, as exploration roadmaps are under study by various space agencies. Nevertheless, several issues related to the health of humans during such a long space mission still must be solved.

Extended-duration space missions expose the crewmembers to microgravity, radiation and a stressful environment due to mission-related factors (*e.g.*, confinement, isolation, anxiety, physiologic stress, sleep deprivation and modifications of their nutrition regimes, circadian rhythms and microbial environments) that affect their physiological status[1]. To properly monitor the crewmembers' health status during a real space mission, a suitable panel of biochemical tests and related analytical instrumentation should be developed, implemented in the space module and validated for its clinical utility and applicability in spaceflight. These tests should be easily performed onboard by the crewmembers on non-invasively collectable biological samples (*e.g.*, saliva, breath expatriate, urine, or stool) and employing compact devices in a point-of-care format.

Among the alterations that might occur in long-term spaceflights, changes in the gastrointestinal (GI) motility and related gut inflammatory states are of particular relevance. The main factors affecting GI motility are the physical properties of the solid and liquid contents of the stomach and intestine and the functional, hormonal and enzymatic changes in those organs. Spaceflight-related changes in GI function, such as fluid shifts, combined with reduced fluid intake, would tend to decrease GI motility. Although GI motility has not been systematically studied in spaceflight, a significant increase in the mouth-to-caecum transit time has been demonstrated in ground simulations (10 d of -6° head-down bed resting[2,3] and water immersion[4]).

Previous studies have demonstrated that adequate nutritional status is critical to maintaining crew health during extended-duration spaceflight[5-8], and a common cause of reduced dietary intake, especially during the first d of a mission, is space motion sickness[9]. The impact of psychological, physical, and immunological stressors on GI motility, duodenal and biliary secretion, epithelial permeability, and inflammation is currently thoroughly documented, and stress has a major influence on digestive diseases. Gastrointestinal motor dysfunctions, mainly caused by stress conditions, alteration of circadian rhythms and nutritional regimen, may also represent themselves as additional stress factors[10,11]. Decreased GI motility will, in turn, result in delayed intestinal absorption, alterations in the intestinal microflora and decreased bioavailability of orally administered drugs[12]. Such possible alterations must be expeditiously and continuously detected to guide the adoption of the actions necessary to avoid negative consequences to the crewmembers' health and, more generally, wellness (and thus to the crew's efficiency).

In this work, we present an integrated approach to the non-invasive monitoring of GI motility and inflammation state that was optimized in the frame of the MARS-500 project. This project was realized by the State Scientific Center of the Russian Federation-Institute of Biomedical Problems of the Russian Academy of Sciences (IBMP), under the auspices of Roscosmos and the Russian Academy of Sciences and in collaboration with the European Space Agency and other space agencies and institutions from all over the world. The project consisted of several isolation experiments, including a final 520-d isolation (the longest spaceflight ground simulation ever conducted) designed to simulate a round-trip manned mission to Mars. The project aimed at obtaining useful information about physical and psychological problems that astronauts might face during a long stay onboard an interplanetary space vehicle and to set up technologies for monitoring their health status with possible application in real space missions.

The integrated approach herein described employed breath tests (BTs) for the evaluation of GI motility. Indeed, ^{13}C- and H_2-BTs based on the oral administration of ^{13}C-labeled or hydrogen-producing substrates followed by the detection of the metabolites of these substrates ($^{13}CO_2$ or H_2, respectively) in the breath represent a convenient, non-invasive and efficient procedure for obtaining information on motor and organ functions of the GI system. Such tests are routinely used for the detection of alterations in GI motility, bacterial overgrowth, and lactose intolerance, among other issues, and for the diagnosis of infection with *Helicobacter pylori*[13-15]. We evaluated the gastric emptying rates of solid and liquid content by ^{13}C-octanoic acid and ^{13}C-acetate BT, respectively, whereas the orocecal transit time was assessed by an H_2-BT that used inulin as the hydrogen-producing substrate (the latter BT was performed simultaneously with the ^{13}C-octanoic acid BT for the measurement of the gastric emptying rate of solids). In addition, a cassette-type lateral flow immunoassay was employed for detecting fecal calprotectin, a biomarker of intestinal inflammation.

Because they are non-invasive and easily self-performed, BTs are potentially transferrable to the space environment, provided protocol standardization and the

development of compact on-board instrumentation. Miniaturized instrumentation based on electrochemical gas sensors is available for H_2-BT, whereas compact instrumentation based on non-dispersive infrared spectroscopy (NDIRS) has been developed as an alternative to isotope ratio mass spectrometry (IRMS) for the measurement of $^{13}CO_2$ in breath[16]. In perspective, miniaturized dedicated analytical instrumentation suitable for on-board operation by the crewmembers will make this integrated approach applicable in real space missions, thus providing a useful tool for the early detection of dysfunctions of the GI system and the adoption of suitable countermeasures, such as diet adjustments or pharmacological interventions.

MATERIALS AND METHODS

Subjects

The crew was composed of six male subjects, who at the beginning of the experiment had a median age of 31 years (range 27-38 years), median body weight of 81 kg (range 74-100 kg), and median body mass index of 26.3 kg/m^2 (range 23.6-32.3 kg/m^2). During the mission simulation, all of the crewmembers received the same diet, the composition of which was almost identical to that of the diet used in the International Space Station[17].

Ethics

All of the scientific investigations performed in the frame of the MARS-500 experiments were reviewed and approved by the IBMP Committee on Bioethics, and all of the volunteers signed the written informed consent for participation in the experiment.

Materials employed for diagnostic tests

A standard muffin meal (EXPIROGer®, manufactured and packaged by Sofar SpA, Milan, Italy) containing 100 mg of ^{13}C-octanoic acid was employed in the ^{13}C-BT for the measurement of the gastric emptying rate of solid meals. The muffin (weight 100 g) had a 378 kcal (1589 kJ) calorie content and the following composition: 5.5 g of proteins, 57.5 g of carbohydrates, 14.0 g of fats (corresponding to 5.8%, 60.8%, and 33.3% of the total calories, respectively), 1.1 g of dietary fiber and 16.7% moisture. Stable ^{13}C-isotope-labeled sodium acetate (99% isotope purity) was purchased from Cambridge Isotope Laboratories (Andover, MA). Inulin (Beneo™ HP-Gel) with a degree of polymerization of 5-60 was obtained from Orafti (Oreye, Belgium). The enteral nutrition solution Nutrizon standard was manufactured by Otsuka Pharmaceutical (Tokyo, Japan) and had (for 100 mL) a 110 kcal (420 kJ) calorie content, 15% of which were from proteins and 55% from carbohydrates. The semiquantitative rapid immunochromatographic test for the detection of calprotectin in feces (PreventID® Cal Detect®) was produced by Preventis GmbH, Wiesenstr, Germany. The test allowed an easy visual evaluation of fecal calprotectin, providing three degrees of positivity: low (< 15 μg/g), medium (15-60 μg/g), and high (> 60 μg/g).

Assay protocols

Breath tests were performed during the Baseline Data Collection period (BDC; before the start of the simulation) and in three separate experimental sessions at approximately d 100, 240 and 475 of the mission simulation. During each experimental session, different ^{13}C-BTs performed on the same subject were staggered by at least 3 d to allow the washout of the administered substrates and the recovery of basal ^{13}C levels.

The combined ^{13}C- and H_2-BT for the measurement of the gastric emptying rate of solids and the orocecal transit time consisted of the simultaneous administration of the EXPIROGer® standard meal and inulin, followed by the measurement of the kinetics of the appearance of $^{13}CO_2$ and H_2 in the breath. In preparation for the test, the crewmembers were requested to refrain from fatty meals or a high intake of dietary fiber the day before the test. Antibiotics, fermented milk products and laxatives were also avoided during the 10-d period preceding the test. After an overnight fast, breath samples were collected to measure the basal levels of $^{13}CO_2$ and H_2. Subsequently, the subjects received the EXPIROGer® standard meal and 5.0 g of inulin dissolved in 200 mL of water. Breath samples for $^{13}CO_2$ analysis were collected up to 240 min after substrate ingestion in 12-mL glass tubes, which were then transferred outside the simulator for analysis. Samples for the evaluation of breath H_2 content were collected in plastic bags up to 440 min after substrate ingestion, and the concentration of H_2 was measured on-board immediately after each breath sample had been collected. During the test, the subjects were allowed to drink water and, after 4 h, to resume their usual dietary regimens.

The ^{13}C-BT for the measurement of the gastric emptying rate of liquids consisted of the administration of sodium ^{13}C-acetate followed by the measurement of the kinetics of the appearance of $^{13}CO_2$ in the breath. After an overnight fast, breath samples were collected to measure the basal level of $^{13}CO_2$. Subsequently, the subjects orally received 150 mg of sodium ^{13}C-acetate dissolved in 500 mL of Nutrizon enteral nutrition solution, and breath samples were collected up to 240 min after substrate ingestion in 12-mL glass tubes, which were then transferred outside the simulator for analysis. After assuming the substrate, the subjects were requested not to ingest any additional food or drink until the end of the test.

The fecal calprotectin test was performed directly by the crewmembers during the BDC and on day 130, 220, and 475 of the mission simulation following the instructions provided by the manufacturer (the test was repeated twice in each experimental session).

Sample analysis

For the measurement of the $^{13}CO_2/^{12}CO_2$ ratio, breath samples were analyzed using a BreathMAT IRMS (Ther-

Table 1 Dynamics of the body mass (kg) of the crewmembers

	Crewmember					
	A	B	C	D	E	F
BDC	81.5	99.5	76.6	86.9	82.5	73.5
Exp. session 1	+3.5	-2.0	+4.3	-1.0	+0.1	+0.2
Exp. session 2	+3.3	-8.2	+4.0	-4.6	-2.0	-4.7
Exp. session 3	+1.5	-20.4	-3.8	-6.9	-1.2	-6.8
End of mission simulation	-1.1	-22.6	-5.4	-9.7	-4.0	-7.2

BDC: Baseline Data Collection.

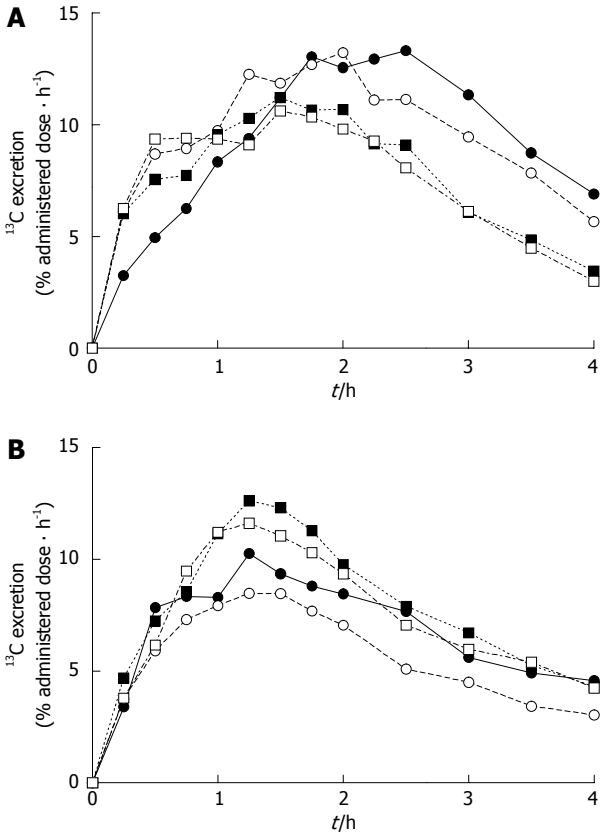

Figure 1 ^{13}C-breath test for the evaluation of gastric emptying rates. Representative $^{13}CO_2$ excretion kinetic profiles obtained in the ^{13}C-breath test for the evaluation of the gastric emptying rates of (A) solids and (B) liquids performed during the Baseline Data Collection period (•) and during the mission simulation (experimental session 1: ◦; experimental session 2: ▪; experimental session 3: ▫).

mo Finnigan MAT GmbH, Bremen, Germany). The measurement of breath H_2 levels was performed onboard by the crewmembers using a portable H_2 analyzer equipped with a miniaturized electrochemical cell (Lactotest 102, Medical Electronic Construction R&D sprl, Brussels, Belgium).

Statistical analysis

The ^{13}C-BT results, given as the $^{13}CO_2$ content of the exhaled CO_2 expressed in δ‰ PDB units (zero δ‰ PDB corresponds to 1.12372% ^{13}C atoms), were processed to evaluate the rate of excretion of $^{13}CO_2$ produced by the metabolism of the ^{13}C-labeled substrate, which was expressed as a percentage of the administered dose per hour. To this purpose, the total expiratory CO_2 production of each subject was assumed to be 300 mmol/m^2 of body surface/h[18], and the body surface was computed as described by Haycock *et al*[19]. For the evaluation of the relevant gastric emptying parameters, the excretion kinetics were analyzed by a least-square fitting procedure using a suitable equation[18], and the gastric emptying half-times were calculated from the coefficients of the equation[20].

The H_2-BT results, given as the H_2 breath concentrations in ppm, were processed for evaluating the enrichment of H_2 in the breath over the basal value due to the fermentation of inulin by the intestinal microflora and then plotted as a function of time; the orocecal transit time was assessed as the time at which the breath hydrogen content rose 10 ppm above the basal value[21].

To assess alterations in GI motility, the results of the breath tests performed during the BDC and during the mission simulation were compared by one-way ANOVA for matched data with Dunnett's post-test using GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA). Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Crew health status

The periodic blood biochemical function tests and clinical examinations during the mission simulation did not show any significant pathology or physiological alteration. Comparison of the body weights of the crewmembers during the BDC and at the end of the mission simulation indicated that one subject (B) displayed a significant reduction in weight (-21%), whereas for the other subjects, the reduction was lower (C, D, E and F) or negligible (A). Although no net increases in body weight were observed, subjects A and C experienced a rise in body mass during the first part of the experiment (Table 1).

^{13}C-BT for gastric emptying rate

Figure 1 shows representative $^{13}CO_2$ excretion kinetic profiles obtained in the ^{13}C-BT for the evaluation of the gastric emptying rates of solids and liquids performed during BDC and in the different experimental sessions during the mission simulation. The gastric emptying half-times obtained for the six crewmembers by analyzing the $^{13}CO_2$ excretion kinetic profiles using the procedure described in the Statistical Analysis section are reported in Table 2.

It can be observed that at the beginning of the simulation (BDC), certain subjects (*i.e.*, A and D) had long gastric emptying half-times of solids (*e.g.*, 4.4 and 5 h for A and D, respectively) and that this behavior was maintained in most of the experimental sessions performed during the mission simulation. As a general rule, long gastric emptying half-times of solids were paralleled (albeit to a lesser extent) by relatively long gastric emptying half-times of liquids, although a large variability in the differences between the two times was observed. Nevertheless,

Table 2 Gastric emptying half-times (h) evaluated by ^{13}C-breath test

Experimental session	Crewmember A	B	C	D	E	F	mean ± SD
Solids							
BDC	4.4	2.8	3.3	5.0	3.2	2.8	3.5 ± 1.0
Exp. session 1	2.7	2.7	2.2	6.2	2.9	2.9	3.2 ± 1.5
Exp. session 2	3.7	2.2	2.9	3.5	2.8	2.5	2.8 ± 0.5
Exp. session 3	4.9	2.2	2.7	4.8	3.4	2.6	3.3 ± 1.2
Liquids							
BDC	2.6	2.3	2.4	3.0	2.8	1.9	2.5 ± 0.4
Exp. session 1	2.6	2.0	2.2	2.8	2.6	2.5	2.5 ± 0.3
Exp. session 2	2.6	2.1	2.7	2.6	2.6	2.5	2.5 ± 0.2
Exp. session 3	2.9	2.0	2.6	4.0	2.8	2.6	2.8 ± 0.7

BDC: Baseline Data Collection.

no evident increasing or decreasing trend in the gastric emptying half-times was detected for any crewmember during the mission simulation; most of the measured gastric emptying half-times were in the range of those reported for healthy subjects[18,22], although in certain cases, rather high values were obtained.

H_2-BT for orocecal transit time

Figure 2 shows the H_2 excretion kinetic profiles obtained in the H_2-BT for the evaluation of the orocecal transit time. The H_2 breath concentrations showed a large variability, sometimes decreasing below the basal level, which increased the difficulty of identifying the H_2 excretion kinetic profiles and evaluating the orocecal transit time by applying the standard criteria reported in the literature (*i.e.*, by identifying the first time at which the breath hydrogen concentration increased by at least 10 ppm above the baseline value).

Although, in several cases, acceptable H_2 excretion profiles were obtained (for example, crewmember D showed high H_2 breath concentrations at long times after substrate ingestion, which were paralleled by a delayed gastric emptying of solids), the overall results suggested that the inulin H_2-BT was negatively affected by external factors, which may have been related to the simulation environment, such as the closed chamber simulating the space station.

Fecal calprotectin test

Table 3 summarizes the results of the fecal calprotectin test for the evaluation of intestinal inflammation performed during the BDC and during the mission simulation. The results are given as scores according to the semi-quantitative evaluation of calprotectin concentration in fecal samples that was performed with the test. Notably, the crewmembers were negative for the fecal calprotectin test during the BDC, but for all of them positive results were obtained in at least one of the tests performed during the mission simulation. The observed degrees of intestinal inflammation varied from low (in two subjects) to high (in four subjects).

Table 3 Results of the fecal calprotectin test[1]

Experimental session	Crewmember A	B	C	D	E	F
BDC	-	-	-	-	-	-
Day 130	-	+++	+	+	-	+++
Day 220	-	-/+[2]	-	-	+++	-/+[2]
Day 475	+++	-/+++[2]	+	-	-/+[2]	+++

[1]Legend: (-) negative, (+) low positivity (< 15 μg/g), (++) medium positivity (15-60 μg/g), (+++) high positivity (> 60 μg/g); [2]The repeated tests gave different results. BDC: Baseline Data Collection.

DISCUSSION

Continuous and non-invasive monitoring of the health status of the crewmembers during space missions requires the development of cutting-edge technologies; their requirements (simple analytical procedures, possibility of self-administration, use of portable point-of-care instrumentation, long shelf-life of reagents) are similar to those faced in critical medicine (*e.g.*, clinical medicine in emergency situations, remote field locations or third-world countries). Thus, new technological solutions that are suitable for the space environment will benefit medical diagnostics for all of us.

In this work, ^{13}C- and H_2-BT were employed for the non-invasive monitoring of GI motility during the MARS-500 project. The accuracy of ^{13}C- and H_2-BT for the measurement of motor functions of the GI system has been demonstrated by several studies[21,23-25]. However, the application of BT in the space environment still requires certain improvements. For example, the ^{13}C-octanoic acid BT is typically performed using extemporaneously prepared meals (*e.g.*, ^{13}C-octanoic acid is incorporated into egg yolk, which is then pan-cooked and consumed with bread and butter), which makes meal standardization difficult and limits test reproducibility. To overcome this drawback, we employed a ready-to-eat test meal (a muffin containing 100 mg of ^{13}C-octanoic acid) with carbohydrate, lipids, proteins and calorie content optimized for the BT performance. The long-term stability of this test meal and its suitability for the measurement of the gastric emptying rate of solids have been evaluated in a multicenter study[26]. Moreover, the muffin is designed for diagnostics; thus, it is gluten-, lactose- and glucose-free to enable its administration to subjects who are affected by celiac disease, lactose intolerance or diabetes, and the unpleasant taste and odor that are characteristic of short-chain fatty acids are efficiently masked. We also combined the ^{13}C-octanoic acid BT for measuring the gastric emptying rate of a solid meal and the inulin H_2-BT for measuring the orocecal transit time into a single test to reduce the number of experimental sessions in the mission simulation and to allow the direct comparison of two different indexes of GI motility, avoiding subject day-to-day variability.

Regarding the instrumentation employed for the an-

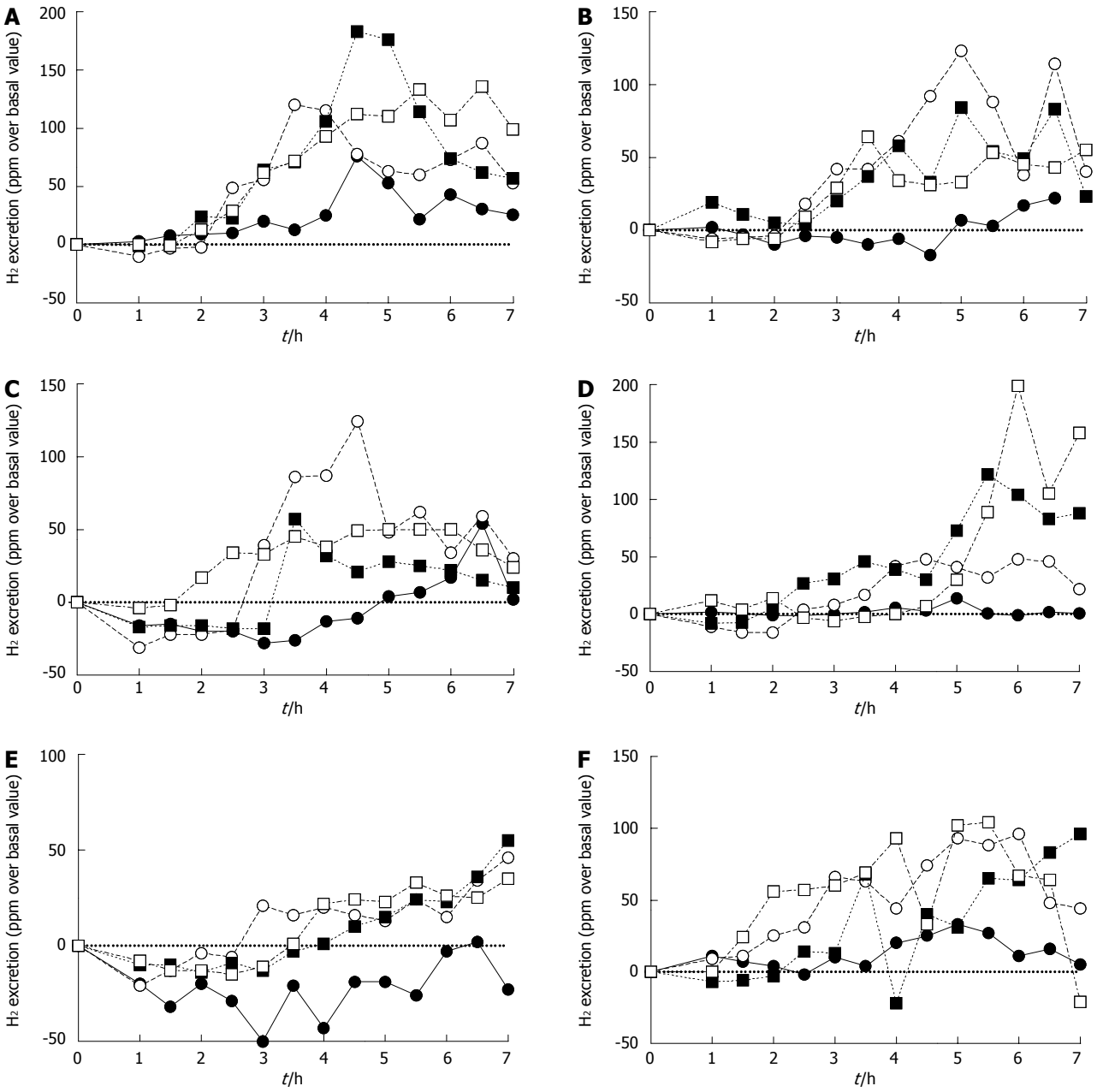

Figure 2 H_2-breath test for the evaluation of orocecal transit time. Hydrogen excretion kinetic profiles obtained in the H_2-breath test (BT) for the evaluation of the orocecal transit time performed during the Baseline Data Collection period (•) and during the mission simulation (experimental session 1: ◦; experimental session 2: ▪; experimental session 3: ▫). This BT was performed simultaneously with the 13C-octanoic acid BT for the evaluation of the gastric emptying rate of solids (A-F).

alysis of the breath samples, the measurement of the $^{13}CO_2/^{12}CO_2$ ratio was performed by IRMS in an external laboratory. However, NDIRS, which is more amenable to miniaturization, could also be used [17,20]. Work is in progress to develop a miniaturized hybrid analytical device combining the NDIRS technology for $^{13}CO_2$ measurement with the fuel cell technology for H_2 measurement employed in the Lactotest 102 H_2 breath analyzer. Such a device will allow the simultaneous onboard measurement of the $^{13}CO_2/^{12}CO_2$ ratio and H_2 concentration in a single breath sample, thus avoiding the need for separate breath sample collection in dual BT.

The results obtained during the MARS-500 experiments did not show significant alterations in the gastric emptying rates of solids and liquids (researchers are currently increasingly inclined to use only gastric emptying half-times when reporting the results of the ^{13}C-octanoic acid BT; therefore, we do not discuss other gastric emptying parameters, such as the lag time). Subjects A and D presented long gastric emptying half-times of solids with high variability, but no unambiguous trends were observed. Moreover, it should be taken into account that Choi *et al*[27,28] suggested that the truncation of the observation period of ^{13}C-octanoic acid BT to four hours could lead to an overestimation of gastric emptying half-times. Therefore, the long half-times measured for subjects A and D could be at least in part ascribed to this factor (indeed, these gastric emptying half-times were

close to or even longer than the observation period).

In contrast, the results of the H_2-BT for the orocecal transit time, performed simultaneously with the ^{13}C-octanoic acid BT, were difficult to interpret because the high variability of the H_2 concentration in the breath samples did not allow a reliable evaluation of the orocecal transit times. Nevertheless, certain results suggested, as expected, a positive correlation with gastric emptying half-times. For example, in subject D, who showed the longest gastric emptying half-times for solids, the highest concentrations of H_2 in the breath were often detected at longer times in comparison with the other subjects. These results suggested that the H_2-BT was strongly affected by external factors, such as the diet of the crewmembers (hydrogen can be produced by the fermentation of other food sugars and related substances, such as dietary fiber) and the environmental conditions (*e.g.*, the possible accumulation of hydrogen in the simulator microenvironment). Indeed, hydrogen concentrations up to 30-40 ppm were recorded inside the simulator, whereas external values remained below 1.0 ppm. Moreover, the portable H_2 analyzer employed in this experiment required manual injection of the breath sample; thus, the reproducibility of the measurement could be improved by implementing automated sample management procedures. Nevertheless, in the absence of further information, it might be concluded that in closed microenvironments, such as the MARS-500 simulator, ^{13}C-BTs should be preferred to H_2-based tests. In particular, the lactose ^{13}C-ureide BT, which has been established as a reliable test for the assessment of orocecal transit time[29,30], could represent an alternative to the inulin H_2-BT.

In contrast to ^{13}C-BTs, the fecal calprotectin test detected significant alterations during the mission simulation: all of the crewmembers were negative for the test during the BDC but showed various degrees of positivity (from low for subjects C and D to high for subjects A, B, E, and F) in at least one of the tests performed during the mission simulation. Calprotectin is a sensitive fecal marker of intestinal inflammation that is used to differentiate between organic intestinal diseases (*e.g.*, chronic inflammatory diseases, infectious diseases, or colon cancer) and functional intestinal diseases (*e.g.*, irritable bowel syndrome)[31,32]. Application of calprotectin test for screening asymptomatic subjects has also been reported[33,34]. Fecal calprotectin can be determined with high specificity and sensitivity using the CalDetect® lateral flow immunoassay[35]. Because it has been already demonstrated in animal models and humans that stress influences the inflammatory response[36,37], the stress conditions experienced by the crewmembers could be responsible for the observed intestinal inflammation, although external factors related to diet and environment, as well as possible alterations in the intestinal microflora, cannot be excluded.

In conclusion, the results obtained in the MARS-500 mission simulation suggested that the stress level experienced by crewmembers during the mission simulation had no significant impact on the GI motility. Because previous experiments performed in microgravity conditions showed alterations in the GI motility[38,39], it could be concluded that microgravity should have a major impact on GI motor functions, whereas stress-related factors might contribute to the onset of motility alterations but are not the primary cause. Nevertheless, useful information on the possible application of BTs in future isolation experiments or real space missions has been obtained. Due to their simplicity of performance, ability to be performed repeatedly, safety, and non-invasiveness, ^{13}C-BTs represent a promising approach for the monitoring of alterations of motor and/or organ functions of the GI system, thus moving space medicine closer to clinical observation systems used on Earth. In the MARS-500 experiments, $^{13}CO_2$ analysis in breath samples was performed by IRMS in an external analysis facility, but portable analytical instruments for $^{13}CO_2$ breath analysis (for example, based on the NDIRS technology) integrated within an informatics framework for data acquisition, analysis, and remote transmission will allow crewmembers to perform such tests autonomously. Regarding H_2-BT, suitable portable H_2 breath analyzers are already available, but the results suggested that the performance of this BT is strongly affected by external factors; thus, it could be concluded that in this type of application, ^{13}C-BTs should be preferred to H_2-based tests. In addition, the measurement of fecal calprotectin by a cassette-type lateral flow immunoassay evidenced a significant degree of intestinal inflammation in all the crewmembers. Although no clinical symptoms associated with intestinal inflammation were reported during the mission simulation, the possibility that a combination of isolation, stress and dietary factors (*i.e.*, prolonged nutrition with canned and preserved foods) could favor the onset of this pathological status should be considered in future mission simulations or real space flights.

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COMMENTS

Background

Extended-duration space missions expose the crewmembers to microgravity, radiation, stress and other factors that can affect their physiological status. For instance, changes in gastrointestinal motility may result in the reduced intestinal

absorption of nutrients, alterations in the intestinal microflora and decreased bioavailability of orally administered drugs. Such possible alterations must be detected expeditiously to avoid negative consequences to the crewmembers' health and, more generally, wellness.

Research frontiers

The evaluation of the gastrointestinal motility during a real space mission requires biochemical tests that can be easily performed onboard by the crewmembers. Biological samples should be easily collectable in a microgravity environment (*e.g.*, saliva or breath expatriate) and analyzed using compact devices in a point-of-care format. Tests and related analytical instrumentation are to be implemented in the space module and validated for its clinical utility and applicability in spaceflight.

Innovations and breakthroughs

In this study, ^{13}C- and H_2-breath tests for the monitoring of gastrointestinal motility have been designed to be self-performed without any external assistance by the subjects participating in the final 520-d isolation experiment in the frame of the MARS-500 project. The reagents for breath test performance have been optimized for long-term storage (no materials could be introduced into the simulator during the isolation period) and minimum preparation required before use; a portable H_2 analyzer equipped with a miniaturized electrochemical cell has been provided to allow the onboard measurement of breath H_2 levels by the crewmembers. A commercially available cassette-type lateral flow immunoassay was also employed for detecting fecal calprotectin, a biomarker of intestinal inflammation.

Applications

The study suggested that breath tests, especially those based on ^{13}C, could be employed for the monitoring of alterations of motor and/or organ functions of the gastrointestinal system in future isolation experiments or real space missions.

Peer review

The authors present an interesting application of non-invasive gastrointestinal (GI) motility and lower intestinal inflammation tests in a closed-chamber space simulation. Although the results overall reveal no significant change in gastric emptying and require additional confirmation, this study represents an interesting demonstration of how GI monitoring may be achieved with very limited resources. This battery of tests could find application not only in outer space but also in bedside testing in a variety of clinical environments, both inpatient and outpatient.

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BRIEF ARTICLE

Evolution of disease phenotype in adult and pediatric onset Crohn's disease in a population-based cohort

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Abstract

AIM: To investigate the evolution of disease phenotype in adult and pediatric onset Crohn's disease (CD) populations, diagnosed between 1977 and 2008.

METHODS: Data of 506 incident CD patients were analyzed (age at diagnosis: 28.5 years, interquartile range: 22-38 years). Both in- and outpatient records were collected prospectively with a complete clinical follow-up and comprehensively reviewed in the population-based Veszprem province database, which included incident patients diagnosed between January 1, 1977 and December 31, 2008 in adult and pediatric onset CD populations. Disease phenotype according to the Montreal classification and long-term disease course was analysed according to the age at onset in time-dependent univariate and multivariate analysis.

RESULTS: Among this population-based cohort, seventy-four (12.8%) pediatric-onset CD patients were identified (diagnosed $\leqslant$ 17 years of age). There was no significant difference in the distribution of disease behavior between pediatric (B1: 62%, B2: 15%, B3: 23%) and adult-onset CD patients (B1: 56%, B2: 21%, B3: 23%) at diagnosis, or during follow-up. Overall, the probability of developing complicated disease behaviour was 49.7% and 61.3% in the pediatric and 55.1% and 62.4% in the adult onset patients after 5- and 10-years of follow-up. Similarly, time to change in disease behaviour from non stricturing, non penetrating (B1) to complicated, stricturing or penetrating (B2/B3) disease was not significantly different between pediatric and adult onset CD in a Kaplan-Meier analysis. Calendar year of diagnosis (P = 0.04), ileal location (P < 0.001), perianal disease (P < 0.001), smoking (P = 0.038) and need for steroids (P < 0.001) were associated with presence of, or progression to, complicated disease behavior at diagnosis and during follow-up. A change in disease location was observed in 8.9% of patients and it was associated with smoking status (P = 0.01), but not with age at diagnosis.

CONCLUSION: Long-term evolution of disease behavior was not different in pediatric- and adult-onset CD patients in this population-based cohort but was associated to location, perianal disease and smoking status.

Key words: Crohn's disease; Age at diagnosis; Disease behavior; Disease course

Lovasz BD, Lakatos L, Horvath A, Szita I, Pandur T, Mandel M, Vegh Z, Golovics PA, Mester G, Balogh M, Molnar C, Komaromi E, Kiss LS, Lakatos PL. Evolution of disease phenotype in adult and pediatric onset Crohn's disease in a population-based cohort. *World J Gastroenterol* 2013; 19(14): 2217-2226 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i14/2217.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i14.2217

INTRODUCTION

Inflammatory bowel disease (IBD) is multifactorial: both genetic and environmental risk factors (*e.g.*, smoking, or appendectomy) contribute to its pathogenesis[1]. During the past two decades, the incidence pattern of IBD has changed significantly[2], showing both common and distinct characteristics. The phenotypic classification of Crohn's disease (CD) plays an important role in patient management, and may help predict the clinical course in CD patients[3]. In 2005, the Montreal revision of the Vienna classification system was introduced[4]. Although the broad categories for CD classification remained the same, changes were made within each category. Upper gastrointestinal (GI) disease is now classified independently of, or alongside, disease at more distal locations. Finally, perianal disease, which occurs independently of small bowel fistulae, is no longer classified as penetrating disease. Instead, a perianal modifier has been introduced, which may coexist with any disease behavior.

Using the Vienna classification system, it has been shown in clinical cohorts that there can be a significant change in disease behavior over time, whereas disease location remains relatively stable[3,5]. In a landmark paper by Cosnes *et al*[6], up to 70% of CD patients developed either penetrating or stricturing disease. Louis *et al*[5] reported similar results in a Belgian study, in which 45.9% of patients had a change in disease behavior ($P < 0.0001$) during 10 years of follow-up, especially from non-stricturing, non-penetrating disease to either stricturing (27.1%; $P < 0.0001$) or penetrating (29.4%; $P < 0.0001$) forms. Age at diagnosis (before or after age 40) had no influence on either disease location or behavior. In contrast, disease location remained relatively stable during follow-up, with only 15.9% of patients exhibiting a change in disease location during the first 10 years. In addition, the probability of change in disease behavior in patients with initially non-stricturing, non-penetrating disease was 30.8% over nine years in a more recent Hungarian study[7], in which data were obtained from referral centers.

More recently, authors from New Zealand[3] showed in a population-based cohort study, that although > 70% percent of CD patients had inflammatory disease at diagnosis, 23% and 40% of patients with initial inflammatory disease progressed to complicated disease phenotypes after five and ten years of follow-up, respectively. This was not associated with age at onset. In contrast, disease location remained stable in 91% of patients with CD. Of note however, the median follow-up of CD patients was only 6.5 years. Similarly, in the IBSEN cohort, 36%, 49% and 53% of CD patients diagnosed between 1990 and 1994 initially had or developed either stricturing or penetrating complications[8]. In addition, recent data suggest a change in the natural history of CD as shown by decreasing surgical rates[9].

According to the available literature, pediatric onset CD runs a more aggressive course, including more extensive disease location, more upper GI involvement, growth failure, more active disease, and need for more aggressive medical therapy, in predominantly referral-center studies[10-12]. However, data so far have been partly contradictory, and pediatric disease behavior seems to parallel that of adults[13]. A Scottish study simultaneously compared disease behavior and location in pediatric and adult onset IBD patients[14]. In childhood-onset patients, there was a clear difference in disease location at onset and after five years; with less ileum- and colon-only location among pediatric-onset patients, but more ileocolonic and upper gastrointestinal involvement ($P < 0.001$ for each). In addition, disease behavior after five years did not differ between the two groups. In contrast, disease phenotype was associated with location. However, the evolution of disease phenotype was not studied.

Because only limited data are available on the evolution of disease phenotype in patients with a pediatric- and adult-onset CD in from a single population-based cohort over a long-term follow-up, the aim of this study was to analyze the evolution of disease behavior and location in a population-based Veszprem province database according to the age-group at diagnosis, which included incident adult- and pediatric-onset CD populations diagnosed between January 1, 1977 and December 31, 2008.

MATERIALS AND METHODS

Patients

A well-characterized Hungarian cohort of 1420 incident cases of inflammatory bowel disease diagnosed between January 1, 1977 and December 31, 2008 were included. In total, 506 CD patients [CD, male: female: 251:255, age at diagnosis: 28.5 years, interquartile range (IQR): 22-38 years] were diagnosed during the inclusion period. Patients were followed until December 31, 2009 or death. All patients had at least one year of follow-up data avail-

Table 1 Clinical characteristics of patients with Crohn's disease

	CD (*n* = 506)
Male/female	251/255
Age at presentation (yr)[1]	28.5 (22-38)
Follow-up (yr)[1]	13.5 (6-19.5)
Familial IBD	12.90%
Location at diagnosis	
L1	32.80%
L2	35.90%
L3	30.60%
L4 only	0.70%
L4	4.80%
Behavior at diagnosis	
B1	56.90%
B2	19.80%
B3	23.30%
Frequent relapse	13.10%
Perianal disease	25.50%
Arthritis	26.70%
PSC	1.80%
Ocular	4.70%
Cutaneous	9.30%
Steroid use	68.60%
Azathioprine use	45.80%
Biological use	10.70%
Resection/re-operation	41.3%/28.2%

[1]Median (interquartile range). L1: Ileal; L2: Colon; L3: Ileocolon; L4: Upper gastrointestinal; B1: Inflammatory; B2: Stenosing; B3: Penetrating; PSC: Primary sclerosing cholangitis.

able. Patients with indeterminate colitis at diagnosis were excluded from the analysis. Patient clinical data is summarized in Table 1. The ratio of urban-to-rural residence was also relatively stable (55% urban).

Methods

Data collected from 7 general hospitals and gastroenterology outpatient units (Internal Medicine Departments, Surgery Departments, Paediatric Departments and Outpatient Units) from Veszprem County (Veszprem, Papa, Tapolca, Ajka, Varpalota, Zirc). A more detailed description of the data collection and case assessment methods used, as well as the geographical and socioeconomic background of the province and the Veszprem Province IBD Group was published in previous epidemiological studies by this group[15].

The majority of patients (94% of CD and 71% of ulcerative colitis patients) were monitored at the Csolnoky F Province Hospital in Veszprem. This hospital also serves as a secondary referral center for IBD patients in the province. Data collection was prospective since 1985; prior to that, only in Veszprem were data collected prospectively. In other sites throughout the province, data for this period (1977-1985) were collected retrospectively in 1985. Both in- and outpatients permanently residing in the area were included in the study. Diagnoses (based on hospitalization records, outpatient visits, endoscopic, radiological, and histological evidence) generated in each hospital and outpatient unit were reviewed thoroughly, using the Lennard-Jones[16] or the Porto criteria[17], as appropriate. At the Veszprem pediatric IBD clinic, all probable cases of IBD are evaluated in a single unit by a pediatric gastroenterologist with experience in the diagnosis and treatment of IBD together with adult gastroenterologists. In addition, all endoscopies for pediatric patients are performed and all follow-up is conducted by two expert adult gastroenterologists, and pediatric cases were followed together by pediatric and adult gastroenterologists. According to the Montreal classification, an age at diagnosis < 17 years was defined as pediatric onset.

Age, age at onset, the presence of familial IBD, presence of extraintestinal manifestations (EIM) including: arthritis, conjunctivitis, uveitis, episcleritis, erythema nodosum, pyoderma gangrenosum, primary sclerosing cholangitis (PSC), and the frequency of flare-ups (frequent flare-up: > 1/year[18]) were registered. Disease phenotype (age at onset, duration, location, and behavior) was determined according to the Montreal classification[4] (based on: age at onset, location, and behavior, with perianal and upper GI disease as additional modifiers). Non-inflammatory behavior was defined as either stricturing or penetrating disease. Perianal disease and behavior change (from B1 to B2 or B3) or location during follow-up was also registered. Every significant flare or new symptom was meticulously investigated by gastroenterology specialists. Morphological investigations included proctosigmoidoscopy, colonoscopy, computed tomography (CT) scan, small-bowel ultrasound and small bowel X-ray. Patients in clinical remission had regular follow-up visits including laboratory and imaging studies (annual abdominal ultrasound). Endoscopy and CT-scans were only occasionally performed in patients in clinical remission. Of note, upper GI symptoms were carefully evaluated. Only indisputable manifestations were classified as upper GI involvement (*e.g.*, stenosis, ulcers), but not small erosions, or even simple gastric or duodenal ulcers, the later occurring shortly after the start of high dose systemic steroid therapy. Upper GI endoscopy was performed regularly at the time on the diagnosis of CD only in the last ten years, earlier only in case of gastroesophageal symptoms.

Medical therapy was thoroughly registered (*e.g.*, steroid, immunosuppressive, or biological use, azathioprine intolerance as defined by the European Crohn's and Colitis Organization, Consensus Report 28), need for surgery or reoperation (resections in CD), development of colorectal and small bowel adenocarcinoma, other malignancies, and smoking habits, were investigated by reviewing medical records during follow-up and by the completion of a questionnaire. Only patients with a confirmed diagnosis for more than one year were enrolled.

In addition, due to Hungarian health authority regulations, a follow-up visit is obligatory for IBD patients at a specialized gastroenterology center every six months. Otherwise, under the conditions of the Hungarian na-

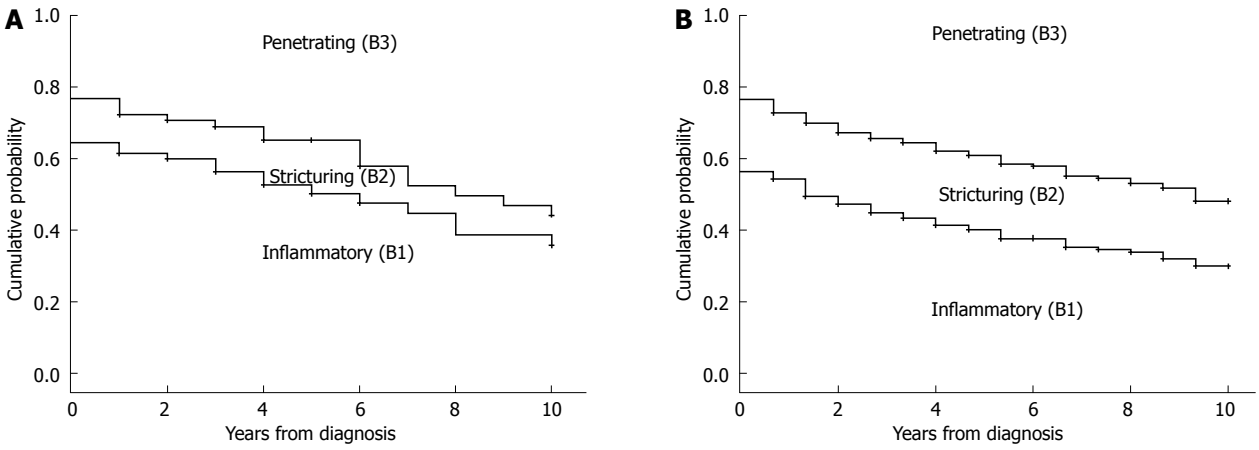

Figure 1 The evolution of disease behavior in patients with Crohn's disease according to the age at diagnosis. A: Pediatric onset; B: Adult onset.

tional health insurance system, patients forfeit their right to ongoing subsidized therapy. Consequently, the relationship between IBD patients and specialists is a close one.

The study was approved by the Semmelweis University Regional and Institutional Committee of Science and Research Ethics and the Csolnoky F Province Hospital Institutional Committee of Science and Research Ethics.

Statistical analysis

Variables were tested for normality by Shapiro Wilk's *W*-test. The distribution of disease behavior at different time points and between subgroups of CD patients was compared by χ^2-test with Yates correction. Odds ratios (OR) were calculated. Kaplan-Meier survival curves were plotted for analysis with LogRank and Breslow tests to determine probability of disease behavior change in patients with inflammatory (B1) behavior at diagnosis. Additionally, Cox-regression analysis using the enter method was used to assess the association between categorical clinical variables and time to disease behavior or location change. Variables with a P value < 0.2 in univariate analysis were included in the multivariate testing results for continuous variables are expressed as median (IQR) unless otherwise stated. Peter Laszlo Lakatos performed all statistical analysis. For statistical analysis, SPSS® 15.0 (SPSS Inc., Chicago, IL) was used. A P value of < 0.05 was considered significant.

RESULTS

Evolution of disease phenotype in CD patients according to age at onset

Five hundred six residents of the Veszprem province were diagnosed with CD during the 32-year period from 1977 to 2008. The clinical characteristics of these patients are shown in Table 1. Sixty-five (12.8%) CD patients were diagnosed < 17 years of age. Follow-up information was collected up to December 31 2009, equaling 5758 patient-years of follow-up.

There was no significant difference in the distribution of disease behavior between pediatric (B1: 62%, B2: 15%, and B3: 23%) and adult onset CD patients (B1: 56%, B2: 21%, and B3: 23%) at diagnosis (P = NS). In addition, the distribution of disease behavior after 1, 3, 5, 7, 10 and 15 years and the probability of developing penetrating or complicated (stenosing/penetrating) disease behavior during follow-up did not significantly differ in patients with pediatric and adult onset disease by χ^2 and Kaplan-Meier analysis (Figure 1, PLogRank = NS, PBreslow = NS) Because the length of follow-up differed between the groups, statistical analysis was not performed using final disease behavior data.

Similarly, the probability and time to change in disease behavior from B1 to B2/B3 disease was not significantly different between pediatric- and adult-onset CD in a Kaplan-Meier analysis (Figure 2). The probability of complicated disease behavior for patients who initially exhibited inflammatory disease behavior was 7.6%, 27.5%, and 42.0% in the pediatric and 12.1%, 26.4%, and 37.5% in the adult-onset patients after 1, 5, and 10 years of follow-up (PLogRank = NS, PBreslow = NS).

In contrast, the distribution of disease location at diagnosis was different between pediatric- and adult-onset CD patients (L3 pediatric-onset: 41.3%, *vs* adult-onset: 28.8% P = 0.05, Figure 3). A change in disease location was observed 8.9% of the CD patients. The probability of change in disease location was 5.2%, 8.9%, and 10.8% after 5, 10, and 15 years of follow-up, respectively. However, this did not differ according to age at onset (PLogRank = NS).

Predictors of progression of disease behavior and location

The calendar year of diagnosis and location were associated with presence of or progress to complicated disease behavior at diagnosis and during follow-up. There was a significant difference in the distribution of disease behavior in patients diagnosed from 1977 to 1998 (n = 273, B1: 50%, B2: 22%, and B3: 28%) and from 1999 to 2008 (B1: 65%, B2: 17% and B3: 18%) at diagnosis (P

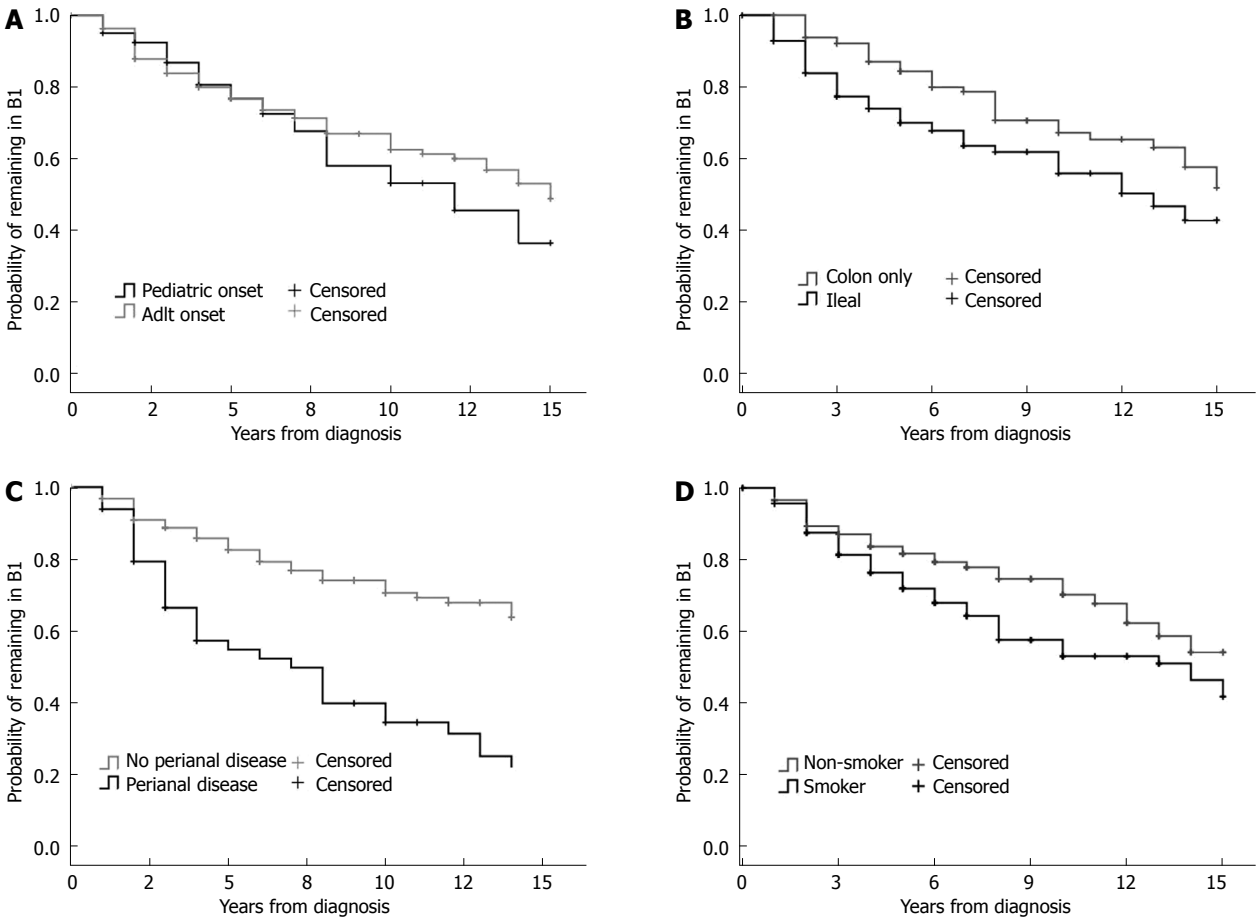

Figure 2 The probability of remaining in inflammatory (B1) disease behavior in patients with Crohn's disease according to the age at diagnosis (A), location (B), presence of perianal disease (C) and smoking status (D). A: *P*LogRank = 0.40, *P*Breslow = 0.62; B: *P*LogRank = 0.013, *P*Breslow = 0.002; C: *P*LogRank < 0.001, *P*Breslow < 0.001; D: *P*LogRank = 0.038, *P*Breslow = 0.051.

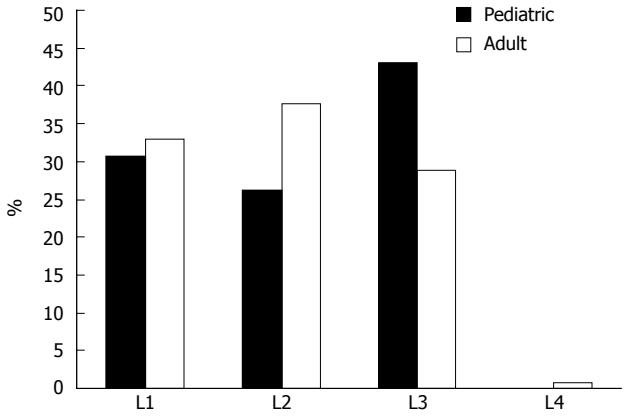

Figure 3 Distribution of disease location in Crohn's disease patients at diagnosis according to the age at onset.

= 0.003) and after one, three, and five years of follow-up ($P_{1\text{-year}}$ = 0.007, $P_{3\text{-years}}$ = 0.002, $P_{5\text{-years}}$ < 0.001 by χ^2 analysis, and in the probability of developing penetrating or complicated (stenosing/penetrating) disease behavior during follow-up in a Kaplan-Meier analysis [*P*LogRank < 0.001, *P*Breslow < 0.001 (Figure 4)]. The probabilities of penetrating or complicated (stenosing/penetrating) disease behavior after three and seven years of follow-up were 37.4% and 44.8%, and 58.4% and 66.2%, in the 1977-1998 cohort, while this was 26.5% and 34.4%, and 43.6% and 50.6%, in the 1999-2008 cohort.

Trends were similar when pediatric-onset and adult-onset patients were analyzed separately. The disease behavior pattern at diagnosis did not differ significantly between the two groups diagnosed in 1977-1998 (pediatric-onset *n* = 33, B1: 51%, B2: 21%, and B3: 28%, adult-onset *n* = 240, B1: 50%, B2: 22%, and B3: 28%) and in 1999-2008 (pediatric-onset *n* = 32, B1: 72%, B2: 9%, and B3: 19%, adult-onset *n* = 201, B1: 64%, B2: 18%, and B3: 18%). The evolution of disease behavior was also similar to the full cohort (data not shown).

In addition, disease location and presence of perianal disease was associated with disease behavior at diagnosis (colon only B1: 73%, B2: 12%, and B3: 15%, *vs* ileal involvement B1: 48%, B2: 24%, and B3: 28%, $P < 0.001$; perianal disease absent B1: 66%, B2: 21%, and B3: 13%, perianal disease present B1: 30%, B2: 15%, and B3: 55%, $P < 0.001$). Probability of change in disease behavior from B1 to B2/B3 disease was significantly higher in patients with ileal involvement (*P*LogRank = 0.013, *P*Breslow = 0.002, $HR_{L1 \text{ or } L3}$ = 2.27, 95%CI: 1.32-3.92)

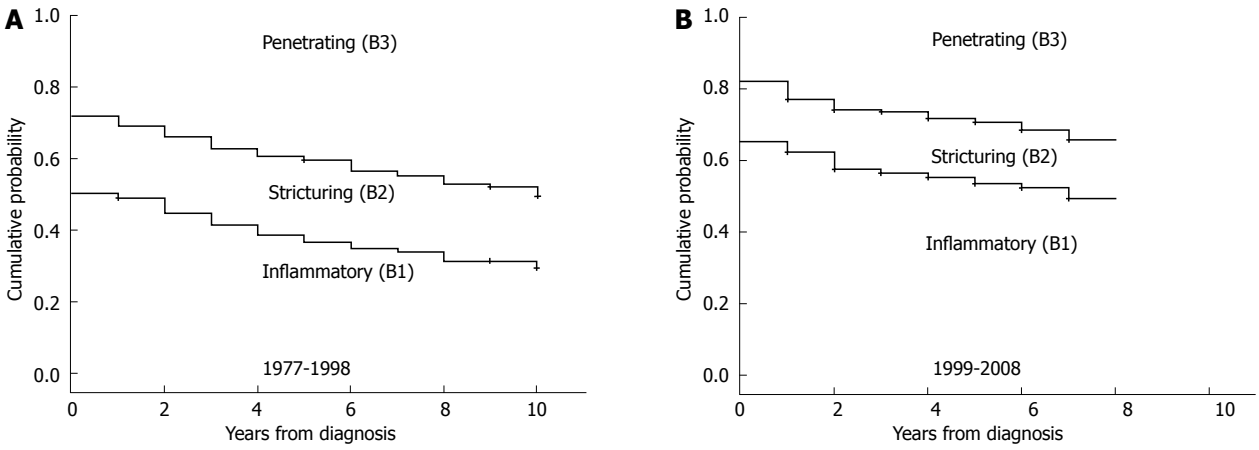

Figure 4 The evolution of disease behavior in patients with Crohn's disease according to the year of diagnosis. A: 1977-1998; B: 1999-2008. *P*LogRank < 0.001, *P*Breslow < 0.001 for complicated behavior between groups A and B.

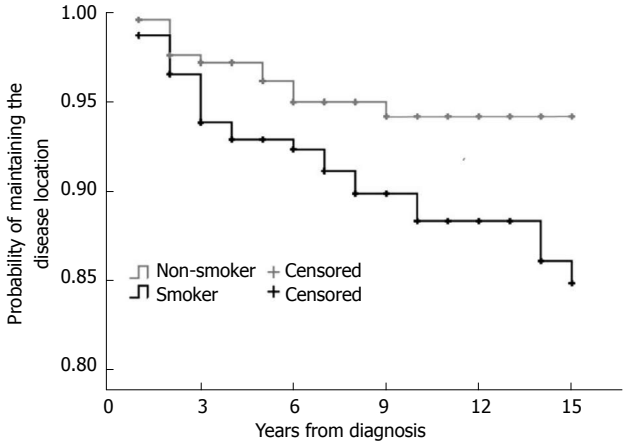

Figure 5 The probability of maintaining the disease location in patients with Crohn's disease according to the smoking status. *P*LogRank = 0.011, *P*Breslow = 0.002.

and perianal disease (*P*LogRank < 0.001, *P*Breslow < 0.001, $HR_{perianal}$ = 2.98, 95%CI: 2.21-4.03) (Figure 2B-D). Similarly, need for steroids, either at diagnosis or during follow-up, was associated with an increased risk of disease progression (*P*LogRank < 0.001, *P*Breslow < 0.001, HR = 3.66, 95%CI: 1.67-8.04), but not early azathioprine exposure. The same trend was observed for smoking (*P*LogRank = 0.038, *P*Breslow = 0.051, $HR_{smoking}$ = 1.482, 95%CI: 0.96-2.37).

In contrast, calendar year of diagnosis was associated with the progression to non-inflammatory disease behavior (*P*LogRank = 0.04, *P*Breslow = 0.04, $HR_{after\ 1998}$ = 0.73, 95%CI: 0.55-0.97) in patients with initially inflammatory disease. The probability of progression to complicated disease behavior after five and seven years was 15.1% and 21.8% in patients diagnosed after 1998 while this was 27.4% and 33.3% in patients diagnosed between 1977 and 1998. In a multivariate Cox regression analysis, after excluding steroid exposure at any time point from the variables, the effect of location ($P < 0.001$; for L1 $P < 0.001$, HR = 2.19, 95%CI: 1.50-3.09; for L3 $P = 0.01$, HR = 1.59, 95%CI: 1.12-2.28), perianal disease ($P < 0.001$, HR = 3.11, 95%CI: 2.23-4.34) and smoking ($P = 0.031$, HR = 1.42, 95%CI: 1.04-1.96) remained significant.

Interestingly, the probability of disease location change differed according to smoking status (*P*LogRank = 0.011, *P*Breslow = 0.03, $HR_{smoking}$ = 2.35, 95%CI: 1.19-4.63, Figure 5), but not according to gender, initial disease location, behavior, presence of perianal disease or calendar year of diagnosis.

DISCUSSION

This current, population-based, prospective study reveals that evolution of disease behavior and location do not differ significantly between CD patients with adult or pediatric onset during long-term follow-up, as the change of disease phenotype is not different significantly in pediatric- and adult-onset CD, in contrast to previous, large, multicenter studies. There were no significant differences in disease behavior between pediatric- and adult-onset patients at the time of diagnosis or during follow-up. In contrast, findings presented here confirm the results of previous studies, namely, that disease location was significantly different according to the age at diagnosis. Pediatric patients presented more frequently with extensive disease. A change in disease location was relatively rare and it was associated with smoking status.

The same, progressive characteristics of CD were described by Cosnes *et al*[6] in a study of patients treated at a French referral center. More than 80% of patients developed complications with time. After 5 and 20 years' disease duration, the risk for stricturing disease complications were 12% and 18% respectively, whereas 40% and 70% of patients developed penetrating complications, respectively. An association was reported, however, with disease location; the probability of complicated disease was as high as 94% after 20 years in patients with ileal disease. Results were comparable in a Belgian study[5]. In this study, 45.9% of patients had a change in disease be-

havior after 10 years of follow-up, especially from non-stricturing, non-penetrating disease to either stricturing (27.1%) or penetrating (29.4%) disease. The frequency of complicated disease was somewhat lower in the present population-based study. The probability of developing penetrating complications was 35.4% and 58.2% after 5 and 20 years' disease duration, while 55.9% and 73.7% of patients diagnosed between 1977-2008 developed either penetrating or stricturing complications. Finally, 53% of patients developed stricturing or penetrating disease during a 10-year follow-up in the population-based prospective IBSEN cohort in CD patients diagnosed between 1990 and 1994[8]. Of note, however, in the study by Cosnes *et al*[6], classification of patients according to disease behavior was a poor predictor of disease activity during the next five years. A similar proportion of patients required immunosuppressive drugs and surgery.

According to previous data, the natural history was reported to be more severe in pediatric CD. Extensive, complicated disease phenotypes were reported to be frequent in a population-based study by Vernier-Massouille *et al*[19] In this study, the prevalence of B2 and B3 phenotypes increased from 25% to 44%, and from 4% to 15%, while the frequency of B1 disease decreased from 71% to 41%, respectively, from diagnosis until approximately 10 years of follow-up. In addition, according to a recent French study by Pigneur *et al*[10] patients with early childhood-onset onset CD often have more severe disease, increased frequency of active periods, and increased need for immunosuppressants. In contrast, in the present study, disease behavior at diagnosis and the rate of progression to complicated disease did not differ between pediatric- and adult-onset CD patients. Similarly, in a population-based cohort in New Zealand[3], age at diagnosis was not predictive of the rate of progression from inflammatory to complicated disease behavior. Until now, this was the only study that investigated the importance of age at onset according to the Montreal classification including pediatric onset patients. However, significant data were collected retrospectively and the median follow-up was 6.5 years which is half of the median follow-up of patients in the present study. In addition, > 70% of CD patients had inflammatory disease at diagnosis, with 23% and 40% of patients with initial inflammatory disease progressing to complicated disease phenotypes after five and ten years of follow-up.

Previous studies suggested that the disease location was different between pediatric and adult onset patients with more ileocolonic and upper GI disease in pediatric patients[3,6,19], in concordance with the present study. In a French population-based pediatric CD study[19] the most frequent location at diagnosis was ileocolonic disease (63%). Disease extension was observed in a surprisingly large proportion of pediatric patients (31%) during follow-up. In addition, in a population-based New-Zealand CD cohort, authors have reported an association between initial disease location and probability of disease extension. Patients with colon-only location progressed more rapidly to ileocolonic disease than those with ileal disease ($P = 0.02$). Of note, the rate of disease location change at 10 years in this study (9%) was in the range reported in the present study (8.9% during a median 13 years), although somewhat higher rates were reported in the study by Louis *et al*[5] (15.9% during 10-years). In the latter study, 20.3% of patients with an initial L1 location changed to another location, while the proportion of patients changing from L2 was 16.7%. In the present study, the probability of disease behavior change was 8.8% and 10.9% after 10 and 15 years of disease duration. The change in disease location was not different between patients with pediatric or adult onset, nor between patients with L1 and L2 disease. In contrast, a novel finding of the present study was that change in disease location was associated with smoking status (HR = 2.35, $P = 0.01$). The probability of a change in disease location was 5.8% and 5.8% in non-smokers, and 11.7% and 15.1% in smokers after 10 and 15 years' disease duration.

Additional predictors of disease behavior change identified in the present study included presence of ileal involvement, perianal disease, smoking and calendar year of diagnosis, with perianal involvement being the most important predictor. The role of initial ileal involvement, extensive disease, and perianal disease as a possible predictor of non-inflammatory behavior was first suggested in a landmark study by Cosnes *et al*[6]. Additionally age < 40 years at diagnosis was associated with the development of penetrating complications (HR = 1.3). Similar findings were presented from the New Zealand cohort[3], where patients with ileal (L1) disease progressed most quickly to non-inflammatory disease behavior, followed by patients with upper GI (L4) or ileocolonic (L3) disease ($P < 0.0001$). The probability of progression to penetrating disease was similar to that of progression to stenosing disease after 10 years. Overall, the proportion of penetrating disease was highest in those with ileocolonic (27%) or ileal disease (21%) compared to patients with colon-only disease (7%, $P = 0.006$). Patients with perianal disease were at risk of a change in disease behavior (HR = 1.62, 95%CI: 1.28-2.05). In a subsequent population-based study from the IBSEN group[8], non-inflammatory disease behavior during follow-up was associated with initial L1 (86%) *vs* L2 (30%, $P < 0.001$) or L3 location (60%, $P < 0.005$). Finally, in a previous publication by our group[7], ileal disease location (HR = 2.13, $P = 0.001$), presence of perianal disease (HR = 3.26, $P < 0.001$), prior steroid use (HR = 7.46, $P = 0.006$), early AZA (HR = 0.46, $P = 0.005$) and smoking (HR = 1.79, $P = 0.032$) were independent predictors of disease behavior change in a referral CD cohort. Data regarding the effect of smoking are equivocal, however. A recent review[20] and previous studies have demonstrated that smoking was associated with complicated disease, penetrating intestinal complications[21], and greater likelihood

of progression to complicated disease, as defined by development of strictures or fistulae, a higher relapse rate, and need for steroids and immunosuppressants[22]. In a recent study by Aldhous *et al*[23], the deleterious effect of smoking was only partially confirmed. Current smoking was associated with less colonic disease, however smoking habits at diagnosis were not associated with time to development of stricturing, penetrating disease, nor with perianal penetrating disease or time to first surgery. Of note, a possible neutralizing effect of immunosuppressant therapy was reported in some studies[24,25].

Conclusions were slightly different if authors assessed the factors associated with the development of disabling disease. In the paper by Loly *et al*[26] stricturing behavior at diagnosis (HR = 2.11, P = 0.0004) and weight loss (> 5 kg) at diagnosis (HR = 1.67, P = 0.0089) were independently associated with time to the development of severe disease in multivariate analysis. The definition of severe, non-reversible damage was, however, much more rigorous. It was defined by the presence of at least one of the following criteria: the development of complex perianal disease, any colonic resection, either two or more small-bowel resections or a single small-bowel resection measuring more than 50 cm, or the construction of a definite stoma. In a similar study by Beaugerie *et al*[27], with a different definition of disabling disease, initial requirement for steroid use (OR = 3.1, 95%CI: 2.2-4.4), an age below 40 years (OR = 2.1, 95%CI: 1.3-3.6), and the presence of perianal disease (OR = 1.8, 95%CI: 1.2-2.8) were associated with the development of disabling disease[27]. The positive predictive value of disabling disease in patients with two and three predictive factors of disabling disease was 0.91 and 0.93, respectively. Concordantly, in the present study, need for steroids was identified as a risk factor for progression of disease behavior (HR = 3.66, $P < 0.001$).

Finally, the calendar year of diagnosis was associated with disease behavior at diagnosis and the progression to non-inflammatory disease behavior (PLogRank = 0.04, PBreslow = 0.04, $HR_{1after\ 1998}$ = 0.73, 95%CI: 0.55-0.97) in patients with initially inflammatory disease in the present study, suggesting a change in the natural history of the disease in the last decade. Trends were similar in the pediatric- and adult-onset patients. However, although azathioprine was started more frequently and earlier in the last decade[28], the change in disease behavior progression was not directly associated with the increased and earlier use of azathioprine, pointing to the fact that probably the change in the patient management is far more complex. Of note, distribution of disease location was not different in patients with a diagnosis before or after 1998. In contrast, presence of perianal disease was less prevalent in the later group (17.9% *vs* 31.5%, $P < 0.001$, OR = 0.48), suggesting and increased awareness and probably earlier diagnosis.

Authors are aware of possible limitations of the present study. The treatment and monitoring paradigm for CD patients has changed significantly over the last three decades. The majority of patients received maintenance therapy with sulfasalazine or a 5-aminosalicylic acid derivative (mesalazine or olsalazine), if tolerated, especially until the mid-1990s. Azathioprine or 6-mercaptopurine were used as maintenance therapy for steroid dependent, steroid-refractory, or fistulizing patients in selected cases, mainly after resective surgery until the late-1980s, but on a more widespread basis and earlier in the disease course only from the mid-to-late 1990s. Short-term oral corticosteroid treatment was used for clinical exacerbations, usually at initial doses of 40-60 mg of prednisone per day, which was tapered and discontinued over 2 to 3 mo. Infliximab (and later adalimumab) has been used for both induction and maintenance therapy in selected cases since the late 1990s. Similarly, small-bowel follow through was replaced by CT or MR-enterography from the 1990s. The strengths of the study include long-term prospective follow-up, the fact that leading IBD specialists were involved during the entire follow-up, and also that the evaluation and monitoring of pediatric-onset patients was managed jointly by pediatric and adult gastroenterologists using similar principles.

In conclusion, the long-term evolution of disease behavior in pediatric- and adult-onset CD patients did not differ in this population-based incident cohort. In contrast location, smoking, and need for steroids were associated with presence of, or progression to, complicated disease behavior at diagnosis and during follow-up, in concordance with previous referral and population-based studies. In addition, there was a change in the evolution of the disease behavior according to the calendar year of diagnosis. Progression to complicated disease phenotype was less likely in patients diagnosed after 1998, however this was at least partly associated with a milder disease phenotype at diagnosis including a decreased prevalence of perianal disease in the later group. A novel finding of the present study was that the change in disease location was associated with smoking status.

COMMENTS

Background

According to the available literature, pediatric onset Crohn's disease (CD) runs a more aggressive course, including more extensive disease location, more upper gastrointestinal involvement, growth failure, more active disease, and need for more aggressive medical therapy, in predominantly referral-center studies.

Research frontiers

Limited data are available on the long-term disease course in pediatric and adult patient cohorts with inflammatory bowel diseases from the same geographic area in population-based cohorts.

Innovations and breakthroughs

Some new data indicate that pediatric disease may parallel that of adults, however data so far are conflictive. The present study reports that the long-term evolution of disease behavior was not different in pediatric- and adult-onset CD patients in this prospective population-based incident cohort from Eastern Europe. Interestingly, change in disease location was associated with smoking status.

Applications

Understanding the evolution of the disease course in CD may lead to more optimized patient managment and follow-up.

Terminology

Disease phenotype is categorized according to the Montreal classification and includes age at onset (A1: < 17 years, A2: 17-40 years and A3: > 40 years) location (L1: Ileal, L2: Colon, L3: Ileocolon, L4: Upper gastrointestinal) and behavior (B1: Inflammatory, B2: Stenosing, B3: Penetrating). While disease location is thought to be more stable, a change in the disease behavior is a rather frequent event.

Peer review

This is a prospective, well-designed study, with a remarkable number of patients with CD reporting that the risk for developing complicated disease phenotype is not different between pediatric onset and adult onset CD patients.

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BRIEF ARTICLE

Comparative analysis of endoscopic precut conventional and needle knife sphincterotomy

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Abstract

AIM: To compare the efficacy, complications and post-procedural hyperamylasemia in endoscopic pre-cut conventional and needle knife sphincterotomie.

METHODS: We performed a retrospective analysis of two pre-cut sphincterotomy (PS) techniques, pre-cut conventional sphincterotomy (PCS), and pre-cut needle knife (PNK). The study included 143 patients; the classic technique was used in 59 patients (41.3%), and the needle knife technique was used in 84 patients (58.7%). We analyzed the efficacy of bile duct access, the need for a two-step procedure, the rates of complications and hyperamylasemia 4 h after the procedure, "endoscopic bleeding" and the need for bleeding control. Furthermore, to assess whether the anatomy of the Vater's papilla, indications for the procedure or the need for additional procedures could inform the choice of the PS method, we evaluated the additive hyperamylasemia risk 4 h after the procedure with respect to the above mentioned variables.

RESULTS: The bile duct access efficacy with PNK and PCS was 100% and 96.6%, respectively, and the difference between the two groups was not significant (P = 0.06). However, the needle knife technique required two-step access significantly more often, in 48.8% *vs* 8.5% of cases (P < 0.0001). The only complication noted was post-ercp pancreatitis (PEP), which was observed in 4/84 (4.8%) and 2/59 (3.4%) patients submitted to PNK and PSC, respectively; the difference between the two procedures was not significant (P = 0.98). An analysis of other consequences of the techniques yielded the following results in the PNK and PCS groups: hyperamylasemia 4 h after the procedure > 80 U/L, 41/84 *vs* 23/59 (P = 0.32); hyperamylasemia 4 h after the procedure > 240 U/L, 19/84 *vs* 11/59 (P = 0.71); pancreatic pain, 13/84 *vs* 7/59 (P = 0.71); endoscopic bleeding, 10/84 *vs* 8/59 (P = 0.97); and the need for bleeding control, 10/84 *vs* 7/59 (P = 0.79). In the next part of the study, we analyzed the influence of the method chosen on the risk of hyperamylasemia with respect to an indication for endoscopic retrograde cholangiopancreatography, papillary anatomy and concomitant procedures performed. We determined that the hyperamylasemia risk was increased by more than threefold [odds ratio (OR) = 3.38; P = 0.027] after PCS in patients with a flat Vater's papilla and more than fivefold (OR = 5.3; P = 0.049) after the PNK procedure in patients who required endoscopic homeostasis.

CONCLUSION: PCS and PNK do not differ in terms of efficacy or complication rates, but PNK is more often associated with the necessity for a two-step procedure.

Key words: Sphincterotomy; Endoscopic; Endoscopic retrograde cholangiopancreatography; Complications; Hyperamylasemia

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INTRODUCTION

Pre-cut sphincterotomy (PS) may increase the efficacy of the ineffective conventional endoscopic cannulation of biliary ducts by 64%-91%, and some studies have reported increases in efficacy of up to 95%-99%[1,2]. However, the efficacy and technical details of PS remain controversial, and the reported complication rates range from 3.78% to 19.2%, with an odds ratio (OR) of 0-2.71[3-7]. Therefore, PS accounts for 0% to 44% of all sphincterotomies at various centers[3,8-10]. The procedure may be performed by one of two methods, using either a non-needle knife or a needle knife[3,5,6,11]. The first procedure is performed using a shallowly anchored conventional structure cannulotome [pre-cut conventional sphincterotomy (PCS)][11-13]; the second procedure (PNK), relies on a needle knife incision of the intramural part of Vater's papilla[1,14]. Both methods are performed with various modifications that can independently influence complications[3]. For example, a variation of PNK proceeds without the distal broadening of the incision to avoid Wirsung's duct orifice damage[15]. In comparison with the conventional incision, this modification produces different anatomical results with an unknown impact on the complication rate and pancreatic juice efflux. The available literature presents only two studies comparing the PCS and PNK methods. However, in these trials, PCS was performed as a trans pancreatic sphincterotomy, and PNK began at the orifice[13,16]. Different approaches to PS indication, different indications for the switch from conventional to PS procedures, technical PS variations, the rules of two-step procedure implementation and different definitions of complications at various centers explain the limited value of data reported by different authors[15]. The lack of interpretable data by different authors prompted retrospective comparison of PCS and PNK (modified, without the distal broadening of the cut). The analysis included the efficacy of access to the common bile duct (CBD), the necessity to initiate a two-step procedure, the frequency of typical complications [post-ercp pancreatitis (PEP), bleeding, and perforation], the frequency of "endoscopic" bleeding, and the need for haemostasis, the influence on impaired pancreatic juice efflux and the need for further hospitalization. Additionally, we assessed the degree to which the effect of the PS method affects on the impaired efflux of pancreatic juice was dependent on papillary anatomy, procedurale indications or concomitant procedures. The aim of the study was to answer two questions: (1) is there a difference between the efficacy and safety of the analyzed pre-cut methods; and (2) should Vater's papilla anatomy, procedural indications and concomitant procedures influence the choice of pre-cut method?

MATERIALS AND METHODS

Inclusion criteria

We included patients with ineffective common bile duct deep cannulation using a conventional cannulotome (Olympus KD 301Q-0729) and guide-wire (MET 35-380 Cook) after a 10 min procedure. Sphincterotomy using a conventional cannulotome was performed if sufficient anchoring in the papilla orifice was possible; the needle knife procedure was used in the remaining patients. Exclusion criteria: Patients with invasive procedures on Vater's papilla in the past, and acute pancreatitis before the endoscopic retrograde cholangiopancreatography (ERCP) procedure were excluded.

Study material

The study included patients submitted to ERCP from one center over 21 mo (February 2010 to November 2011). Papillotomy was performed on 402 patients, during that time frame, of whom 165 qualified for the pre-cut procedure. However, 22 were excluded from analysis because of a previous endoscopic attempt on the papilla or symptoms of acute pancreatitis before ERCP. Finally, 143 (35.6%) patients were admitted to the study. A conventional cannulotome was used in 59 (41.3%) of the patients in this group, the needle knife technique was used in 84 patients (58.7%).

Technique

The conventional pre-cut procedure was performed with a cannulotome (Olympus KD 301Q-0320). A 2 to 3 mm long incision was made after anchoring the cannulotome, and the end of the device was continuously repositioned toward the CBD orifice for deep cannulation. The patient was referred to a two-step procedure, if, a maximum of five trials of cannulation were ineffective. The next ERCP was performed 4-7 d later, after the tissue edema had regressed. The needle knife technique was performed with a KD-441Q Olympus cannulotome at the midway point between the papilla's orifice and the transverse fold. Catheterization was performed when the CBD orifice was exposed, and the sphincterotomy was proximally broadened with a conventional cannulotome with the distal fragment left intact. The procedure was postponed 4-7 d in cases with five ineffective trials of cannulation. For each PS technique, we used prosthesis if a randomly contrasted Wirsung's duct exhibited impaired retrograde contrast efflux. We performed endoscopic hemostasis with an HES solution (hypertonic 5.6% NaCl solution with adrenaline 1:20 000) for cases of bleeding for more than 2 min, which made further cannulation trials impossible. Serum amylase levels were measured in every patient to assess pancreatic juice efflux impairment 4 h after the procedure. Pancreatic pain requiring analgesics was assessed 24 h after the procedure, and pancreatic pain was an indication for subsequent serum amylase level assessment. The analysis: The PCS and PNK techniques were assessed according to the efficacy of CBD access, the necessity of a two-step procedure, pancreatic juice efflux impairment (amylase level > 80 UI after 4 h), hospitalization indications (amylase level > 240 IU after 4 h)[3,17-19], and PEP, which was defined as an amylase level three times the upper limit with concomitant pancreatic

Table 1 Patient characteristics-risk factor

	Needle-knife PNK (n = 84)	Conventional PCS (n = 59)	P value
Female	43	38	0.16
Age < 50 yr	16	7	0.36
Bilirubin level (norm)	8	10	0.30
Concomitant systemic diseases	20	6	0.06
Neoplasms	26	17	0.96
Retention cause			
Choledocholithiasis	24	23	0.26
Papillar stenosis	14	10	0.85
Distal stenosis	18	13	0.70
Middle stenosis	3	5	0.37
Hilum stenosis	5	4	0.88
Anatomy of the papilla			
Flat	28	23	0.60
Prominent	32	23	0.94
In diverticulum	12	10	0.84
Tumor	18	5	0.06
Biliary duct diameter			
(mean)	14.49	14.07	0.42
(< 9 mm)	18	13	0.90
Accesory procedures			
Prosthesis implantation			
CBD	65	51	0.83
Wirsung	11	2	0.08
Prosthesis in CBD diam. (mean)	6.01	5.89	0.59
Pathological sampling	12	3	0.13

CBD: Common bile duct; PCS: Pre-cut conventional sphincterotomy; PNK: Pre-cut needle knife.

pain requiring analgesics 24 h after the procedure[19-22]. We also analyzed bleeding, which was defined as the presence of clinical symptoms of blood extravasation into the alimentary tract[20], the frequency of "endoscopic" bleeding (without clinical symptoms), and the necessity of hemostasis (no spontaneous regression 2 min after the incision). We compared perforations, which were defined as contrast extravasation out of the duodenal lumen during ERCP or gas in the retroperitoneal space on imaging[8,9,22,23]. The PCS and PNK methods were submitted to logistic regression analysis to assess their influence on pancreatic juice efflux and the effect of Vater's papilla anatomy (flat, prominent, inside diverticulum, or tumor), ERCP indications (choledocholithiasis, distal stenosis, or CBD diameter) and concomitant procedures (prosthesis, hemostasis,or pathological specimen sampling)[19,23].

Statistical analysis

Frequency tables as well as χ^2 and Mann-Whitney U tests were used for the statistical analyses where appropriate. Unifactor and multifactor models of logistic regression were used to assess the probability that the analyzed parameters influenced the presence of hyperamylasemia. The measure of hyperamylasemia risk was expressed as an odds ratio (OR) with 95% confidence intervals. P values less than 0.05 were considered to be statistically significant, and the statistical analyses were performed using MedCalc ver. 12.3.

Table 2 Comparison of the efficacy and complication rates of pre-cut conventional sphincterotomy and pre-cut needle knife procedures

Procedure	PNK (n = 84)	PCS (n = 59)	P value
Two-step access	41	5	< 0.0001
Efficacy	84	57	0.58
Amylase level (after 4 h)			
> 80 U/L	41	23	0.32
> 240 U/L	19	11	0.71
Pancreatic pain (after 24 h)	13	7	0.71
PEP	4	2	0.98
"Endoscopic" bleeding	10	8	0.97
Endoscopic homeostasis	10	7	0.79
Perforation	0	0	-

PEP: Post endoscopic pancreatitis; PCS: Pre-cut conventional sphincterotomy; PNK : Pre-cut needle knife.

RESULTS

The study included 143 patients; the conventional pre-cut technique was used in 59 patients (41.3%), and the needle knife was used in 84 patients (58.7%). The clinical characteristics and risk factors were compared between the PCS and PNK groups, and the parameters are presented in Table 1. There were no significant differences between the groups with respect to the following parameters: sex, age < 50 years, percentage of patients with normal bilirubin levels, concomitant systemic and tumor diseases, cause of icterus (choledocholithiasis, or distal CBD stenosis), Vater's papilla anatomy (flat, prominent, inside a diverticulum, or tumor) or the diameter of the common biliary duct. The frequency of prosthesis implantation in the CBD and pancreatic duct, the diameter of the prosthesis introduced to the biliary duct and the pathological specimen sampling did not differ between the groups. In the second part of the study, we assessed both techniques according to the necessity of introducing a two-step procedure, the efficacy of the endoscopic approach to the bile ducts and the consequences of the pre-cut procedures (Table 2). A two-step procedure was performed significantly more frequently in the PNK group, than in the PCS group (48.8% *vs* 8.5%, $P < 0.0001$). The two-step procedure allowed for CBD catheterization in all 45 patients in the PNK group, and 2 out of 5 patients in the PCS group. Biliary tree access was achieved in all patients treated with PKN and in 96.6% of patients treated with the PCS technique. Differences in the efficacies of the two methods were not statistically significant; however, in the PNK group, successful access was more frequently associated with a two-step procedure. There were no significant differences in the number of patients with elevated amylase levels exceeding 80 IU and 240 IU 4 h after the procedure. Pancreatic pain was observed 24 h after the procedure in 15.5% and 11.9% of patients in the PNK and PCS groups, respectively, and the differences were not statistically significant. Moreover, there were no notable differences in the rates of PEP. PEP was observed in 4.8% of the patients in the PNK group, and 3.4% in the PCS group. All PEP cases exhibited a mild or mod-

Table 3 Logistic regression-Hyperamylasemia (> 80 U/L) 4 h after the procedure and its association with indication, Vater's papilla anatomy and additional procedures

Parameter	Conventional (PCS)		Needle knife (PNK)	
	P	OR	*P*	OR
Indications				
Lithiasis	1.4	0.73	0.19	0.52
Distal stenosis	0.98	1.01	0.98	0.96
Middle stenosis	0.38	0.52	0.55	0.67
CBD diam. < 9 mm	0.96	0.97	0.9	1.06
Bilirubin level - N	0.43	1.72	0.42	1.8
Vater's papilla anatomy				
Flat	0.027[1]	3.38	0.22	0.56
Prominent	0.034	0.28	0.86	1.0
In diverticulum	0.52	0.62	0.93	1.05
Tumor	0.96	1.04	0.9	1.06
Additional procedures				
CBD prosth. diam. < 6 Fr	0.13	5.3	0.56	0.7
Endoscopic haemostasis	0.82	1.2	0.049	5.4[2]
Specimen sampling	0.84	0.78	0.92	1.06

[1]Statistically significant only in unifactor logistic regression; [2]Statistically significant only in multifactor logistic regression. CBD: Common bile duct; OR: Odds ratio; PCS: Pre-cut conventional sphincterotomy; PNK: Pre-cut needle knife.

erate course and were not treated surgically. All bleeding events observed in both groups were qualified as "endoscopic". There were no significant differences between the two groups. The pre-cut procedure was not complicated by perforation or bleeding associated with clinical symptoms in any patient in either group. Additionally, the risk of hyperamylasemia 4 h after the procedure was evaluated for any association with procedural indications, papillary anatomy, common bile duct prosthesis, prosthesis diameter, bleeding hemostasis, or the collection of pathological specimens. Logistic regression analysis (Table 3) revealed a three fold increase in the risk of hyperamylasemia after the PCS technique in patients with a flat papilla (OR = 3.38), whereas the hyperamylasemia risk in PNK patients was 5 times higher (OR = 5.38) after endoscopic hemostasis.

DISCUSSION

The pre-cut procedure is used following an unsuccessful conventional cannulation attempt of the biliary tree. The procedure is performed using a variety of techniques that can roughly by divided into two major groups: PNK and PCS[15]. Some authors believe that the conventional pre-cut procedure is the method of choice, and in case of its failure, needle cannulotome is recommended[12]. PCS is widely believed to offer better direction and depth for the incision, which should decrease the risk of perforation and bleeding. However, each consecutive unsuccessful cannulation increases the risk of post-endoscopic pancreatitis (OR = 1.39), and can reach OR = 9.4 after more than 15 ineffective cannulations[4,7]. PNK may be modified in the manner in which the incision is made, halfway between the orifice and the transverse fold with no distal

Table 4 Frequency of pre-cut sphincterotomy with a two-step approach, and efficacy of common bile duct cannulation (pre-cut conventional sphincterotomy and pre-cut needle knife procedures)

Ref.	PS freq.	PS technique	Two-step	Efficacy
Slot *et al*[1]	16.5%	PNK	12%	99%
Kasmin *et al*[14]	18.0%	PNK	32%	93%
Huigbregtse *et al*[21]	19.2%	PNK	47%	91%
Dowsett *et al*[25]	12.8%	PNK	54%	96.2%
Shakoor *et al*[26]	3.8%	PNK	13%	85%
Leung *et al*[27]	3.9%	PNK	15%	95%
Own material	20.9 %	PNK	48%	100%
	14.7%	PCS	8.5%	94.4%
Binmoeller *et al*[11]	38%	PCS	9%	100%
Goff *et al*[12]	44.0%	PCS	14%	97%

PS: Pre-cut sphincterotomy; PCS: Pre-cut conventional sphincterotomy; PNK: Pre-cut needle knife.

elongation. This technique avoids manipulations in the vicinity of Wirsung's duct orifice, which is believed to decrease the risk of PEP. Nevertheless, inferior maneuverability in the direction and depth of the cut may increase the perforation rate[14,15]. We found only two publications directly comparing the efficacy and safety of needle knife and non-needle knife PS methods[16,13]. Therefore, we aimed to compare PCS and PNK (modified, without the distal broadening cut) in the present work. The limitations of the present study are the small number of patients and the retrospective format of the study. At least 8422 patients need to be analyzed to assess the lack of difference in PEP frequency between the two groups; however, this would be difficult to accomplish in a single study. The second limitation of the present analysis is the retrospective format of the study, which restrains the exclusion of all of the parameters that indirectly influence the results. This retrospective design explains the difference in the frequency of Vater's papilla tumor in the analyzed groups 18 (PNK) *vs* 5 (PCS) patients, which may suggest that the needle-knife technique was used more often in patients with Vater's papilla tumor. The difference was not statistically significant ($P = 0.06$); however, the possibility that the difference may become significant in larger sample sizes cannot be excluded. These conditions suggest the necessity of performing larger, prospective, randomized and multi-center studies.

There is a significant discrepancy in the frequencies of PS among various centers.The percentage of patients submitted to PNK in our study was comparable to other reports; however, conventional pre-cut papillotomy is rare. Nevertheless, both PS techniques were employed relatively often, (35.6% of all patients with sphincterotomy) (Table 4). This relatively high frequency was most likely the result of an early switch from the conventional method to the needle knife technique to lower the risk of PEP after multiple ineffective cannulation attempts[24]. In the present study, the the efficacy results demonstrated no significant differences between the PCS and PNK methods, nevertheless, needle knife incision more often

Table 5 Complication rates after pre-cut sphincterotomy

Ref.	Pts. No.	PS type	Start of cut	All complications	PEP	Bleeding	Perforation
Slot *et al*[1]	-	PNK	Orifice	12%	0.5%	5.5%	3%
Kasmin *et al*[14]	72/398	PNK	Centre	11%	3.8%	3.8%	3.8%
Huibregtse *et al*[21]	190/987	PNK	Orifice	2.6%	1.0%	1.5%	0%
Dowsett *et al*[25]	96/748	PNK	Orifice	5.20%	1.0%	4%	0%
Shakoor *et al*[26]	53/1367	PNK	Orifice	11%	5.5%	3.7%	1.8%
Leung *et al*[27]	20/510	PNK	Centre	20%	0%	20%	0%
Donnellan *et al*[28]	352/2603	PNK	Centre	4.8%	1.0%	4.2%	0.3%
Our data	84/402	PNK	Centre	4.80%	5.4%	0%	0%
	59/402	PCS	-	3.4%	3.4%	0%	0%
Binmoeller *et al*[11]	123/327	PCS	-	5.3%	2.7%	2.4%	0%
Goff *et al*[12]	32/110	PCS	-	12%	12%	0%	0%

PS: Pre-cut sphincterotomy; PEP: Pos-ercp pancreatitis; PCS: Pre-cut conventional sphincterotomy; PNK : Pre-cut needle knife.

requires a two-step implementation procedure. The overall efficacy of cannulation did not depend on the technique used and was not influenced by the higher frequency of the two-step procedure in PNK, which is similar to data from other centers (Table 4). The aim of the second part of the study was to compare the groups with respect to typical complications (PEP, bleeding, and perforation) and additional parameters, including increased amylase levels 4 h after the procedure, "endoscopic" bleeding confirmed in ERCP and the necessity for endoscopic hemostasis. We did not observe significant differences in any of the above mentioned variables. Similarly, investigators in Helsinki and a multicenter trial performed in China also reported no differences in the complication rates in a direct comparison of needle knife and non-needle knife PS[16,13]. It should be noted that this study compared two different technical modifications of PCS and PNK. The pre-cut method with conventional sphincterotomy was performed after anchoring the cannulotome in Wirsung's duct; meanwhile, the PNK method the cut initiated in the orifice. These technical modifications explain why a direct comparison with the present trial is impossible. The data presented above also demonstrate a unique distribution of complications compared with other available reports (Table 5). We noted one complication, PEP, that fulfilled Cotton's consensus criteria[20]. A credible reason for the absence of clinically significant bleeding may stem from frequent endoscopic haemostasis in extravasation observed during the procedure. In contrast, there are various definitions of bleeding after ERCP, which may result in discrepancies in the presented data and make reliable comparisons impossible.

The most probable reason for the lack of perforation in all analyzed patients might be the frequent use of a two-step procedure. This method avoids of further cannulation trials in regions of edematous tissues with altered anatomy. It appears that repeating the procedure after edema regression, 4-7 d after the first procedure, may be safer than repeated cannulation trials, and the visible bile streak may facilitate proper localization of the CBD orifice. This idea is only partially supported by data from different centers. Dowsett *et al*[25] and Huibregtse *et al*[21] did not report perforation using a two-step procedure after PN in almost half their patients; Shakoor *et al*[26], Donnellan *et al*[27] and Bruins Slot *et al*[1], who described the use of a two-step procedure relatively rarely, reported perforation rates in 1.8% and 3% of their patients, respectively. However, Kazimin *et al*[14] and Leung *et al*[28] reports, did not report a similar relationship, which may be the result of their relatively low rates of PNK and the technical modifications in their methodology (Tables 4 and 5). One example of such a modification is Doswett's suggestion[25] to elevate the upper part of the papilla with the needle knife during PNK cutting, which should lower the risk of duodenal wall penetration. The lack of a standard procedure precludes reliable comparisons of results. Nevertheless, our data seem to validate the statement that the PCS and PNK methods do not differ in terms of complication rates, and that the PNK technique is more often associated with a two-step procedure, justifying the strategy to attempt PCS first and switch to PNK in case of PCS failure. It should be noted that the switch to PNK from PCS was performed relatively early in the presented material, because many ineffective cannulation trials may increase the risk of PEP[24]. In contrast, the PCS procedure is not feasible in all patients including; for example, in cases of duodenum lumen stenosis in presence of a pancreatic head tumor or obstructed papillary orifice due to the deposit. Other situations that may indicate the use of different types of the pre-cut procedure may depend on papilla anatomy, procedural indications or concomitant actions. In the third part of our study, we attempted to determine which factors should impact the choice of the type of pre-cut procedure. For this reason, we assessed the procedural differences with respect to impaired post-procedural pancreatic juice efflux. The study revealed an additional risk (OR = 3.38) of impaired pancreatic juice efflux 4 h after the procedure in PCS patients with a flat papilla (Table 3). This finding suggests that specific anatomy should prompt special precautions in multiple cannulations, and that specific anatomy indicates an early switch to the needle knife technique, if feasible. However, a flat papilla is a contraindication for the PNK method due to the unsatisfactory depth control during the incision and the higher risk of duodenal wall penetration. We have performed the PNK procedure in 23 patients with flat

papillae without any perforations. It appears that in these patients, the omission of the PCS step and the direct conversion to PNK is reasonable. The second indication of a high risk (OR = 5.4) of hyperamylasemia 4 h after the procedure was hemostasis in patients after analysed PNK pre-cut modification. This result suggests that the necessity for hemostasis requires the distal upper part of the papilla to be incised to ensure proper pancreatic juice efflux in an environment of hemostatic edema. However, the relatively small sample of patients and retrospective character of the present study require further prospective research.

COMMENTS

Background

The pre-cut procedure allows access to the bile ducts in cases of conventional technique failure. However, there are variations in the detailed technique for this procedure, which are generally divided into the non-needle knife procedure using a cannulotome with a conventional structure, and the needle knife using a needle-shaped device. Both techniques have been widely modified, and there are no firm rules defining the indications for the type of technique of choice.

Research frontiers

Author assessed the efficacy and safety profiles of both techniques and estimated their influence on pancreatic juice efflux (on the basis of amylase levels 4 h after the procedure) according to papillary anatomy, indications and the type of concomitant procedures.

Innovations and breakthroughs

In the first part of the study, they compared two modifications of pre-cut sphincterotomy. In contrast to Halttunen's and Wang's studies, the conventional incision was performed without Wirsung's duct cannulation; In addition, another difference was the needle knife incision was initiated from the middle of the intramural portion without a distal incision. The second part of the study concerned the influence of the procedural technique used on pancreatic juice efflux impairment depending on papillary anatomy, indications for the procedure and concomitant procedures. The risk of hyperamylasemia is three times higher after the conventional precut technique in patients with flat papilla. In the group of patients treated with the precut needle knife technique, the risk of hyperamylasemia was five times higher after endoscopic hemostasis.

Applications

The primary part of the study revealed that both analyzed methods may be used interchangeably, as they exhibit no differences in complication rates. Nevertheless, the needle knife technique often requires two endoscopic retrograde cholangiopancreatography (ERCP) procedures and should be the method of choice in cases of conventional pre-cut incision failure. The reason for the increased risk of hyperamylasemia is hemostasis after the needle knife procedure. This finding suggests that leaving the distal papilla intact may impair pancreatic juice efflux. This could be addressed by an incision in the distal part of the papilla, which requires further study.

Terminology

Pre-cut conventional sphincterotomy describes the incision performed with a cannulotome in cases of unfeasible deep cannulation of bile ducts. Pre-cut needle knife procedure is performed with a cannulotome a protruding distal portion responsible for intramural incision of the papilla. The incision may be initiated from the orifice of the papilla (orifice cut) or in the middle segment of the intramural part (middle cut). In the second modification, the distal part may be dissected or intact (as in their data). Two-step procedure-describes the situation after the pre-cut procedure and failure to access bile ducts. The second ERCP is performed 4-7 d after the first one and avoids cannulation in the region of oedematous tissues with altered anatomy.

Peer review

In this retrospective paper, the authors compared safety and efficacy of two different precut technique for biliary access. The authors conclude that the two techniques are basically similar concerning biliary cannulation success and complication rate, except for the need of a second intervention which was more needed in the needle knife group. This is an interesting paper.

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BRIEF ARTICLE

Epithelial markers of colorectal carcinogenesis in ulcerative colitis and primary sclerosing cholangitis

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Abstract

AIM: To evaluate the expression of epithelial markers of colorectal carcinogenesis in patients with long-term ulcerative colitis (UC) and primary sclerosing cholangitis (PSC) before and after transplantation.

METHODS: Eight patients with UC and PSC prior to liver transplantation (PSC-UC), 22 patients with UC after liver transplantation for PSC (OLT), 9 patients with active ulcerative colitis without PSC (UCA), 7 patients with UC in remission (UCR) and 10 controls (N) underwent colonoscopy with multiple biopsies. Specimens were analysed histologically and semi-quantitatively immunohistochemically for p53, Bcl-2 and cyclooxygenase-2 (COX-2) markers. Statistical analysis was performed by Kruskal-Wallis and Fisher's exact tests.

RESULTS: PSC-UC had a statistically significantly higher expression of p53 in the nondysplastic mucosa as compared to OLT, UCA, UCR and N ($P < 0.05$). We also found a statistically significant positive correlation between the incidence of PSC and the expression of p53 ($P < 0.001$). UCA had a higher p53 expression as compared to UCR. OLT had a significantly lower expression of p53 as compared with PSC-UC ($P < 0.001$). Bcl-2 had a significant higher bcl-2 expression as compared with controls. No difference in COX-2 expression between PSC-UC, UCR and UCA was found. UCA had higher COX-2 expression as compared to UCR. We also found a statistically significant positive correlation between the expression of COX-2 and p53. Patients after liver transplantation for PSC had a statistically significantly lower expression of the p53 compared with PSC-UC ($P < 0.001$). PSC-UC had the same inflammatory endoscopic activity as OLT and UCR when evaluated with the Mayo score.

CONCLUSION: Our study shows that the nondysplatic mucosa of UC patients with PSC is characterised by a higher expression of the tumour suppressor gene p53, suggesting a higher susceptibility of cancer. This p53 overexpression correlates with the presence of PSC whilst it is not present in patients with UC after liver transplantation for PSC.

Key words: Imunohistochemistry; Ulcerative colitis; Primary sclerosing cholangitis; Colorectal carcinoma; Liver transplantation; p53; bcl2; Cyclooxygenase-2

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INTRODUCTION

Patients with ulcerative colitis (UC) have an increased risk of colorectal cancer (CRC). Primary sclerosing cholangitis (PSC) is a chronic inflammatory disease often associated with inflammatory bowel disease (IBD)[1]. PSC patients have a greater risk of potential malignant impact[1]. IBD may be diagnosed at any time during the course of PSC but, in most cases, IBD is recognised first[2]. Although both diseases run distinct courses with no direct relationship between their severities there are some features that distinguish patients with PSC and UC. Some authors have even suggested that PSC with UC represents a distinct disease phenotype[3,4].

The increased risk of CRC is associated with long-term UC, the activity of the disease, the extent of the disease, the presence of PSC and the family incidence of CRC[5,6]. In a population-based Swedish study, the cumulative incidence of CRC in UC patients with PSC was 33% at 20 years[7]. Moreover, dysplasia and cancer in patients with a combined diagnosis of PSC-UC have recently been found even in patients with a shorter duration of the disease[8]. Liver transplantation for PSC in UC patients has also been shown as a risk factor for CRC[9-12]. However, some reports have not yet confirmed this data[2,13].

Colitis-associated carcinoma (CAC) has several distinguishing clinical features when compared with sporadic colorectal carcinoma (SCC). CAC progresses to invasive adenocarcinoma from flat and nonpolypoid dysplasia more frequently than SCC. CAC may also be multifocal, likely due to the broad field-effect of mucosal inflammation contributing to the development of neoplasia[14]. Standard colonoscopy is thus insufficient in detecting flat dysplasia and regenerative changes in colonic mucosa. Consequently, multiple biopsies or advanced endoscopic techniques such as chromoendoscopy, narrow band imaging or autofluorescence have been used[14,15]. Recently, it has been indicated that early detection of premalignant changes in the nondysplastic mucosa of UC by immunohistochemistry and polymerase chain reaction methods might be possible[8] Epithelial histopathological markers of colorectal carcinogenesis, which have thus far been utilised especially in advanced dysplastic changes, may also now have clinical impact in nondysplastic mucosa[8]. To the best of our knowledge, PSC-UC patients at present have not been studied in the context of histopathological markers in nondysplastic mucosa.

The tumour suppressor gene p53 is a 53 kDa nuclear protein involved in the control of the cell cycle, apoptosis and the maintenance of genomic stability[16-18]. p53 plays an active role in both DNA repair and the induction of apoptosis[19]. It is mutated in a variety of cancers including colorectal carcinoma[20-22]. Abnormal p53 expression, detected by immunohistochemistry, is often used as a marker of p53 mutation and thus found in dysplastic or cancerous tissue. Surprisingly, high p53 expression has been found in chronic UC patients with severe disease without cancer[14,23]. Interestingly, alterations of p53 were reported to occur early in the carcinogenesis of CAC compared to SCC, where they seem to be a late event. Bcl-2 is an important antiapoptotic gene. Some of the effects of p53 may be at least partially mediated by the downregulation effect on bcl-2. Bcl-2 has been shown to be overexpressed in SCC; however, its role in CAC is uncertain.

Cyclooxygenase-2 (COX-2) is an important inflammatory mediator which might play a role in the pathophysiologic processes of inflammatory bowel disease and the development of neoplasia as well[24]. COX-2 is induced upon cellular activation by hormones, proinflammatory cytokines, growth factors and tumour promoters[25]. COX-2 overexpression occurs early in UC-associated neoplasia and the COX-2 increase cannot be explained only by inflammatory activity alone[26].

Liver disease (PSC) might influence colonic mucosa by an unknown mechanism. One of the possible explanations of this mechanism is bile acid.

The aim of this study was to evaluate the expression of epithelial markers of colorectal carcinogenesis (p53, bcl-2, COX-2) in UC patients with or without the presence of PSC and after liver transplantation for PSC and correlate this expression with clinical and histopathological parameters.

MATERIALS AND METHODS

Patients

Eight patients with UC and PSC without liver transplantation (PSC-UC), 22 patients with UC after liver transplantation for PSC (OLT), 9 patients with active ulcerative pancolitis (UCA), 7 patients with UC in remission (UCR) and 10 controls (N) were included into the study (Table 1). UC activity was evaluated by the endoscopic Mayo score (0-remission, 1-mild, 2-moderate, 3-severe). The diagnosis of PSC was confirmed by ERCP or MRCP and liver biopsy. All subjects gave their informed consent with the study protocol which had been reviewed and approved by the local ethics committee. The study was performed in accordance with the Helsinki Declaration and Title 45, Code of Federal Regulations, Part 46, Protection of Human Subjects.

Histopathology evaluation

All patients underwent a colonoscopy with a standard white light endoscope. All UC patients, regardless of PSC diagnosis, that were included into the study suffered pan-

Table 1 Demographic features of participating patients

	n	Age (yr)	Sex (M/F)	Duration of UC (yr)	PSC	Duration after OLT (yr)	Histology	Endoscopy score (Mayo)
N	10	52.2 ± 14.09	4/6	0	No	0	0	0
UCR	7	41.57 ± 13.35	3/4	9.57 ± 1.59	No	0	0.42 ± 0.4	0.71 ± 0.45
UCA	9	45.88 ± 17.62	5/4	9.56 ± 2.41	No	0	2.7 ± 0.4	2.5 ± 0.49
PSC-UC	8	37.12 ± 6.8	5/3	8.75 ± 1.56	Yes (*n* = 8)	0	2.1 ± 0.59	1.12 ± 0.33
OLT	22	43.33 ± 12.11	11/11	12.4 ± 5.24	No	5.19 ± 2.61	1.4 ± 0.49	1.09 ± 0.29

N: Controls; M: Male; F: Female; UC: Ulcerative colitis; N: UCR: UC in remission; PSC: Primary sclerosing cholangitis; OLT: UC after liver transplantation for PSC; UCA: UC active disease.

Table 2 Monoclonal and polyclonal antibodies used in this study

Specificity	Origin	Company	Antigen retrieval	Dilution of antibodies
bcl-2 Oncoprotein	Mouse	DakoCytomation, Denmark	Buffer EDTA, pH 8	20 ×
p53	Mouse	DakoCytomation, Denmark	Tris/EDTA, pH 9	40 ×
COX-2	Mouse	Cayman, Michigan, United States	Citrate buffer, pH 6	40 ×

COX-2: Cyclooxygenase-2.

colitis. Biopsies were taken from the entire colon in 10cm intervals (approximately 40 samples). Neither dysplasia nor cancer was detected. Semiquantitative evaluation of p53, bcl-2 and COX-2 immunoreactivity was performed independently by two the hispathologists (Eva H, Eva S) in a blinded fashion. There was a general agreement between these observers. For the few discrepancies, a second evaluation was undertaken to find an agreement. Biopsies were analysed histologically and semi-quantitatively immunohistochemically for p53, bcl-2 and COX-2 with a scoring scale comparable to other studies[19,21,26,27]. The expression of antigens was analysed on 4 μm thick sections by a two-step indirect immunoperoxidase method. Slides were deparaffinised in xylene and rehydrated in graded ethanol. After deparaffinisation and rehydratation, the slides were cooked in a microwave oven (buffers used for antigen retrieval are listed in Table 2). Endogenous peroxidase was blocked by 0.3% H_2O_2 in 70% methanol for 30 min. Next, the specimens were incubated with a primary antibody for 30 min. The antibody was detected by incubation with a secondary antibody (Histofine Simple Stain MAX PO, Nichirei, Japan) for 30 min and incubation with Dako Liquid DAB+ Substrate-Chromogen System (DakoCytomation, Denmark). Afterwards, the specimens were counterstained with Haematoxylin and mounted in Entellan (Merck, Germany). Monoclonal antibodies (Ab) used in this study are listed in Table 2. p53 was evaluated in the intranuclear region, whereas bcl-2 and COX-2 were examined by imunohistochemistry in colonic cytoplasmatic region of the epithelial cells.

The immunohistochemistry scoring scale was based on the evaluation of the percentage of staining of positive cells, 0, no staining, 1+, mild 1%-32% of epithelial cell population, 2+, moderate from 33% to 66% of cell population and 3+, the highest staining from 67% to 100%. A positive result was considered as staining of more than 33% of the epithelial cells. Staining intensity was evaluated as weak, moderate and strong. Histological and endoscopical disease activity (Mayo score, also known as the Mayo Clinic Score and the Disease Activity Index) were evaluated (0-no inflammation, 1, mild, 2, medium and 3, severe inflammation)[28,29].

Statistical analysis

The data were evaluated using a robust Kruskal-Wallis test followed by Dunn's multiple comparison with Bonferroni correction. The relationships between positivity in epithelial markers was evaluated by Fisher's exact test (P value < 0.05 was considered significant). The relationships between the continuous variables were evaluated using Spearman´s correlation.

RESULTS

p53

PSC-UC had a significantly higher expression of p53 in the nondysplastic mucosa as compared to OLT, UCA, UCR and N ($P < 0.05$) (Figure 1A). We also found a statistically significant positive correlation between the presence of PSC and the expression of p53 ($P < 0.001$) (Table 3). UCA had a higher p53 expression as compared to UCR ($P < 0.05$). Correlation between p53 expression and duration of UC did not reach significance (r = -0.014, P = 0.917, n = 55).

bcl-2

UCA had a significantly higher bcl-2 expression as compared to controls (Figure 1B).

COX-2

The expression of COX-2 did not differ in PSC-UC as compared to OLT, UCA and UCR. UCA had a higher COX-2 expression as compared to UCR ($P < 0.05$) (Figure 1C). We also found a statistically significant positive correlation between the expression of COX-2 and p53 ($P < 0.05$) (Table 3).

Disease activity

PSC-UC, UCA and OLT did not significantly differ in histological disease activity. However, their histological activity was significantly higher when compared with

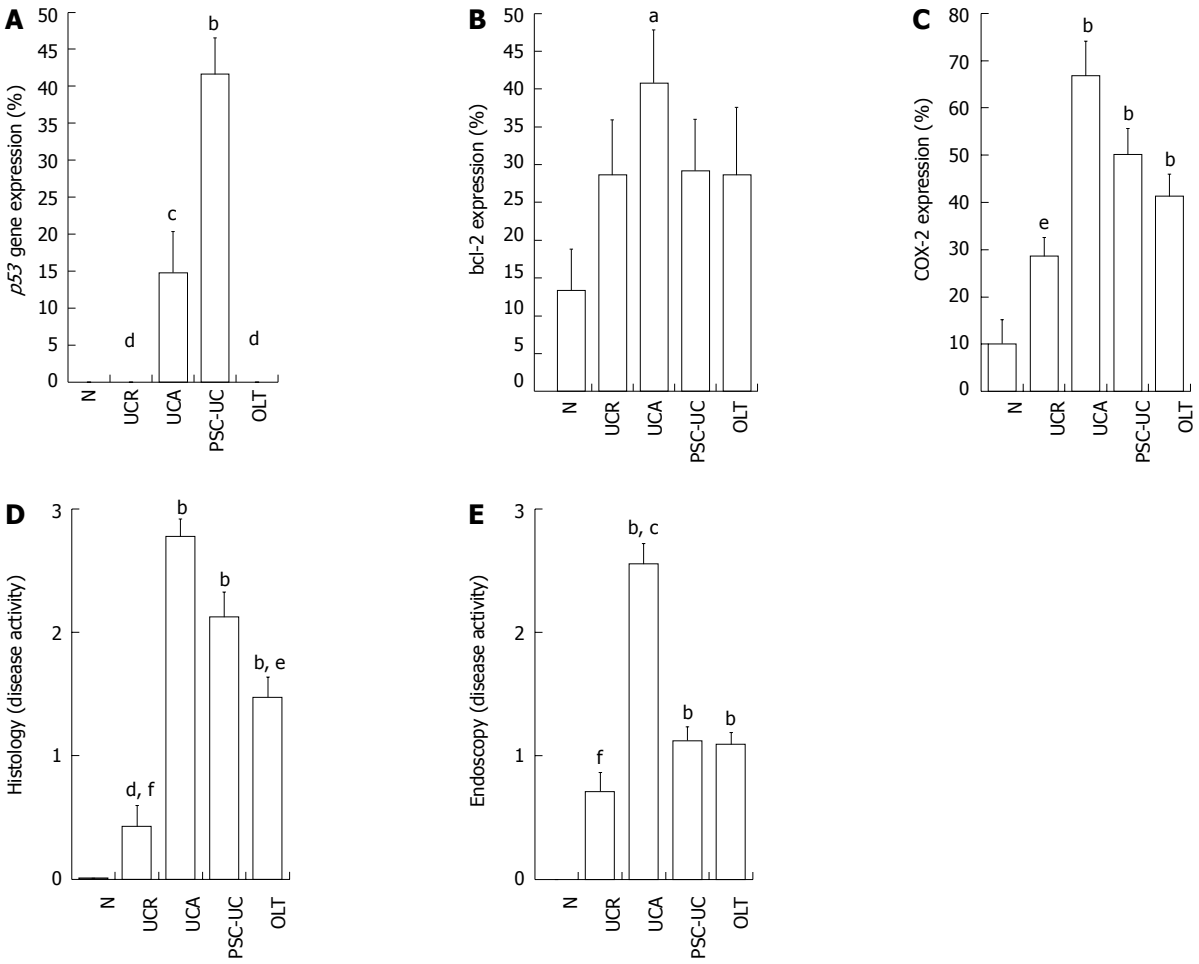

Figure 1 Comparison of findings in percent (%) in all tested group. A: Comparison of intranuclear *p53* gene expression in percent (%) in all tested group; B: Comparison of bcl-2 expression in percent (%) in all tested group; C: Comparison of COX-2 expression by immunohistochemistry in percent (%) in all tested group; D: Comparison of inflammatory disease activity by histology (0 = nonactive, 1 = mild, 2 = moderate, 3 = severe) in all tested group; E: Comparison of endoscopic findings by Mayo score in all tested group in all tested group. The bars with error bars represent group means with SEM. Differences between groups were evaluated using Dunn's multiple comparisons with Bonferroni correction. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* N group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* PSC-UC group; $^{e}P < 0.05$, $^{f}P < 0.01$ *vs* UCA group. UC: Ulcerative colitis; UCR: UC in remission; PSC: Primary sclerosing cholangitis; OLT: UC after liver transplantation for PSC; UCA: UC active disease; COX-2: Cyclooxygenase-2.

UCR and N ($P < 0.001$) (Figure 1D). PSC-UC had the same inflammatory endoscopic activity as OLT and UCR when evaluated with the Mayo score but this activity was lower when compared with UCA ($P < 0.05$) (Figure 1E).

Liver transplantation

Patients after liver transplantation for PSC had a statistically significantly lower expression of the p53 gene compared with PSC-UC. These two groups of patients did not differ in the other tested parameters (bcl-2, COX-2, histology and endoscopy) (Table 4).

DISCUSSION

The presence of PSC in UC patients is generally considered as a risk factor for colorectal cancer. However, comprehension of the specific mechanisms involved in CAC pathogenesis in PSC patients remains limited. The role of colonic mucosal markers such as p53, bcl-2 and COX-2 based on immunohistochemistry evaluation in PSC-UC patients has not yet been reported.

Our study shows that PSC-UC is characterised by a higher expression of the tumour suppressor gene p53 in nondysplastic mucosa as compared with OLT, UCA, UCR and controls which suggests a higher neoplastic potential of PSC-UC. Moreover, we found a statistically significant positive correlation between the incidence of PSC and p53 expression. The observed expression of p53 is driven mainly by inflammation while it did not correlate either with histological or endoscopical activity. Surprisingly, we found a lower p53 expression in OLT when compared to PSC-UC. To our knowledge, this finding has not been previously described in the literature and suggests the hypothesis that liver disease (PSC) is associated by an unknown mechanism with increased expression of p53 in the intestinal mucosa. In addition, p53 expression correlates with higher COX-2 expression suggesting that inflammation may contribute to the amount of *p53* gene expression. On the other hand, the expression of COX-2 did not differ between PSC-UC

Table 3 Relationship between p53, cyclooxygenase-2 and primary sclerosing cholangitis *n* (%)

	PSC-	PSC+	Row total	COX-2-	COX-2+	Row total
p53-	43 (78.18)	0 (0)	43 (78.18)	34 (61.82)	5 (9.09)	39 (70.91)
p53+	4 (7.27)	8 (14.55)	12 (21.82)	9 (16.36)	7 (12.73)	16 (29.09)
Column total	47 (85.45)	8 (14.55)	55 (100)	43 (78.18)	12 (21.82)	55 (100)

Statistical significance (Fisher's exact test) $P < 0.001$. PSC: Primary sclerosing cholangitis; COX-2: Cyclooxygenase-2.

Table 4 Comparison of primary sclerosing cholangitis-ulcerative colitis and ulcerative colitis after liver transplantation for primary sclerosing cholangitis

	p53	COX-2	bcl-2	Histology score	Endoscopy score (Mayo)
PSC-UC	↑[a]	↔	↔	↔	↔
OLT	↓	↔	↔	↔	↔
P value	$P < 0.001$	NS	NS	NS	NS

[a]$P < 0.05$ *vs* PSC-UC group. COX-2: Cyclooxygenase-2; UC: Ulcerative colitis; PSC: Primary sclerosing cholangitis; OLT: UC after liver transplantation for PSC.

and OLT, UCA and UCR. This finding might advance the hypothesis that the COX-2 mediated inflammatory pathway could play a similar role in PSC-UC and UC patients irrespective of the presence of PSC. We have also confirmed the previously described higher expression of p53 and COX-2 in the active disease[23,30,31].

The importance of the p53 tumour suppressor gene in PSC associated carcinogenesis has been demonstrated for hepatobiliary malignancies including cholangiocarcinoma and CAC without PSC[17,32,33]. Increased *p53* gene expression in the colonic mucosa in UC patients has been reported; however, patients with PSC have not been evaluated in these studies[14,15,17,29,34-38]. Our data clearly show a positive correlation between the presence of PSC and the level of expression of p53 in the intestinal nondysplastic mucosa of UC patients. These results thus support the hypothesis that PSC plays a role in UC associated colorectal carcinogenesis. We suggest that this happens, at least in part, through the overexpression of p53.

Alterations in the *p53* gene predispose to colonocytes dysplasia[25]. Mutations of the *p53* gene seem to occur at an early stage in CAC carcinogenesis compared to it being a late event in CRC[19,27]. Previously, we confirmed this by showing p53 overexpression in nondysplastic mucosa in a disease with high risk of cancer development. There is an ongoing debate in the literature whether p53 alterations can occur in nondysplatic epithelium. Patients with longstanding UC without dysplasia showing p53 overexpression may develop neoplasia 5 times more likely than those without[17]. Other studies reported p53 mutations in nondysplastic epithelium in patients with or without colorectal cancer[35,39]. In contrast, p53 was found only in dysplastic mucosa by others[25,36-37]. Accordingly, p53 expression clearly preceded dysplasia. It also appeared earlier in the course of the disease than previously reported[38]. However, we did not confirm this data in our study. One explanation is the small number of the tested group. In addition, the early expression of p53 in nondysplastic mucosa might make it a high risk marker of premalignant epithelium. The hypothesis of a p53 driven carcinogenesis in PSC-UC is further supported by the fact that we found a difference between p53 expression in PSC-UC patients and in patients after liver transplantation. Surprisingly, we found no p53 expression in the OLT group. Liver transplantation may contribute to the reduction of *p53* gene expression by eliminating the causative liver disease (PSC?). Liver transplantation could thus be viewed as having a temporary protective effect in the CAC pathogenesis. However, this finding needs to be verified in further studies. The mechanism of this phenomenon remains unknown. It would be interesting to see whether p53 expression diminishes in the same patients following transplantation or whether it comes back as in the case of the recurrence of PSC. In a 6 year follow up of our tested group, we observed no PSC recurrence. Another factor that may contribute to the different expression is the use of immunosuppressive therapy in patients after transplantation.

The mechanisms involved in the pathogenesis of CAC may be different in patients with PSC as compared to UC alone[10,40]. The effects of hepatobiliary factors may be one explanation. Bile acids play an important role in PSC-UC[1,2,17,40]. Secondary bile acids have been shown to result in hyperproliferation and thus play a role in PSC-UC and CAC pathogenesis[1,2,17]. Reduction of the incidence of CAC was achieved in PSC-UC with the use of ursodeoxycholic acid[41,42]. Unfortunately, the effects of ursodeoxycholic acid cannot be judged from our study as all our patients with PSC, prior or after transplantation, received it.

The inflammatory theory is still considered to be important in the process of colorectal carcinogenesis in UC[14,22]. The mechanisms of COX-2 driven carcinogenesis are still not fully understood, though studies suggest that an increased expression of COX-2 as a consequence of inflammation reduces apoptosis and increases angiogenesis[26,31]. In our study, we confirmed higher COX-2 expression in UCA compared to UCR. PSC-UC did not

differ in the expression of COX-2 when compared with OLT, UCA and UCR. However, PSC-UC was identical in histological inflammatory activity to UCA, but had a higher activity in comparison to UCR despite similar COX-2 expression. The COX-2 expression thus did not fully correlate with histological inflammatory activity alone. Interestingly, in the study of Agoff *et al*[26], COX-2 overexpression occurred early in UC-associated neoplasia; however, the cancer risk increase could not be explained solely by inflammatory activity alone. In their study, overall neoplastic change explained the majority of the variation in COX-2 expression, whereas inflammatory activity explained only 11%[26].

Bcl-2 is considered as an important antiapoptotic gene which is in reciprocal relation with p53[43]. Ilyas *et al*[34] have shown that bcl-2 plays an important role in UC associated carcinogenesis. We found a higher bcl-2 expression in UCA as compared to controls. Inflammation could be one possible explanation. In contrast to p53, no association with the presence of PSC was observed. We also did not find negative regulation of bcl-2 and p53 as previously described in breast cancer or adenomas. Thus, the impact of bcl-2 on colorectal cancer pathogenesis of PSC-UC based on our findings is still unclear.

We could also suggest, as other authors have, that PSC-UC might be a subgroup of UC[3,4,44]. PSC-UC is characterised by the same histological inflammatory activity as UCA but differs from UCR and N. PSC-UC had a higher p53 expression as compared to UCA, UCR, OLT and N; however, no difference between these groups was observed in COX-2 expression. PSC-UC thus shows signs of both UCA and UCR characteristics. Because of the known mild clinical course of PSC-UC as compared to UC alone, it may be underdiagnosed with unfavourable clinical consequences. Accordingly, regular colonoscopy has been recommended for all PSC patients. For that reason, p53 overexpression might be a useful predictor of potential carcinogenesis of colorectal mucosae in PSC-UC patients. In addition, according to our study, routine clinical and endoscopic indexes of colitis without PSC (*e.g.*, Mayo, UCDAI) cannot be used in PSC-UC. The presence of PSC in patients with UC should be taken into account especially in clinical and experimental studies.

Our study has several limitations. We investigated only a small group of subjects and used the immunohistochemical method for detection of mucosal markers. It should be noted, however, that immunohistochemical investigations and mutation analysis rely on samples of mucosa obtained by colonoscopic biopsy and thus are subject to the same sampling error[27]. In addition, we may have missed some non-sense mutations resulting in a truncated protein[23]. We also did not detect dysplasia in any of our patients. However, it might have been interesting to compare the expression of these markers in nondysplastic and dysplastic mucosa. Moreover, it would have been better to analyse the same patients with PSC and UC before and after OLT. This was not possible since the PSC-UC patients had not yet undergone OLT, but these patients will be included into a subsequent study.

In conclusion, PSC-UC was characterised by a higher expression of the tumour suppressor gene *p53* in nondysplatic mucosa explaining, at least in part, the higher neoplastic potential of PSC-UC. Furthermore, this overexpression was not present in UC patients who underwent liver transplantation for PSC. The expression of p53 thus correlated with the presence of PSC, suggesting a carcinogenic effect of the liver disease on colonic mucosa. The presence of p53 expression in nondysplastic mucosa may support its use as a marker of increased susceptibility to cancer that may enable detection of premalignant epithelium. It may be the use of epithelial markers of carcinogenesis which may in the future be used to better predict the risk preneoplastic lesions and CAC in UC patients with PSC and after liver transplantation. Our results need to be verified in larger future studies.

COMMENTS

Background

The presence of primary sclerosing cholangitis (PSC) in ulcerative colitis (UC) patients is generally considered a risk factor for colorectal cancer. However, comprehension about the specific mechanisms involved in colitis associated carcinoma (CAC) pathogenesis in PSC patients remains limited. The aim of this study was to evaluate the expression of epithelial markers of colorectal carcinogenesis in patients with long-term UC and PSC before and after transplantation.

Research frontiers

CAC has several distinguishing clinical features when compared with sporadic colorectal carcinoma (SCC). CAC progresses to invasive adenocarcinoma from flat and nonpolypoid dysplasia more frequently than SCC. CAC may also be multifocal likely due to the broad field-effect of mucosal inflammation contributing to the development of neoplasia. Standard colonoscopy is thus insufficient in detecting flat dysplasia and regenerative changes in colonic mucosa. Recently, it has been indicated that early detection of premalignant changes in the nondysplastic mucosa of UC by immunohistochemistry and polymerase chain reaction methods might be possible. Epithelial histopathological markers of colorectal carcinogenesis, which have thus far been utilised especially in advanced dysplastic changes, may also now have clinical impact in nondysplastic mucosa. The role of colonic mucosal markers such as p53, bcl-2 and cyclooxygenase-2 (COX-2) based on immunohistochemistry evaluation in UC and PSC prior to liver transplantation (PSC-UC) patients has not yet been reported.

Innovations and breakthroughs

Data clearly show a positive correlation between the presence of PSC and the level of expression of p53 in the intestinal nondysplastic mucosa of UC patients. These results thus support the hypothesis that PSC plays a role in UC associated colorectal carcinogenesis. The authors suggest that this happens, at least in part, through the overexpression of p53.

Applications

Because of the known mild clinical course of PSC-UC as compared to UC alone, it may be underdiagnosed with unfavourable clinical consequences. Accordingly, regular colonoscopy has been recommended for all PSC patients. For that reason, p53 overexpression might be a useful predictor of potential carcinogenesis of colorectal mucosae in PSC-UC patients. In addition, according to their study, routine clinical and endoscopic indexes of colitis without PSC (*e.g.*, Mayo, UCDAI) cannot be used in PSC-UC. The presence of PSC in patients with UC should be taken into account especially in clinical and experimental studies. The presence of p53 expression in nondysplastic mucosa may support its use as a marker of increased susceptibility to cancer that may enable detection of premalignant epithelium. It may be the use of epithelial markers of carcinogenesis which may in the future be used to better predict the risk preneoplastic lesions and CAC in UC patients with PSC and after liver transplantation.

Peer review

The study shows that the nondysplatic mucosa of UC patients with PSC is characterised by a higher expression of the tumour suppressor gene p53, suggesting a higher susceptibility of cancer. This p53 overexpression correlates with the presence of PSC, whilst it is not present in patients with UC after liver transplantation for PSC.

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BRIEF ARTICLE

Therapeutic efficacy of transarterial chemo-embolization with a fine-powder formulation of cisplatin for hepatocellular carcinoma

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Abstract

AIM: To evaluate the efficacy of transarterial chemoembolization (TACE) using a suspension of a fine-powder formulation of cisplatin (DDPH) in lipiodol (LPD) in the treatment of hepatocellular carcinoma (HCC).

METHODS: The subjects were 262 HCC patients treated with TACE using a DDPH-LPD suspension. The DDPH-LPD suspension was prepared by mixing 50 mg of DDPH into 10 mL of LPD. TACE was repeated when treated lesions relapsed and/or new hepatic lesions were detected. These patients received additional TACE using the same agent. TACE was repeated until complete regression of the tumor was obtained. The primary efficacy endpoint of the current study was the objective early response rate. Secondary efficacy endpoints were progression-free survival (PFS) and overall survival.

RESULTS: The objective early response rate was 43.6%. Cumulative PFS rates were 56.7% at 6 mo, 23.1% at 12 mo, 13.4% at 18 mo, and 10.5% at 24 mo. The median PFS was 6.6 mo. Cumulative survival rates were 90.6% at 6 mo, 81.9% at 12 mo, 70.5% at 24 mo, and 58.8% at 36 mo. Median survival time was 46.6 mo. All adverse reactions were controllable by temporary suspension of treatment. No serious complications or treatment-related deaths were observed.

CONCLUSION: TACE using a suspension of DDPH in LPD may be a useful treatment for HCC.

Key words: Cisplatin; DDPH; Hepatocellular carcinoma; Portal vein tumor thrombosis; Transarterial chemoembolization

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a common primary liver cancer with a rising incidence worldwide[1]. In Japan, more than 30 000 people die of HCC each year[2]. Curative therapies such as resection, liver transplantation, and local ablative treatments may offer a chance of improved life expectancy, but these treatment modalities are applicable to only a small proportion of HCC patients. As a result, in patients with advanced HCC who are not

eligible for these curative therapies, transarterial chemoembolization (TACE) has been the mainstay treatment option with proven survival benefits[3,4]. Many studies of TACE have been reported; a method using lipiodol (LPD), an oily contrast medium used as a drug delivery system, is now widely used, and anticancer drugs such as doxorubicin (ADM), epirubicin, and other anthracyclines are often used[5,6]. However, the tumors have a high frequency of recurrence after TACE[5,7]. Moreover, HCC is not necessarily sensitive to these drugs[8,9]. Therefore, the therapeutic results of TACE for HCC should improve as anticancer drugs become more effective.

Cisplatin (CDDP), a platinum compound, is an effective anticancer agent used in the treatment of various malignancies[10]. Researchers have reported that TACE using a suspension of CDDP powder in LPD may be more effective against unresectable HCC than TACE using an ADM-LPD emulsion[11,12]. However, only a few institutions have used this for TACE because it is difficult to refine the CDDP powder. Since 2004, a fine-powder formulation of CDDP (DDPH, IA-call; Nippon Kayaku, Tokyo, Japan) has been available as a therapeutic agent for intra-arterial infusion in Japan. As a result, TACE using DDPH has become widespread in Japanese institutions. We have already used TACE with DDPH for HCC patients and reported favorable results[13]. The aim of this study was to elucidate the efficacy of this therapy by analyzing the clinical results of 262 HCC patients treated in this manner.

MATERIALS AND METHODS

Study design and patient eligibility

This clinical investigation was approved by the ethics committee of our institution, and informed consent was obtained from all patients. The study was designed as a single-institution, open clinical study. The primary efficacy endpoint of the current study was the objective response rate. Secondary efficacy endpoints were progression-free survival (PFS) and overall survival (OS).

Eligibility criteria were as follows: (1) Eastern Cooperative Oncology Group performance status of 0-2; (2) age over 20 years; (3) diagnosis of HCC based on imaging or histological findings; (4) no indication for surgical resection or local ablation therapy such as radiofrequency ablation (RFA); (5) bidimensionally measurable hepatic lesions; (6) adequate hepatic function (serum total bilirubin < 3.0 mg/dL), and adequate renal function (serum creatinine < the upper normal limit); (7) no extrahepatic metastasis; (8) no tumor thrombus in the main trunk of the portal vein; or (9) no HCC treatment for 4 wk before study entry.

Enrolled were patients with HCC suitable for curative treatments such as surgical resection and local ablation therapy but who were of high risk for these therapies. A total of 262 consecutive patients who were to undergo TACE using DDPH between January 2006 and May 2011 were enrolled. All of the enrolled patients met the inclusion criteria.

Diagnosis

The diagnostic criteria for HCC *via* imaging were based on hyperattenuation in the arterial phase and hypoattenuation in the portal phase on dynamic computed tomography (CT) or magnetic resonance imaging (MRI), and tumor stain on angiography. When HCC could not be diagnosed by imaging alone, fine-needle biopsy using abdominal ultrasonography (US) was performed to obtain histological proof. Further assessment of HCC was conducted by measuring levels of α-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP).

Liver function was evaluated according to the Child-Pugh classification[14]. Tumor stage was assessed based on the tumor node metastasis (TNM) staging system of the Liver Cancer Study Group of Japan[15]. Portal vein tumor thrombosis (PVTT) grade was classified as follows: Vp0, no invasion of the portal vein; Vp1, invasion of the third or more distal branch of the left or right portal vein; Vp2, invasion of the second branch of the portal vein; Vp3, invasion of the first branch of the portal vein; and Vp4, invasion of the trunk of the portal vein.

Preparation of the agents for TACE

DDPH was mixed with LPD (iodized oil, Lipiodol Ultra-Fluide; Andre Guerget, Aulnay-sous-Bois, France). The DDPH-LPD suspension was prepared by mixing 50 mg of DDPH into 10 mL of LPD. The dosage of DDPH-LPD suspension was adjusted depending on the tumor size, number of tumors, degree of liver impairment, and renal function, but the maximum dose of DDPH-LPD suspension was not allowed to exceed 10 mL.

Treatment procedures

In all TACE procedures, hepatic angiography was performed by the femoral approach using a 4-Fr catheter and a 1.8-Fr to 2.4-Fr microcatheter. After confirming the hepatic arteries supplying the target tumor, a catheter was selectively inserted into the hepatic artery supplying the target tumor, and the DDPH-LPD suspension was injected. In patients with several tumors in the liver, superselective catheterization was performed for each lesion. If superselective catheterization was not possible, the DDPH-LPD suspension was injected into the right and left main hepatic arteries distal to the origin of the cystic artery. After the injection, arterioembolization was performed used porous gelatin particles (Gelpart; Nippon Kayaku, Tokyo, Japan) mixed with contrast medium.

All patients were followed up with US, CT, and/or MRI after 1 mo and then every 3 mo thereafter. Treatment was repeated by TACE alone when treated lesions relapsed and/or new hepatic lesions were detected. These patients received additional TACE using the same agent during the follow-up period unless the tumors progressed. TACE was repeated until complete regression of the tumor was obtained.

Evaluation of therapeutic efficacy

Tumor response was assessed by US, CT, and/or MRI at 1 mo from the start of treatment and every 3 mo thereaf-

Table 1 Baseline characteristics of the 262 patients

Characteristics		
Enrolled patients		262
Age (yr)	Median (range)	70 (32-92)
Sex	Male/female	176/86
Etiology	HBV/HCV/NBNC	30/170/62
Child Pugh classification	A/B/C	147/93/22
Number of tumors	< 10/≥ 10	114/148
Maximum tumor size (mm)	Median (range)	32.5 (8.0-300.0)
Stage[1]	Ⅰ/Ⅱ/Ⅲ/Ⅳ	17/45/136/64
PVTT grade	Vp0/Vp1-2/Vp3	202/27/33
Total bilirubin (mg/dL)	mean ± SD	1.0 ± 0.7
Albumin (g/dL)	mean ± SD	3.4 ± 0.6
Prothrombin time (%)	mean ± SD	91.1 ± 8.4
Platelet count (× 10^4/L)	mean ± SD	8.9 ± 5.0
AFP (ng/mL)	median (range)	31.6 (1.0-1 000 000)
AFP-L3 (ng/mL)	median (range)	4.0 (0-91.8)
DCP (mAU/mL)	median (range)	60 (0-928 900)
Previous treatment	Yes/no	107/155

Data are expressed as median values with ranges, mean ± SD, or number of patients. [1]According to the modified RECIST (mRECIST) criteria. HBV: Hepatitis B virus; HCV: Hepatitis C virus; NBNC: Negative for hepatitis B surface antigen and HCV antibody; PVTT: Portal vein tumor thrombosis; AFP: α-fetoprotein; AFP-L3: Lens culinaris agglutinin-reactive fraction of α-fetoprotein; DCP: Des-γ-carboxy prothrombin.

ter. The response was classified according to the modified RECIST (mRECIST) criteria[16], which take into account only the viable (arterially enhancing) component of the target tumors, and grade tumor response as follows: complete response (CR)-disappearance of any intratumoral arterial enhancement in all target lesions; partial response (PR)-at least a 30% decrease in the sum of diameters of viable target lesions, taking as reference the baseline sum of the diameters of target lesions; progressive disease (PD)-increase (at least 20%) in the sum of the diameters of viable target lesions, taking as reference the smallest sum of the diameters of viable (enhancing) target lesions recorded since treatment started, or appearance of new lesions; stable disease (SD)-all other cases.

Toxicity was evaluated using the National Cancer Institute-Common Terminology Criteria for Adverse Events, version 3.0 (CTCAE v3.0).

Statistical analysis

Baseline data are expressed as means ± SD or as medians and range. Statistical analysis was performed in September 2011. The cumulative survival rate and PFS were calculated from the date of therapy initiation and assessed by the Kaplan-Meier life-table method, and differences were evaluated using the log-rank test. Univariate analysis of predictors for survival of patients was assessed using the Kaplan-Meier life-table method, and differences were evaluated using the log-rank test. Multivariate analysis of predictors for survival was assessed by the Cox proportional hazards model. Significance was accepted at $P < 0.05$. All analyses were performed using SPSS version 11 software (SPSS, Chicago, IL, United States).

RESULTS

Patient characteristics

The characteristics of the 262 patients are listed in Table 1. There were 176 male and 86 female patients, ranging in age from 32 to 92 years (median age, 70 years). There were 147 (56.1%), 93 (35.5%), and 22 (8.4%) patients with Child-Pugh Stages A, B, and C, respectively. The median diameter of the largest tumor was 32.5 mm (range, 8-300 mm). Serum AFP levels were > 10 ng/mL in 182 patients, and 146 patients were DCP-positive (> 40 mAU/mL).

Clinical efficacy

The median duration of follow-up was 17.0 mo (range, 2.0-64.0 mo). A total of 682 TACE procedures were performed in 262 patients. The median number of TACE procedures was 2 cycles (range, 1-13 cycles). Early response status in the 262 patients was assessed after the first course of therapy. As a result, 34 patients (13.0%) had CR, 80 patients (30.6%) had PR, 69 patients (26.3%) had SD, and 79 patients (30.1%) had PD [response rate (CR + PR/all cases) = 43.6%]. The disease control rate (CR + PR + SD/all cases) was 69.9%.

PFS

The median PFS was 6.6 mo. The PFS rates at 6, 12, 18, and 24 mo were 56.7%, 23.1%, 13.4%, and 10.5%, respectively (Figure 1).

Survival

The median survival time (MST) was 46.6 mo. The cumulative survival rates at 6, 12, 24, and 36 mo were 90.6%, 81.9%, 70.5%, and 58.8%, respectively (Figure 1).

Cumulative survival rates of patients with no PVTT were 96.3% at 6 mo, 90.4% at 12 mo, 79.7% at 24 mo. On the other hand, cumulative survival rates of patients with PVTT were 68.6% at 6 mo, 49.0% at 12 mo, 33.7% at 24 mo. The survival rate was significantly higher in patients with no PVTT than in patients with PVTT ($P < 0.001$, Figure 2A).

Moreover, cumulative survival rates were determined by PVTT grade in 60 patients with PVTT. Cumulative survival rates of patients with Vp1-2 were 87.6% at 6 mo, 67.0% at 12 mo, 52.8% at 24 mo. On the other hand, cumulative survival rates of patients with Vp3 were 51.0% at 6 mo, 32.5% at 12 mo, 16.2% at 24 mo. The survival rate was significantly higher in patients with Vp1-2 than in patients with Vp3 ($P = 0.009$, Figure 2B).

Prognostic factors affecting patient survival were analyzed by examining 17 potential parameters (Table 2). Univariate analysis revealed 12 significant prognostic factors related to survival: stage ($P < 0.001$), Child Pugh classification ($P = 0.005$), JIS score ($P < 0.001$), total bilirubin ($P = 0.031$), albumin ($P = 0.012$), number of tumors ($P < 0.001$), maximum tumor size ($P < 0.001$), PVTT grade ($P < 0.001$), tumor distribution ($P < 0.001$),

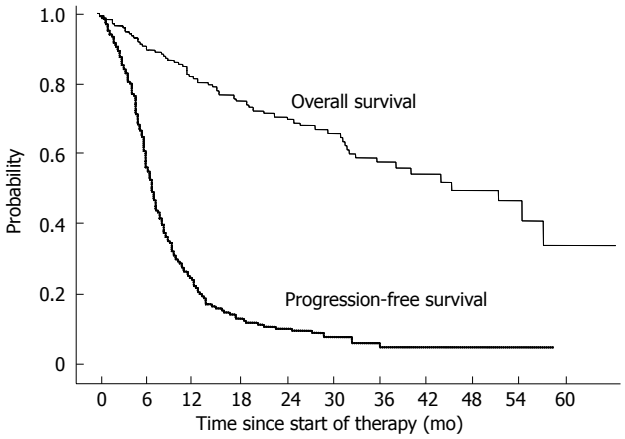

Figure 1 Overall survival and progression-free survival curves of 262 patients treated with transarterial chemoembolization using DDPH.

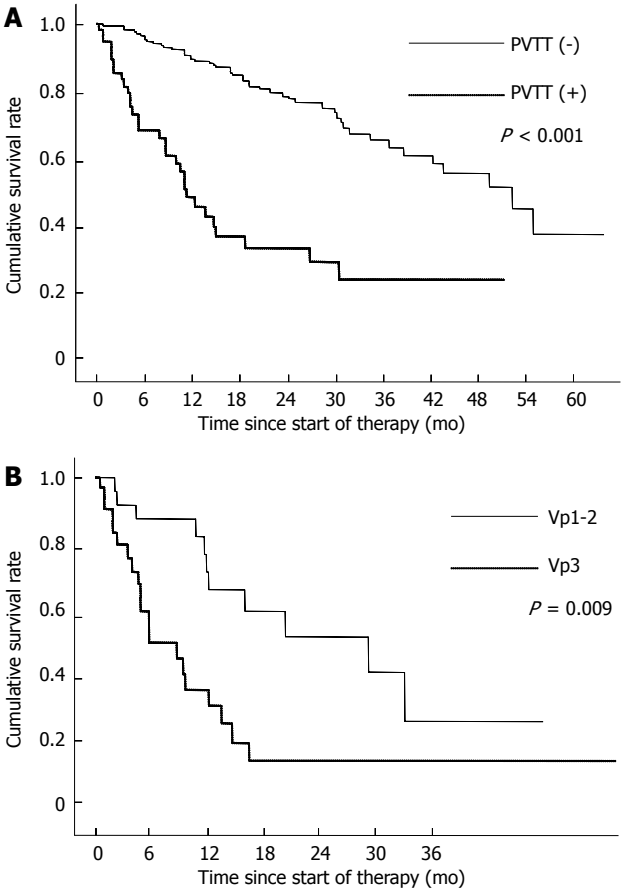

Figure 2 Cumulative survival rates. A: Comparison of cumulative survival rates between patients with no portal vein tumor thrombosis and those with portal vein tumor thrombosis; B: Comparison of the cumulative survival rates between patients with Vp1-2 and patients with Vp3. PVTT: Portal vein tumor thrombosis.

AFP ($P < 0.001$), DCP ($P < 0.001$), and therapeutic effect ($P < 0.001$).

Multivariate analysis showed 3 significant prognostic factors related to survival: PVTT grade ($P = 0.010$), AFP ($P = 0.002$), and therapeutic effect ($P < 0.001$).

Adverse effects

Table 3 summarizes the adverse effects. No treatment-related deaths occurred, and no grade 4 treatment-related adverse effects were noted. Fever and nausea were seen transiently in 50% or more patients, but both were mild. Leucopenia and thrombocytopenia occurred in 15 (5.7%) and 18 (6.9%) patients, respectively; these were also mild and transient. Although grade 2 or higher liver abscess and hepatic/renal failure were observed in 4 (1.4%) and 1 (0.4%) patients, respectively, these adverse reactions were controllable by medical treatment. In addition, hepatic arterial damage (HAD) after TACE was observed in one patient. Although one patient was observed to have slight wall irregularity of the hepatic artery, HAD associated with TACE did not interfere with catheterization at the next TACE session.

Table 2 Univariate and multivariate analysis of predictors of survival

Variable	Hazard ratio	95%CI	*P* value
Univariate analysis of predictors of survival			
Age (≤ 65 *vs* > 65 yr)	1.085	0.772-1.525	0.638
Gender (M *vs* F)	0.861	0.607-1.222	0.403
Previous treatment (no *vs* yes)	1.258	0.911-1.738	0.164
HCV antibody (negative *vs* positive)	0.951	0.673-1.344	0.776
Stage (Ⅰ, Ⅱ, Ⅲ *vs* Ⅳ)	2.705	1.919-3.814	< 0.001
Child Pugh classification (A *vs* B or C)	1.584	1.149-2.184	0.005
JIS score (0-2 *vs* 3-5)	2.285	1.638-3.189	< 0.001
Total bilirubin (≤ 1.5 mg/dL *vs* > 1.5 mg/dL)	1.578	1.044-2.385	0.031
Albumin (> 3.5 mg/dL *vs* ≤ 3.5 mg/dL)	1.527	1.100-2.119	0.012
Number of tumors (< 10 *vs* ≥ 10)	1.920	1.378-2.675	< 0.001
Maximum tumor size (≤ 50 mm *vs* > 50 mm)	2.052	1.404-2.998	< 0.001
PVTT grade (Vp0, 1, 2 *vs* Vp3)	4.142	2.754-6.230	< 0.001
Tumor distribution (Unilateral *vs* Bilateral)	2.237	1.464-3.420	< 0.001
AFP (≤ 100 ng/mL *vs* > 100 ng/mL)	2.131	1.539-2.949	< 0.001
AFP-L3 (≤ 50% *vs* > 50%)	1.664	0.957-2.894	0.071
DCP (≤ 100 mAU/mL *vs* > 100 mAU/mL)	2.201	1.588-3.051	< 0.001
Therapeutic effect (CR + PR *vs* SD + PD)	3.419	2.382-4.909	< 0.001
Multivariate analysis of predictors of survival			
PVTT grade (Vp0, 1, 2 *vs* Vp3)	2.310	1.217-4.384	0.010
AFP (≤ 100 ng/mL *vs* > 100 ng/mL)	1.856	1.265-2.724	0.002
Therapeutic effect (CR + PR *vs* SD + PD)	3.392	2.240-5.135	< 0.001

The JIS score is obtained by simply adding both scores for the tumor, lymph node, metastasis (TNM) stage and Child-Turcotte-Pugh stage. HCV: Hepatitis C virus; JIS: Japan integrated staging; PVTT: Portal vein tumor thrombosis; AFP: α-fetoprotein; DCP: Des-γ-carboxy prothrombin; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

DISCUSSION

TACE plays a crucial role in the treatment of HCC without surgical resection or RFA. The survival benefit of TACE has also been confirmed by randomized, controlled trials and a meta-analysis[3,4]. The most commonly

Table 3 Adverse effects among the 262 patients *n* (%)

Adverse effect	Grade 1	Grade 2	Grade 3	Grade 4
Nausea/vomiting	160 (61.1)	48 (18.3)	- (-)	- (-)
General fatigue	28 (10.7)	17 (6.5)	1 (0.4)	- (-)
Fever	168 (64.1)	27 (10.3)	- (-)	- (-)
Abdominal pain	121 (46.1)	54 (20.6)	- (-)	- (-)
Leucopenia	13 (4.9)	2 (0.7)	- (-)	- (-)
Thrombocytopenia	16 (6.1)	2 (0.7)	- (-)	- (-)
AST/ALT	154 (58.8)	46 (17.6)	- (-)	- (-)
Liver abscess	- (-)	2 (0.7)	2 (0.7)	- (-)
Hepatic/Renal failure	- (-)	- (-)	1 (0.4)	- (-)

Data are expressed as number of patients with percentages in parentheses. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

used agent used in TACE for HCC treatment is ADM-LPD emulsion, followed by embolization with a gelatin sponge[12,13,17,18]. The MST ranged from 18 to 34 mo with the use of TACE with ADM-LPD emulsion, but there is no clear evidence identifying the best chemotherapeutic agent for TACE.

CDDP is an effective anticancer agent used in the treatment of various malignancies[10]. CDDP has been reported to exert its actions by binding to the DNA in cancer cells, inhibiting DNA synthesis and subsequent cellular division. The antitumor activity of CDDP is closely associated with the serum concentration of the drug[19].

The key point of intra-arterial infusion chemotherapy is the selective retention of anticancer drugs at a high concentration in the HCC for a long time. LPD shows very selective deposition within HCC, in which it remains for several months after intra-arterial injection, whereas it disappears more rapidly from the nontumorous parenchyma[20]. Consequently, augmented antitumor efficacy and milder side effects were expected with the use of this substance for TACE. In fact, Morimoto *et al*[21] investigated the pharmacological advantages of TACE using DDPH for hypervascular hepatic tumors in animal experiments. They reported that the tumor concentration of the platinum agent in the DDPH-LPD-TACE group was about 14 times higher than that in the DDPH-hepatic arterial infusion (HAI) group. In addition, they reported that the plasma concentrations of the platinum agent were lower in the DDPH-LPD-TACE group than in the DDPH-HAI group.

Ono *et al*[12] reported that TACE using a suspension of CDDP powder in LPD was more effective against unresectable HCC than that using ADM-LPD emulsion. However, because CDDP was only available as a solution, it was difficult to prepare a high-dose CDDP suspension using LPD.

A fine-powder formulation of CDDP, namely DDPH, for intra-arterial infusion has been available for HCC treatment since 2004 in Japan. Dispensing of CDDP powder improved with the development of DDPH, and DDPH has now come to replace CDDP powder. Since DDPH-LPD suspension for TACE in HCC patients was expected to yield better therapeutic outcomes, TACE using DDPH-LPD suspension became widespread in Japanese institutions. We have already used TACE with DDPH-LPD suspension for HCC patients and reported favorable results[13]. This article focused on the efficacy of this therapy by analyzing the clinical results of 262 HCC patients treated in this manner.

The MST in the current study was 46.6 mo. The cumulative survival rates at 6, 12, 24, and 36 mo were 90.6%, 81.9%, 70.5% and 58.8%, respectively. The outcome in the present study was superior to previous trials of TACE using ADM, epirubicin, and other anthracyclines[5,6,13]. This could be explained as being due to the fact that TACE with ADM cannot be repeated as required because of the high frequency of adverse effects of ADM, such as leucopenia, severe vascular changes, and hepatic artery occlusion[12,13,22]. In the current study, leucopenia and HAD were observed in only 15 (5.7%) and 1 (0.4%) patients, respectively. Considering that TACE is often repeated in most patients, longer patency of the hepatic artery is preferable for properly deploying the lipiodol mixture and embolic agents into the tumor. In addition, we concluded that anthracyclines such as ADM may be relatively less effective against HCC; this is because of the high expression level of P-glycoprotein, which transports antitumor agents such as anthracyclines or vinca alkaloids from cells with a high active efflux mechanism in HCC tumors[23].

Moreover, survival in the present study was superior to previous trials of TACE using drug-eluting beads[24-29]. In these outcomes of previous trials of TACE using drug-eluting beads, the response rates were superior to the current study. Nevertheless, the cumulative survival rates of the patients in the current study were higher than those of the patients in the previous trials. Drug-eluting beads are known to give more distal vessel occlusion for a long-term period[30]. Therefore, it is possible that TACE with drug-eluting beads could have a greater embolizant effect than TACE with DDPH-LPD suspension, and this would lead to increased tumor growth factor release in response to hypoxia, with a consequent probability of recurrence and reduced overall survival.

The presence of PVTT has traditionally been considered a contraindication for transarterial therapy[31]. However, a recent study has revealed that TACE for patients with PVTT had survival benefits over conservative treatment[32]. Compared with this recent study, cumulative survival rates of patients with PVTT in the present study were better. On the other hand, cumulative survival rates of patients with Vp3 in subgroup analysis of the present study were 51.0% at 6 mo, 32.5% at 12 mo, and 16.2% at 24 mo. In our previous study of hepatic arterial infusion chemotherapy (HAIC) with 5-fluorouracil (5-FU) and pegylated IFN-α2b (PEG-IFNα-2b) for HCC patients with Vp3/4, cumulative survival rates were 83.8% at 6 mo, 77.8% at 12 mo, 55.6% at 24 mo[33]. Although it is impossible to compare the results of TACE using a suspension of DDPH in LPD and HAIC using 5-FU and PEG-IFNα-2b for HCC patients with Vp3, we think that a randomized controlled study comparing these therapies in patients with Vp3/4 will be needed in the future.

The prognosis of HCC patients depends on many factors, such as tumor stage and liver function. In the current study, the prognostic factors in patients treated with TACE with DDPH-LPD suspension were investigated. Among the variables examined, PVTT grade (Vp0-2), AFP (≤ 100 ng/mL), and therapeutic effect (CR+PR) were identified as being significantly associated with longer survival times on multivariate analysis. These results were similar to the result of a nationwide prospective cohort study by Takayasu *et al*[34], which was performed in 8510 patients with unresectable HCC who underwent TACE using an emulsion of lipiodol and anticancer agents followed by gelatin sponge particles.

Considering these facts, we conclude that TACE using DDPH-LPD suspension could be a useful treatment strategy for HCC patients. To confirm these results, randomized controlled trials comparing TACE using DDPH-LPD suspension with TACE using ADM-LPD emulsion or TACE using drug-eluting beads for patients with HCC are mandatory. Moreover, we think that a randomized controlled study comparing these therapies and HAIC for HCC patients with PVTT will be needed in the future.

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COMMENTS

Background

In recent years, transcatheter arterial chemoembolization (TACE) using an emulsion of doxorubicin (ADM) with lipiodol (LPD) (ADM-LPD emulsion) followed by embolization with a gelatin sponge has been commonly employed for hepatocellular carcinoma (HCC) treatment. However, HCC is not necessarily sensitive to these drugs.

Research frontiers

Cisplatin, a platinum compound, is an effective anticancer agent used in the treatment of various malignancies. Recently, a fine-powder formulation of cisplatin (DDPH, IA-call; Nipponkayaku, Tokyo, Japan) has also been available since 2004 as a therapeutic agent for intra-arterial infusion in Japan. Researchers have recently reported that TACE using a suspension of cisplatin powder in LPD may be more effective against unresectable HCC as compared with that using ADM-LPD emulsion. Therefore, TACE using DDPH has become widespread in Japanese institutions.

Innovations and breakthroughs

In this article, the authors evaluated the effectiveness of TACE using DDPH-LPD for 262 HCC patients. The objective early response rate was 43.6%. Cumulative survival rates were 90.6% at 6 mo, 81.9% at 12 mo, 70.5% at 24 mo, and 58.8% at 36 mo. Median survival time was 46.6 mo. All adverse reactions were controllable by temporary suspension of treatment. No serious complications or treatment-related deaths were observed. The outcome in the present study was superior to previous trials of TACE using ADM-LPD. Moreover, survival in the present study was superior to previous trials of TACE using drug-eluting beads.

Applications

Although randomized, controlled trials comparing TACE using DDPH-LPD suspension with TACE using ADM-LPD emulsion or TACE using drug-eluting beads for patients with HCC are mandatory, the authors conclude that TACE using DDPH-LPD suspension could be a useful treatment strategy for HCC patients.

Terminology

TACE is a minimally invasive medical procedure to restrict a tumor's blood supply. TACE is an interventional radiology procedure. The procedure involves gaining percutaneous access to the hepatic artery. When a blood vessel supplying tumor has been selected, alternating aliquots of the chemotherapy dose and of embolic particles, or particles containing the chemotherapy agent, are injected through the catheter. CDDP is a chemotherapy drug. It was the first member of a class of platinum-containing anti-cancer drugs that now also includes carboplatin and oxaliplatin. These platinum complexes react *in vivo*, binding to and causing crosslinking of DNA, which ultimately triggers apoptosis.

Peer review

This paper is well written. The clinical results are appropriately described. The authors present clinical evaluation of TACE of DDPH in lipiodol in the treatment of HCC. The data indicate that the treatment in HCC patients resulted in significantly better early response rate, overall survival, progression free survival and cumulative survival rates.

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BRIEF ARTICLE

Effects of Lizhong Tang on cultured mouse small intestine interstitial cells of Cajal

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Abstract

AIM: To investigate the effects of Lizhong Tang, an herbal product used in traditional Chinese medicine, on mouse small intestine interstitial cells of Cajal (ICCs).

METHODS: Enzymatic digestions were used to dissociate ICCs from mouse small intestine tissues. The ICCs were morphologically distinct from other cell types in culture and were identified using phase contrast microscopy after verification with anti c-kit antibody. A whole-cell patch-clamp configuration was used to record potentials (current clamp) from cultured ICCs. All of the experiments were performed at 30-32 ℃.

RESULTS: ICCs generated pacemaker potentials, and Lizhong Tang produced membrane depolarization in current-clamp mode. The application of flufenamic acid (a nonselective cation channel blocker) abolished the generation of pacemaker potentials by Lizhong Tang. Pretreatment with thapsigargin (a Ca^{2+}-ATPase inhibitor in the endoplasmic reticulum) also abolished the generation of pacemaker potentials by Lizhong Tang. However, pacemaker potentials were completely abolished in the presence of an external Ca^{2+}-free solution, and under this condition, Lizhong Tang induced membrane depolarizations. Furthermore, When GDP-β-S (1 mmol/L) was in the pipette solution, Lizhong Tang still induced membrane depolarizations. In addition, membrane depolarizations were not inhibited by chelerythrine or calphostin C, which are protein kinase C inhibitors, but were inhibited by U-73122, an active phospholipase C inhibitors.

CONCLUSION: These results suggest that Lizhong Tang might affect gastrointestinal motility by modulating pacemaker activity in interstitial cells of Cajal.

Key words: Interstitial cells of Cajal; Lizhong Tang; Motility; Gastrointestinal tract; Whole-cell patch clamp configuration

Core tip: The gastroprokinetic effects of Lizhong Tang are mediated by the induction of pacemaker potentials in interstitial cells of Cajal (ICCs). Taken together, our data suggest that the gastroprokinetic effects of Lizhong Tang are mediated by the induction of pacemaker potentials in ICCs. Considering the effects of this drug on ICCs, further research is required to identify the compounds responsible for the effects of Lizhong Tang and to determine their mechanisms of action.

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INTRODUCTION

Lizhong Tang, was first reported 1800 years ago in "Shanghan Lun", and it remains a classical herbal product in traditional Chinese medicine. Lizhong Tang is composed of Radix Ginseng (Panax ginseng C.A. Meyer), Rhizoma Zingiberis (Zingiber officinale Roscoe), Rhizoma Atractylodis Macrocephalae (Atractylodes macrocephala Koidz.) and Radix Glycyrrhizae (Glycyrrhiza uralensis Fisch)[1], and it is widely used in traditional medicine to treat spleen deficiency patterns in many diseases with common symptoms, such as vomiting, diarrhea, stomach pain, poor appetite, cold limbs, and stomach bleeding which, are caused by cold and weak organs[1,2]. However, little is known of the molecular basis of the effects of Lizhong Tang on gastrointestinal (GI) motility.

Interstitial cells of Cajal (ICCs) are pacemaker cells in GI muscles that generate rhythmic oscillations in membrane potentials known as slow waves[3-5]. Slow waves propagate within ICC networks and are conducted into smooth muscle cells *via* gap junctions. Furthermore, they initiate phasic contractions by activating Ca^{2+} entry through L-type Ca^{2+} channels. Pacemaker activity in the murine small intestine is mainly due to periodic activations of nonselective cation channels[6,7] or Cl^- channels[8,9]. ICCs also mediate or transduce inputs from the enteric nervous system. However, the effects of Lizhong Tang in mouse small intestine ICCs have not been investigated, and therefore, we undertook this study to investigate the characteristics of Lizhong Tang in mouse small intestine ICCs.

MATERIALS AND METHODS

Preparation of cells and cell cultures

Balb/c mice (3-7 d old) of either sex were anesthetized with ether and sacrificed by cervical dislocation. The small intestines, from 1 cm below the pyloric ring to the cecum, were removed and opened along the mesenteric border. The luminal contents were removed by washing with Krebs-Ringer bicarbonate solution. The tissues were then pinned to the base of a Sylgard dish, and the mucosae were removed by sharp dissection. Small tissue strips of intestinal muscle (consisting of both circular and longitudinal muscles) were equilibrated in Ca^{2+}-free Hanks solution (containing the following in mmol/L: KCl 5.36, NaCl 125, NaOH 0.34, Na_2HCO_3 0.44, glucose 10, sucrose 2.9, and HEPES 11) for 30 min, and then, the cells were dispersed using an enzyme solution containing collagenase (Worthington Biochemical Co., Lakewood, NJ, United States) 1.3 mg/mL, bovine serum albumin (Sigma Chemical Co., St. Louis, MO, United States) 2 mg/mL, trypsin inhibitor (Sigma) 2 mg/mL and ATP 0.27 mg/mL. The cells were plated onto sterile glass coverslips coated with murine collagen (2.5 μg/mL, Falcon/BD, Franklin Lakes, NJ, United States) in a 35-mm culture dish and then cultured at 37 ℃ in a 95% O_2, 50 mL/L CO_2 incubator in smooth muscle growth medium (Clonetics Corp., San Diego, CA, United States) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, United States) and murine stem cell factor (SCF 5 ng/mL, Sigma). ICCs were identified immunologically using an anti-c-kit antibody (phycoerythrin-conjugated rat anti-mouse c-kit monoclonal antibody; eBioscience, San Diego, CA, United States) at a dilution of 1:50 for 20 min[10]. The ICCs were morphologically distinct from other cell types in culture and were identified using phase contrast microscopy after verification with anti c-kit antibody.

Patch-clamp experiments

A whole-cell patch-clamp setup was used to record the membrane potentials (current clamp) of cultured ICCs. An axopatch ID (Axon Instruments, Foster, CA, United States) was used to amplify membrane currents and potentials. The command pulse was applied using an IBM-compatible personal computer running pClamp software (version 6.1; Axon Instruments). The data obtained were filtered at 5 kHz displayed on an oscilloscope and a computer monitor, and printed using a pen recorder (Gould 2200, Gould, Valley View, OH, United States). The results were analyzed using pClamp and Origin (version 6.0) software. All of the experiments were performed at 30-32 ℃.

Solutions and drugs

The physiological salt solution used to bathe cells (Na^+-Tyrode) contained the following (in mmol/L): KCl 5, NaCl 135, $CaCl_2$ 2, glucose 10, $MgCl_2$ 1.2 and HEPES 10, adjusted to pH 7.4 with NaOH. The pipette solution contained the following (in mmol/L): KCl 140, $MgCl_2$ 5, K_2ATP 2.7, NaGTP 0.1, creatine phosphate disodium 2.5, HEPES 5 and EGTA 0.1, adjusted to pH 7.2 with KOH. Lizhong Tang was purchased from I-WORLD Pharmaceuticals (South Korea). Lizhong Tang is composed of Radix Ginseng (Panax ginseng C.A. Meyer), Rhizoma Zingiberis (Zingiber officinale Roscoe), Rhizoma Atractylodis Macrocephalae (Atractylodes macrocephala Koidz.) and Radix Glycyrrhizae (Glycyrrhiza uralensis Fisch.). The adult dosage is 10-15 g (crude material) per day. More information about Lizhong Tang can be obtained at the I-WORLD Pharmaceuticals Homepage (http://i-pharm.koreasme.com). The pills were dissolved with distilled water at a concentration of 0.5 g of crude drug/ml and stored in the refrigerator. All of the drugs were obtained from Sigma (Sigma Chemical Co., United States). The drugs were dissolved in distilled water, and added to the bathing solution to the desired concentrations immediately prior to use. The addition of these chemicals to the bathing solution did not alter its pH. Thapsigargin, U-73122, and U-73343 were dissolved in dimethyl sulfoxide (DMSO) to produce 50 and 100 mmol/L stock solutions and added at 1000 times dilutions to the bathing solution on the day of the experiment. The final concentration of DMSO in the bathing solution was always < 0.1%, and we confirmed that this

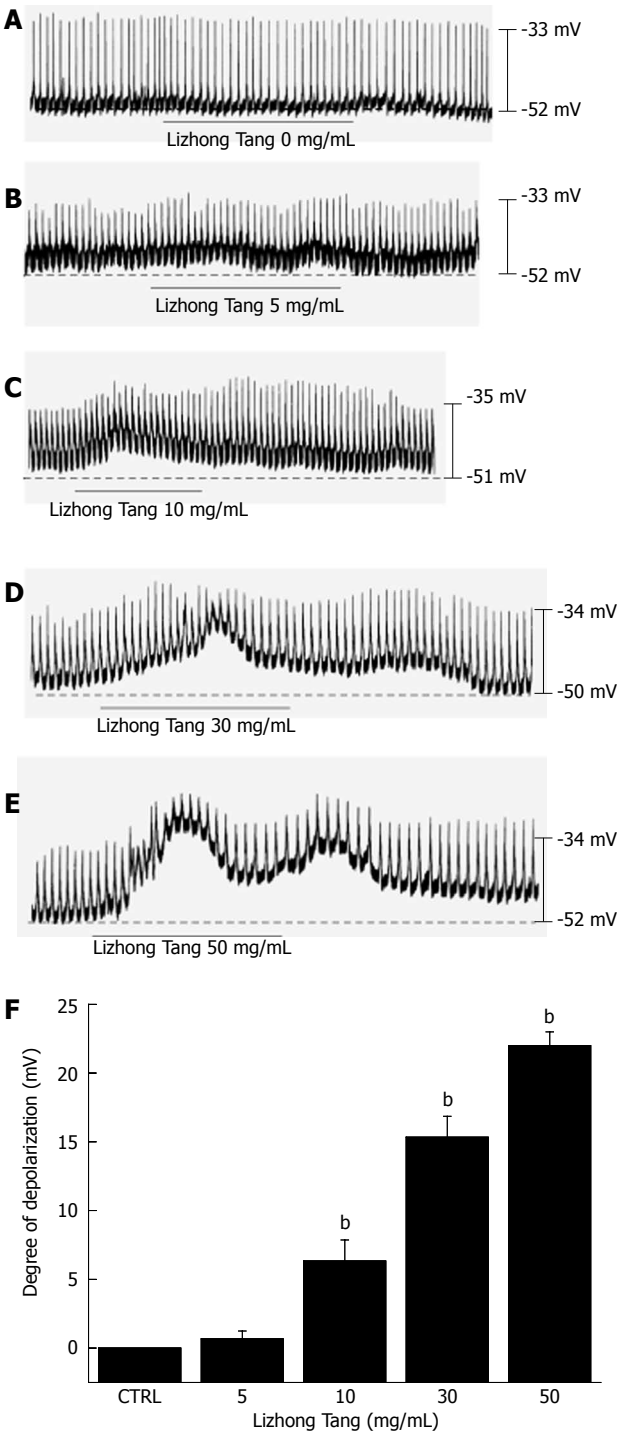

Figure 1 Effects of Lizhong Tang on pacemaker potentials in cultured interstitial cells of Cajal from murine small intestines. A-E: Pacemaker potentials in interstitial cells of Cajal exposed to Lizhong Tang (0-50 mg/mL) in current-clamp mode (I = 0); F: The responses to Lizhong Tang are summarized. Bars represent mean ± SE. $^{b}P < 0.01$ *vs* control group. CTRL: Control.

concentration did not affect the results.

Statistical analysis

All of the data are expressed as the mean ± SE. Student's *t*-test for unpaired data was used to compare the control and experimental groups. Statistical significance was accepted for P values < 0.05.

RESULTS

Effect of Lizhong Tang on the pacemaker potentials of cultured ICCs

The patch-clamp technique was tested on ICCs, which had formed network-like structures in culture after 2-4 d. Spontaneous rhythms were routinely recorded from cultured ICCs under current- and voltage-clamp conditions; ICCs within networks displayed more robust electrical rhythms. Tissue-like spontaneous slow waves have been previously recorded from these cells[11]. To understand the relationship between Lizhong Tang and the modulation of pacemaker activity in ICCs, we examined the effects of Lizhong Tang on pacemaker potentials. Recordings from cultured ICCs under current-clamp mode (I = 0) showed spontaneous pacemaker potentials. The mean resting membrane potential was -52 ± 1.3 mV, and the mean amplitude was 23 ± 2 mV. In the presence of Lizhong Tang (0-50 mg/mL), the membrane potentials were depolarized to 0.6 ± 0.5 mV at 5 mg/mL, 6.3 ± 1.5 mV at 10 mg/mL, 15.2 ± 1.3 mV at 30 mg/mL, and 22.1 ± 1.2 mV at 50 mg/mL (Figure 1A-E). Summarized values and a bar graph of the effects of Lizhong Tang on pacemaker potentials are provided in Figure 1F (n = 4).

Effects of non-selective cation channel blocker or Cl^- channel blocker on Lizhong Tang-induced pacemaker potentials in cultured ICCs

To determine the characteristics of the membrane depolarizations induced by Lizhong Tang, flufenamic acid (a non-selective cation channel blocker)[12,13] and niflumic acid (a Cl^- channel blocker)[12,14] were used. In the presence of flufenamic acid (5 μmol/L), pacemaker potentials were abolished and the subsequent application of Lizhong Tang (30 mg/mL) did not produce membrane depolarization (Figure 2A). In the presence of flufenamic acid, the membrane depolarizations produced by Lizhong Tang were 0.6 ± 0.4 mV, which was significantly different from the control values obtained in the absence of flufenamic acid (n = 4, Figure 2C). Pacemaker potentials were also abolished in the presence of niflumic acid (5 μmol/L), but Lizhong Tang still produced membrane depolarization (Figure 2B). In the presence of niflumic acid, the mean membrane depolarization produced by Lizhong Tang was 15.3 ± 0.4 mV, which was not significantly different from the control condition (n = 4, Figure 2C).

Effects of external Ca^{2+}-free solution and Ca^{2+}-ATPase inhibitor in the endoplasmic reticulum on Lizhong Tang-induced pacemaker potentials in cultured ICCs

External Ca^{2+} influx is necessary for GI smooth muscle contractions and is essential for the generation of pacemaker potentials by ICCs. The generation of pacemaker currents is known to be dependent on intracellular Ca^{2+} oscillations[15]. To investigate the roles of external and of internal Ca^{2+}, Lizhong Tang was tested under external Ca^{2+}-free conditions and in the presence of thapsigargin, an inhibitor of Ca^{2+}-ATPase in the endoplasmic reticu-

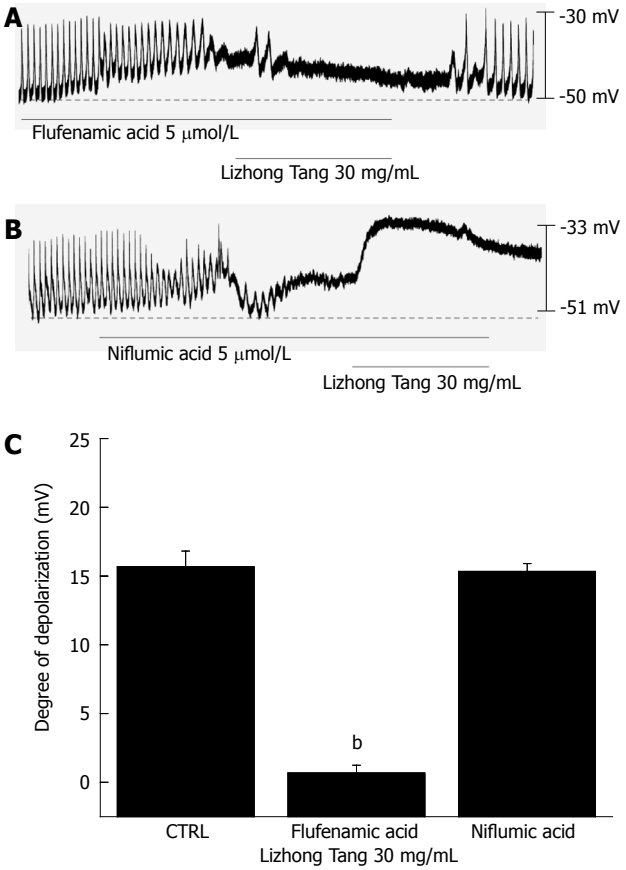

Figure 2 Effects of flufenamic acid (a nonselective cation channel blocker) or niflumic acid (a Cl^- channel blocker) on Lizhong Tang-induced pacemaker potentials in cultured interstitial cells of Cajal from murine small intestines. A: The application of flufenamic acid (5 μmol/L) abolished the generation of pacemaker potentials, and in the presence of flufenamic acid, Lizhong Tang (30 mg/mL) did not cause membrane depolarization; B: In contrast, although niflumic acid (5 μmol/L) abolished the generation of pacemaker potentials, it did not block Lizhong Tang-induced (30 mg/mL) membrane depolarization; C: The responses to Lizhong Tang in the presence of flufenamic acid or niflumic acid are summarized. Bars represent mean ± SE. $^bP < 0.01$ *vs* control group. CTRL: Control.

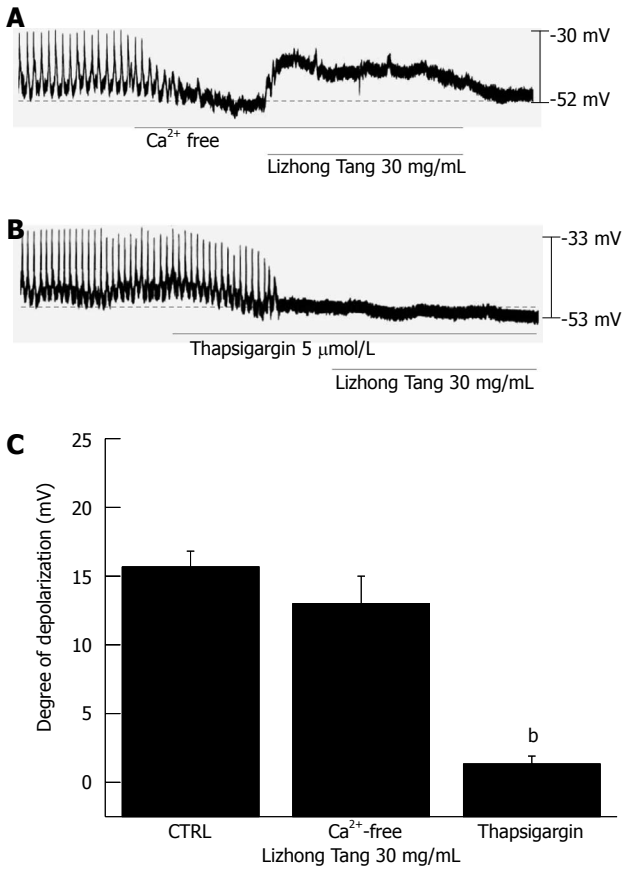

Figure 3 Effects of an external Ca^{2+}-free solution, thapsigargin (an inhibitor of Ca^{2+}-ATPase in the endoplasmic reticulum), or U-73122 (an active phospholipase C inhibitor) on Lizhong Tang-induced pacemaker potentials in cultured interstitial cells of Cajal. A: External Ca^{2+}-free solution abolished the generation of pacemaker potentials, but failed to block Lizhong Tang-induced (30 mg/mL) membrane depolarization; B: Thapsigargin (5 μmol/L) abolished the generation of pacemaker potentials, and blocked Lizhong Tang-induced (30 mg/mL) membrane depolarization; C: The responses to Lizhong Tang in external Ca^{2+}-free solution in the presence of thapsigargin are summarized. Bars represent mean ± SE. $^bP < 0.01$ *vs* control group. CTRL: Control.

lum[6,12]. Pacemaker potentials were completely abolished in the presence of an external Ca^{2+}-free solution, and under this condition, Lizhong Tang induced membrane depolarizations (n = 4, Figure 3A). However, under external Ca^{2+}-free conditions, membrane depolarizations by Lizhong Tang (30 mg/mL) were not significantly different from the depolarizations induced by Lizhong Tang (30 mg/mL) under normal Ca^{2+} conditions (n = 4, Figure 3C). In addition, Lizhong Tang-induced membrane depolarizations were inhibited by thapsigargin pretreatment (Figure 3B). Furthermore, the membrane depolarizations induced by Lizhong Tang were significantly affected by the presence of thapsigargin (n = 4, Figure 3C).

The involvement of G protein on Lizhong Tang-induced pacemaker potentials in cultured ICCs

The effects of GDP-β-S (a non-hydrolysable guanosine 5'-diphosphate analogue that permanently inactivates G-protein binding proteins[16]) were examined to determine whether G-proteins are involved in the effects of Lizhong Tang on ICCs. When GDP-β-S (1 mmol/L) was in the pipette solution, Lizhong Tang (30 mg/mL) still induced membrane depolarizations (Figure 4A). However, the membrane depolarizations induced by Lizhong Tang were not significantly affected by the presence of GDP-β-S (1 mmol/L) in the pipette solution (n = 4, Figure 4C).

Effects of phospholipase C inhibitor on Lizhong Tang-induced pacemaker potentials in cultured ICCs

Because membrane depolarizations induced by Lizhong Tang are related to intracellular Ca^{2+} mobilization, we examined whether the effects of Lizhong Tang on pacemaker potentials required phospholipase C (PLC) activation. To test this possibility, Lizhong Tang-induced membrane depolarizations were measured in the absence or presence of U-73122 (an active PLC inhibitor[17]). Pacemaker membrane depolarizations currents were completely abolished by U-73122 (5 μmol/L), and under these conditions, Lizhong Tang-induced (30 mg/mL)

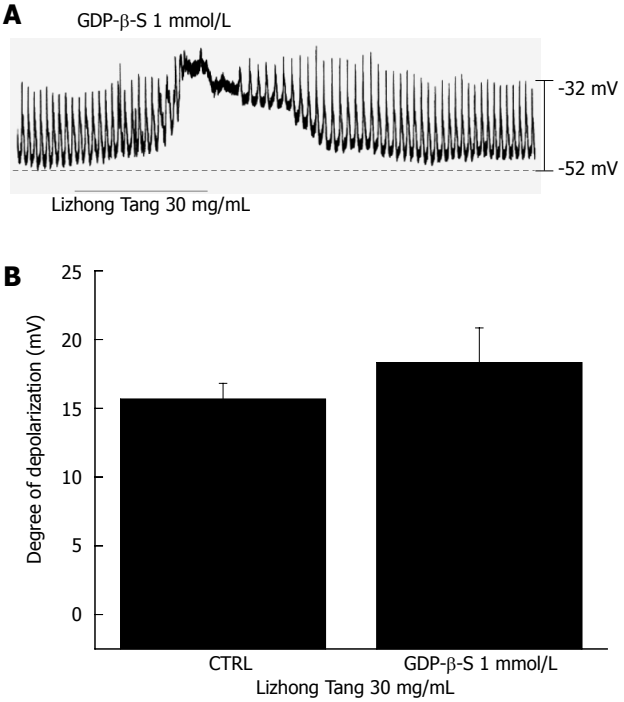

Figure 4 Effects of GDP-β-S in the pipette on Lizhong Tang-induced pacemaker potentials in cultured murine small intestine interstitial cells of Cajal. A: Pacemaker potentials in interstitial cells of Cajal exposed to Lizhong Tang (30 mg/mL) in the presence of GDP-β-S (1 mmol/L) in the pipette. Under these conditions, Lizhong Tang (30 mg/mL) caused membrane depolarization; B: The responses to Lizhong Tang in the presence of GDP-β-S in the pipette are summarized. Bars represent mean ± SE. CTRL: Control.

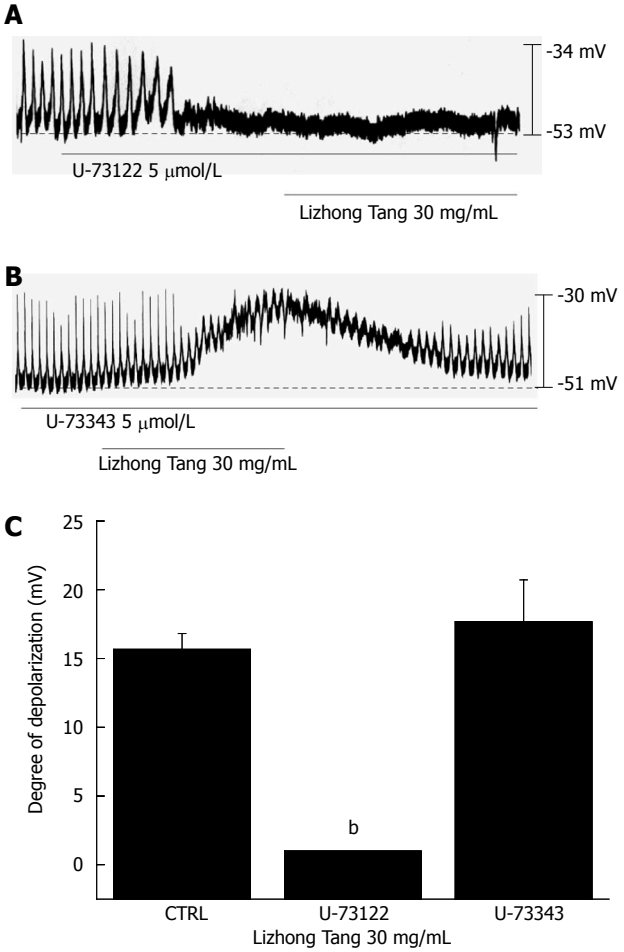

Figure 5 Effects of phospholipase C inhibitors on Lizhong Tang-induced potentials in cultured interstitial cells of Cajal. A: U-73122 (5 μmol/L; a phospholipase C inhibitor) abolished the generation of pacemaker potentials, and blocked Lizhong Tang-induced (30 mg/mL) membrane depolarization; B: The application of U-73343 (5 μmol/L) did not influence the generation of pacemaker currents or block Lizhong Tang-induced (30 mg/mL) membrane depolarization; C: The responses to Lizhong Tang in the presence of phospholipase C inhibitors are summarized. Bars represent mean ± SE. $^{b}P < 0.01$ *vs* control group. CTRL: Control.

membrane depolarizations were suppressed (n = 4, Figure 5A). In the presence of U-73122, the mean membrane depolarization produced by Lizhong Tang was 0.5 ± 0.3 mV, and this was significantly different than the depolarization observed in the absence of U-73122 (n = 4, Figure 5C). Treatment with U-73343 (5 μmol/L; an inactive analog of U-73122) had no influence on Lizhong Tang-induced pacemaker potentials, and Lizhong Tang-induced (30 mg/mL) membrane depolarizations were not suppressed by U-73343 (Figure 5C).

Effects of protein kinase C inhibitor on Lizhong Tang-induced pacemaker potentials in cultured ICCs

We tested the effects of chelerythrine and of calphostin C (both inhibitors of protein kinase C (PKC)[12,18]) to investigate whether Lizhong Tang-induced pacemaker potential responses are mediated by the activation of PKC. Neither chelerythrine (1 μmol/L) nor calphostin C (1 μmol/L) had any effect on membrane depolarizations induced by Lizhong Tang (30 mg/mL; Figure 6) and the value was also not significantly different when compared with the membrane depolarizations induced by Lizhong Tang in the absence of chelerythrine or calphostin C (n = 5, Figure 6C).

DISCUSSION

The GI tract exhibits spontaneous mechanical contractions that are mediated by the periodic generation of electrical pacemaker potentials, which are the basic determinant of GI smooth muscle activity[3]. Recent studies have shown that the ICCs act as the pacemakers and conductors of electrical slow waves in GI smooth muscles[3-5]. Moreover, evidence indicates that endogenous agents, such as, neurotransmitters, hormones, and paracrine substances modulate GI tract motility by influencing ICCs[5-7,19,20]. Therefore, one of the best ways to investigate the role of GI motility is to use ICCs. Many types of ICCs with different immunohistochemical and electrical properties, including myenteric ICCs (ICC-MY), intramuscular ICCs (ICC-IM), deep muscular plexus ICCs (ICC-DMP), and submucosal ICCs (ICC-SM), are distributed throughout the GI tract[16]. In animal models lacking ICC-MY, slow waves in the small intestine are strongly attenuated, which shows that these cells are indeed essential for pacemaker activity in the GI tract[21]. Furthermore, ICCs are involved in physiological GI mo-

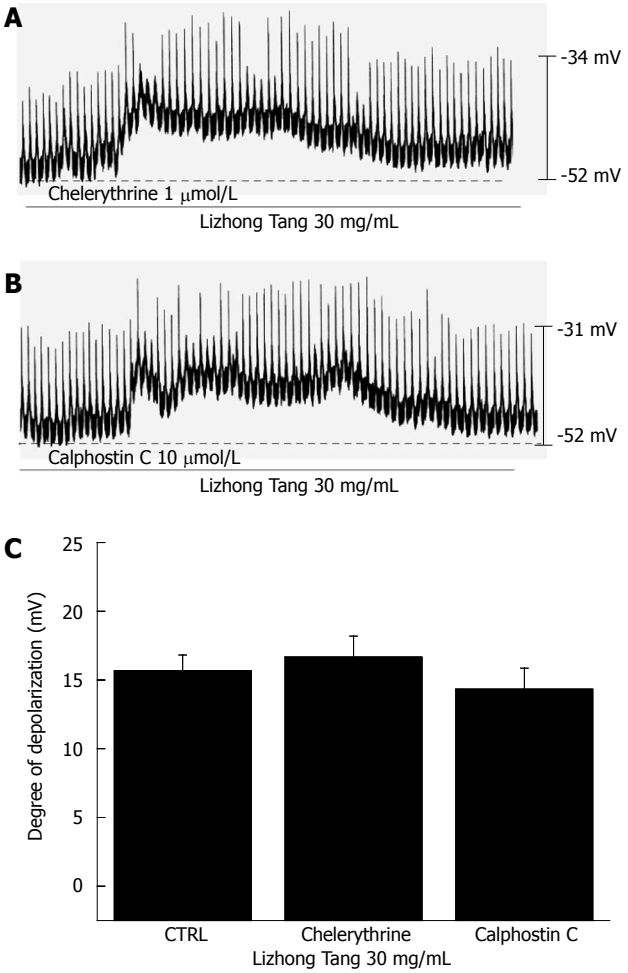

Figure 6 Effects of chelerythrine or calphostin C (inhibitors of protein kinase C) on Lizhong Tang-induced pacemaker potentials in cultured interstitial cells of Cajal. A, B: Pacemaker potentials in interstitial cells of Cajal exposed to Lizhong Tang (30 mg/mL) in the presence of chelerythrine (1 μmol/L) or calphostin C (10 μmol/L). Lizhong Tang caused membrane depolarization in the presence of both inhibitors; C: The responses to Lizhong Tang in the presence of chelerythrine or calphostin C are summarized. Bars represent mean ± SE. CTRL: Control.

tility and are therefore clinically important in many bowel disorders, including inflammatory bowel disease, chronic idiopathic intestinal pseudo-obstruction, intestinal obstruction with hypertrophy, achalasia, Hirschsprung's disease, juvenile pyloric stenosis, juvenile intestinal obstruction, and anorectal malformation[16].

Lizhong Tang warms the liver and spleen and strengthens the spleen and stomach. It has been widely used as treatment from deficiency, diarrhea with watery stool, nausea and vomiting. In addition, it also has ameliorative effects on loss of appetite, abdominal pain, acute or chronic gastritis gastric or duodenal ulcers, irritable bowel syndrome, chronic colitis, chronic bronchitis, oral herpes, and functional uterine bleeding[1,22,23]. However, the effects of Lizhong Tang on GI tract motility and ICCs have not been investigated.

In this study, Lizhong Tang produced membrane depolarization in current-clamp mode, and the application of flufenamic acid (a non-selective cation channel blocker), but not niflumic acid (a Cl^- channel blocker), abolished the generation of the pacemaker potentials induced by Lizhong Tang, suggesting that the Lizhong Tang-induced membrane depolarizations may be mediated by non-selective cationic channels. In addition, pretreatment with a Ca^{2+}-free solution or with thapsigargin (a Ca^{2+}-ATPase inhibitor in the endoplasmic reticulum), abolished the generation of pacemaker potentials. Under Ca^{2+}-free conditions, Lizhong Tang also showed membrane depolarization; however, in the presence of thapsigargin, Lizhong Tang did not show membrane depolarization, suggesting that intracellular calcium release is necessary. Furthermore, pacemaker membrane depolarizations were inhibited by U-73122 (PLC inhibitor), but not by GDP-β-S, which permanently binds G-binding proteins. In addition, the PKC inhibitors chelerythrine and calphostin C did not block Lizhong Tang-induced pacemaker potentials, suggesting that PLC is involved in the induction of the pacemaker potentials, but that PKC is not. In summary, Lizhong Tang affects GI motility by modulating pacemaker activity in ICCs, and this activation is associated with non-selective cationic channels via phospholipase C activation, and Ca^{2+} release from internal storage in an external Ca^{2+}-, G-protein-, and PKC-independent manner.

Taken together, our data suggest that the gastroprokinetic effects of Lizhong Tang are mediated by the induction of pacemaker potentials in ICCs. Considering the effects of this drug on ICCs, further research is required to identify the compounds responsible for the effects of Lizhong Tang and to determine their mechanisms of action.

COMMENTS

Background

Interstitial cells of Cajal (ICCs) are the pacemaker cells that generate slow waves in the gastrointestinal (GI) tract. Lizhong Tang is a classic herbal product in traditional Chinese medicine. However, the effects of Lizhong Tang in mouse small intestine ICCs have not been investigated.

Research frontiers

The gastroprokinetic effects of Lizhong Tang are mediated by the induction of pacemaker potentials in ICCs.

Innovations and breakthroughs

Lizhong Tang affects GI motility by modulating pacemaker activity in ICCs, and this activation is associated with non-selective cationic channels via phospholipase C activation, and Ca^{2+} release from internal storage in an external Ca^{2+}-, G-protein-, and protein kinase C-independent manner.

Applications

Lizhong Tang may be a new target for pharmacological treatment of GI motility disorders.

Peer review

In their manuscript, authors studies the effect of Lizhong Tang, an herbal product used in traditional Chinese medicine, on the pacemaking activity of mouse small ICCs. It was well written.

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BRIEF ARTICLE

Disease progression in chronic hepatitis C patients with normal alanine aminotransferase levels

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Abstract

AIM: To investigate whether the disease progression of chronic hepatitis C patients with normal alanine aminotransferase (ALT) levels differs by ALT levels.

METHODS: A total of 232 chronic hepatitis C patients with normal ALT (< 40 IU/L) were analyzed. The patients were divided into "high-normal" and "low-normal" ALT groups after determining the best predictive cutoff level associated with disease progression for each gender. The incidence of disease progression, as defined by the occurrence of an increase of $\geqslant$ 2 points in the Child-Pugh score, spontaneous bacterial peritonitis, bleeding gastric or esophageal varices, hepatic encephalopathy, the development of hepatocellular carcinoma, or death related to liver disease, were compared between the two groups.

RESULTS: Baseline serum ALT levels were associated with disease progression for both genders. The best predictive cutoff baseline serum ALT level for disease progression was 26 IU/L in males and 23 IU/L in females. The mean annual disease progression rate was 1.2% and 3.9% for male patients with baseline ALT levels $\leqslant$ 25 IU/L (low-normal) and > 26 IU/L (high-normal), respectively (P = 0.043), and it was 1.4% and 4.8% for female patients with baseline ALT levels $\leqslant$ 22 IU/L (low-normal) and > 23 IU/L (high-normal), respectively (P = 0.023). ALT levels fluctuated during the follow-up period. During the follow-up, more patients with "high-normal" ALT levels at baseline experienced ALT elevation (> 41 IU/L) than did patients with "low-normal" ALT levels at baseline (47.7% *vs* 27.9%, P = 0.002). The 5 year cumulative incidence of disease progression was significantly lower in patients with persistently "low-normal" ALT levels than "high-normal" ALT levels or those who exhibited an ALT elevation > 41 U/L during the follow-up period (0%, 8.3% and 34.3%, P < 0.001).

CONCLUSION: A "high normal" ALT level in chronic hepatitis C patients was associated with disease progression, suggesting that the currently accepted normal threshold of serum ALT should be lowered.

Key words: Alanine aminotransferase; Upper limits of normal; Disease progression; Hepatitis C virus; Hepatocellular carcinoma

Core tip: Recent studies have indicated that the upper limit of normal for the serum alanine aminotransferase (ALT) level should be lowered. However, outcome studies based on the development of adverse events during long-term follow-up are limited. In this present study, among patients infected with chronic hepatitis C virus who had normal ALT levels, the risk of disease progression differed between patients with "high-normal" and "low-normal" ALT levels, even within the currently ac-

cepted normal levels. This finding suggests that lowering the normal threshold of ALT levels may be necessary to better identify patients who are at increased risk for disease progression.

Sinn DH, Gwak GY, Shin J, Choi MS, Lee JH, Koh KC, Paik SW, Yoo BC. Disease progression in chronic hepatitis C patients with normal alanine aminotransferase levels. *World J Gastroenterol* 2013; 19(14): 2256-2261 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i14/2256.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i14.2256

INTRODUCTION

Serum alanine aminotransferase (ALT) is an easily available, low-cost screening tool for detecting hepatocellular disease[1,2]. Currently, the upper limit of normal (ULN) of ALT has been set at a mean value ± 2SD in a group of healthy individuals[1], usually at approximately 40 IU/L in many hospitals, including our hospital, although this value varies slightly between laboratories. However, several recent studies have demonstrated that the ULN of ALT should be lower than the currently accepted thresholds[3-7]. In these studies, the ULN of ALT was assessed in the standard manner (set at a mean value ± 2SD for healthy individuals); however, by defining a "new" healthy reference population, largely by excluding metabolically abnormal individuals[6]. If the ULN of ALT, often interchangeably used with the healthy level, is defined in this manner, it will vary according to the chosen reference class.

Another way of defining the ULN of ALT, or healthy levels, involves outcome studies, which are based on the development of adverse events during long-term follow-up[8-11]. The ULN of ALT can be set at a level that places individuals at increased risk of adverse consequences. In fact, a "high-normal" ALT level, even within the currently accepted normal range, has been associated with increased liver disease-related mortality[12,13], suggesting that the "healthy" ALT level should be lower than the currently accepted thresholds. In the present study, we aimed to assess whether a "high-normal" ALT level is associated with an increased risk of disease progression among patients with chronic hepatitis C.

MATERIALS AND METHODS

Patients

Previously, we reported the incidence and risk factors of disease progression in 1137 patients with chronic hepatitis C virus (HCV) infections[14]. The present study is a subgroup analysis of our previous study. In our previous study, we enrolled 1137 chronic hepatitis C patients who had no history or evidence of advanced liver disease. The detailed inclusion and exclusion criteria are described in our previous report[14]. Briefly, patients exhibited evidence of chronic HCV infection but had no history or evidence of cirrhotic complications, including a Child-Pugh score of > 5 points, esophageal or gastric variceal bleeding, spontaneous bacterial peritonitis, hepatic encephalopathy, and HCC. For the present study, out of a total of 1137 patients, we selected 232 patients who did not receive antiviral therapy for chronic HCV infection and exhibited normal ALT levels (< 40 IU/L) at enrollment.

Follow-up and endpoint assessment

Follow-up data collection and endpoint assessment followed the protocol of our previous study[14]. Briefly, all patients were followed-up at least every 3-6 mo, or more frequently as required, for at least 1 year. Follow-up tests included conventional biochemical tests and abdominal ultrasonography screening. Endoscopic examination was performed when patients exhibited any symptoms or signs suggesting gastrointestinal bleeding, such as hematemesis, melena, hematochezia or sudden drops in blood hemoglobin levels. If patients did not exhibit any indications of gastrointestinal bleeding, endoscopic examination was not performed routinely or regularly. Patients who dropped out during the follow-up or who died without reaching the endpoint were classified as either withdrawals or censored cases, respectively. All ALT levels during the follow-up were obtained from each patient, and the changes in ALT levels during the follow-up were also assessed. Blood samples of the patients were collected after > 8 h of fasting and analyzed within 24 h. Plasma concentrations of ALT were measured using an autoanalyzer (Hitachi Modular D2400, Roche, Tokyo, Japan).

The primary endpoint was the time to disease progression, as defined by the first occurrence of any of the following: an increase of at least 2 points in the Child-Pugh score, spontaneous bacterial peritonitis, bleeding gastric or esophageal varices, hepatic encephalopathy, the development of HCC or death related to liver disease[14]. HCC was diagnosed by histological evaluation or was diagnosed clinically according to the 1st edition of guidelines for the diagnosis of HCC of the Korean Association for the Study of the Liver[15]. As one of the potential risk factors for disease progression, alcohol consumption was assessed as all-or-none from available medical records. The Institutional Review Board at Samsung Medical Center reviewed and approved this study protocol.

Statistical analysis

The cumulative incidence rate of disease progression was calculated and plotted by using the Kaplan-Meier method. Differences in the incidence rate between the groups were analyzed using a log-rank test. A receiver operating curve (ROC) analysis was performed for ALT levels to estimate the best predictive cut-off values. Multivariate analysis was performed using the Cox proportional hazard model for variables with *P*-values of < 0.05 for univariate analysis to identify factors associated with disease progression. *P*-values less than 0.05 were considered significant.

Table 1 Baseline characteristics of the 232 patients

Characteristics	(*n* = 232)
Age (yr, mean ± SD)	57.2 ± 10.7
Gender, male/female	89 (38):143 (62)
Weight (kg, mean ± SD)	61.6 ± 9.3
Alcohol consumption	32 (14)
Diabetes	29 (13)
Estimated duration of infection (mo, median, range)	18 (0–398)
Alanine aminotransferase (IU/L, median, quartile)	25 (19–32)
Aspartate aminotransferase (IU/L, median, quartile)	30 (23–48)
Platelet (10^3/mm^3, median, quartile)	179 (13–224)
Aspartate aminotransferase: platelet ratio index (median, range)	0.4 (0.1–7.5)
> 1	50 (22)
Genotype[1]	
1b	12 (57)
1 others	1 (5)
2	8 (38)

[1]Percent value refers to percentage within studied patients. Data are expressed as absolute numbers (percentage) or mean ± SD.

RESULTS

Patient characteristics and incidence of disease progression

Table 1 presents the clinical features of the patients at study entry. Disease progression was noted in 33/232 patients (14.2%) during the median follow-up of 54.1 mo (range: 12-151 mo). The mean annual incidence rate of disease progression was 3.1%. The cause of disease progression (first occurrence) was HCC in 27 patients (11.6%), bleeding varices in 2 patients (0.9%), ≥ a 2 point increase in the Child-Pugh score in 2 patients (0.9%), and hepatic encephalopathy in 2 patients (0.9%).

Disease progression according to the baseline serum ALT levels

Because previous studies have suggested differing normal ALT thresholds in males and females[16-18], we analyzed data separately by gender. The ALT level (tested as a numeric variable) was significantly associated with the disease progression in both males [hazard ratio (HR) = 1.09; 95%CI: 1.01-1.21, P = 0.048] and females (HR = 1.07; 95%CI: 1.01-1.13, P = 0.040). When ALT levels were stratified, the incidence rates of disease progression were 5%, 11%, and 21% in male patients with ALT levels of < 20 IU/L (n = 20), 20-39 IU/L (n = 27), and 30-39 IU/L (n = 42), respectively (P = 0.191) (Figure 1A). In female patients, the incidence rates were 2%, 18%, and 20% with ALT levels of < 20 IU/L (n = 42), 20-39 IU/L (n = 56), and 30-39 IU/L (n = 45), respectively (P = 0.034) (Figure 1B).

We performed ROC curve analysis to determine the best ALT cutoff value associated with disease progression. The best cutoff value was 26 IU/L in males (area = 0.722, P = 0.011, sensitivity = 0.85, specificity = 0.53) and 23 IU/L in females (area = 0.634, P = 0.055, sensitivity = 0.80, specificity = 0.47). The cumulative incidence of disease progression was significantly higher in patients with "high-normal" ALT levels than in those with "low-normal" ALT levels in both males and females (Figure 2).

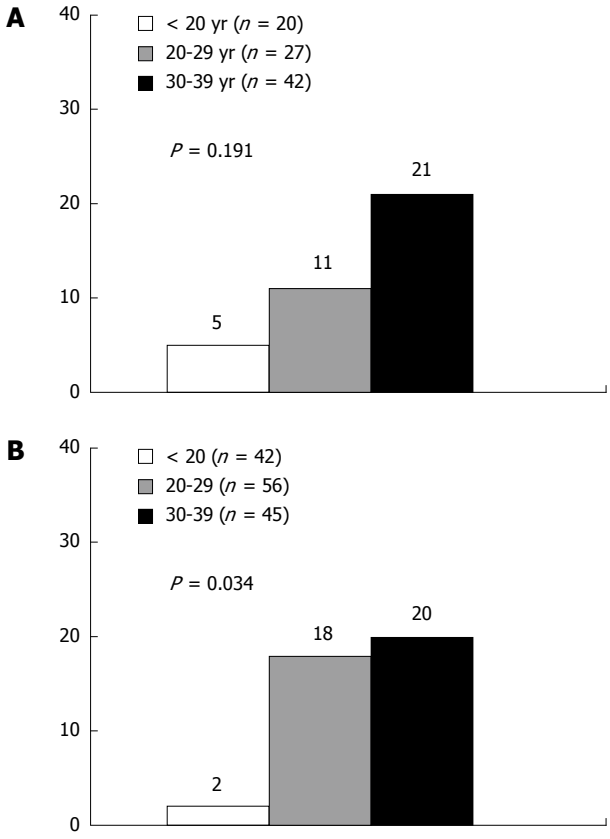

Figure 1 Incidence of disease progression according to serum alanine aminotransferase levels. There was an increase in the incidence of disease progression along with an increase in serum alanine aminotransferase (ALT) values for males (A) and for females (B). White, gray and black bars represent patients with ALT levels < 20 IU/L, 20-29 IU/L, and 30-39 IU/L, respectively.

Risk of disease progression according to baseline ALT levels

The potential risk factors assessed for disease progression included the following variables: age, gender, diabetes mellitus, alcohol intake, body weight, estimated duration of infection, platelet levels, ALT levels, aspartate aminotransferase (AST) levels, and α-fetoprotein levels. Because HCV RNA quantitation and genotype data were available for only a few patients, these variables were not included in our analysis.

Univariate Cox proportional-hazard regression analyses revealed that age, platelet levels, AST levels, and ALT levels were significantly associated with disease progression in males (Table 2). In females, age and diabetes as well as platelet, AST, and ALT levels were associated with disease progression (Table 2). Multivariate Cox proportional-hazard regression analyses were performed for the above-mentioned variables. After adjusting for potential confounders, the baseline ALT levels remained a significant factor associated with disease progression in both genders (Table 2).

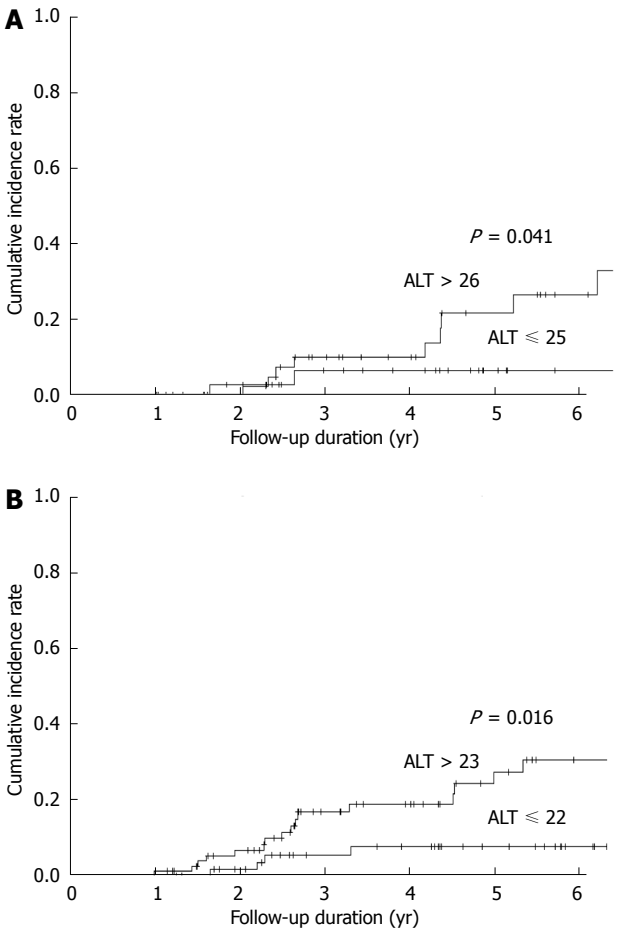

Figure 2 Cumulative incidence of disease progression according to alanine aminotransferase levels. The incidence rate differed between patients with "high-normal" and "low-normal" alanine aminotransferase (ALT) levels. A: Males; B: Females.

Change of ALT level during follow-up and risk of disease progression

ALT levels fluctuated during the follow-up period. During the follow-up, ALT remained "low-normal" in 41 patients (17.7%), "high-normal" in 101 patients (43.5%), and elevated over > 41 IU/L in 90 patients (38.8%). More patients with "high-normal" ALT levels at baseline experienced an ALT elevation (> 41 IU/L) during follow-up than did patients with "low-normal" ALT levels at baseline (47.7% *vs* 27.9%, $P = 0.002$). The 5-year cumulative incidence of disease progression was significantly lower in patients whose ALT levels remained "low-normal" than in those patients whose ALT levels were "high-normal" or who exhibited ALT elevation > 41 U/L during follow-up (0%, 8.3% and 34.3%, $P < 0.001$, Figure 3).

DISCUSSION

The present study demonstrated a significant difference in the disease progression rate in chronic hepatitis C patients with "high-normal" and "low-normal" ALT levels for both genders. Because the long-term prognosis significantly differs between patients with "high-normal" ALT values and patients with "low-normal" ALT values, these findings strongly suggest that the currently used normal ALT range warrants further stratification[19-22]. This finding is consistent with findings by Lee *et al*[13], who investigated the incidence of HCC in a prospective cohort of chronic HCV-infected patients. The authors reported that elevated serum ALT levels were an independent risk factor for the development of HCC and that the risk begins to rise in patients with ALT levels of 15 IU/L, far below the currently used ULN for ALT levels[13]. Thus, patients with "high-normal" ALT (26 to 40 IU/L in male and 23 to 40 IU/L in female in this study) should not be considered "normal" or "healthy", and lowering the 'healthy' ULN of ALT is advisable.

In this study, we enrolled patients who exhibited normal ALT levels at baseline. However, during the follow-up, many patients experienced ALT elevation. Overall, 90 of 232 patients (38.8%) exhibited ALT elevation > 41 IU/L during the follow-up. Patients with "high-normal" ALT levels exhibited a higher incidence of ALT flare

Table 2 Disease progression hazard ratio for each factor according to gender

	Univariate		Multivariate	
	Hazard ratio (95%CI)	*P* value	Hazard ratio (95%CI)	*P* value
Male				
ALT (IU/L) > 26 *vs* ≤ 25	4.67 (1.03-21.1)	0.045	5.35 (1.05-27.3)	0.043
Platelet (10^3/mm^3)	0.98 (0.97-0.99)	0.002	0.98 (0.96-0.99)	0.012
Age (yr)	1.07 (1.01-1.13)	0.030	1.06 (0.98-1.14)	0.12
AST (IU/L)	1.02 (1.01-1.03)	< 0.001	1.00 (0.99-1.02)	0.77
Female				
ALT (IU/L) > 23 *vs* ≤ 22	3.51 (1.17-10.5)	0.025	4.40 (1.12-15.8)	0.023
Platelet (10^3/mm^3)	0.97 (0.96-0.98)	< 0.001	0.97 (0.96-0.98)	< 0.001
Age (yr)	1.06 (1.01-1.10)	0.017	1.04 (0.98-1.09)	0.18
AST (IU/L)	1.02 (1.01-1.03)	< 0.001	1.00 (0.99-1.01)	0.97
Diabetes (yes *vs* no)	3.23 (1.24-8.41)	0.016	2.57 (0.83-7.92)	0.10

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

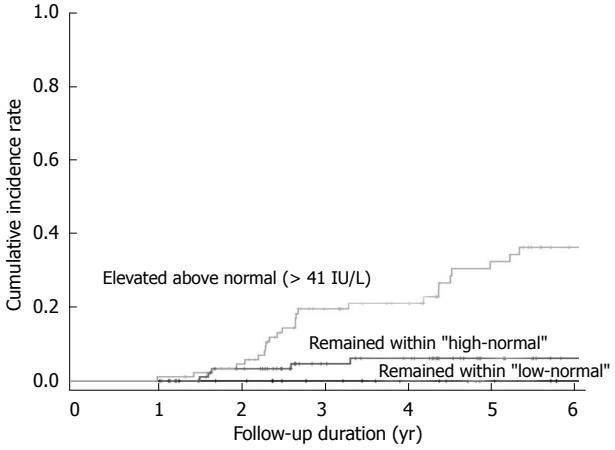

Figure 3 Cumulative incidence of disease progression according to changes in alanine aminotransferase levels. The incidence rate differed among patients who exhibited persistently "low-normal" alanine aminotransferase (ALT) levels or "high-normal" ALT levels and patients who exhibited ALT elevation (> 41 IU/L) during follow-up.

(> 41 IU/L) than did patients with "low-normal" ALT levels. During follow-up, only 17.7% of patients with initially "low-normal" ALT levels persistently expressed "low-normal" ALT levels. The risk of disease progression differed significantly among patients who exhibited ALT elevation (> 41 IU/L), patients who persistently exhibited "high-normal" ALT, and patients who persistently exhibited "low-normal" ALT levels (34.3%, 8.3% and 0%, respectively). This finding emphasizes the importance of serial ALT follow-ups[20,23,24], even for patients with normal ALT levels, because ALT levels can change and disease can progress in these patients.

It is noteworthy that best cutoff value of ALT for disease progression differed according to gender in this study. Several factors influence serum ALT values, including age, race, gender and body mass index[16-18,25]. Currently, many laboratories do not assign different ULN of ALT by gender; however, several studies support the consideration of gender when setting the ULN of ALT[3,5]. In this study, we also observed different cutoff values according to gender (26 IU/L in males and 23 IU/L in females) when the ULN of ALT was calculated in terms of predicting adverse consequences. Gender-specific ULN values of ALT appear more reasonable and should be applied in clinical practice.

There are limitations to this study that require careful interpretation of our results. The mean annual incidence rate of disease progression in this study was 3.09%. Previous studies on the natural history of HCV have used various outcome measures, making it difficult to compare to this study. Nevertheless, the annual progression rate to cirrhosis in chronic HCV infection has been reported to be 0.1% to 1%[26-28]. As the endpoint of this study was advanced cirrhotic complications, the reported incidence rate in this study was very high. This study is a retrospective study that was performed at a tertiary referral center. Hence, selection bias may account for the high incidence rate of disease progression. Furthermore, a considerable proportion of the patients may have had significant fibrosis at baseline. Although baseline liver biopsies were not performed in most patients, an aspartate aminotransferase: platelet ratio index > 1, a noninvasive marker that can predict fibrosis in chronic hepatitis C patients[29], was noted in 22% of the patients at baseline.

In summary, the present study demonstrated that patients with "high-normal" ALT levels, even within the currently accepted normal range, exhibit a significantly higher risk of ALT elevation and disease progression. The optimal ALT cutoff value to predict adverse outcomes differed by gender. Thus, gender-specific and lower ALT cutoffs seem more appropriate than the currently used ALT cutoff (40 IU/mL, regardless of gender).

COMMENTS

Background

Serum alanine aminotransferase (ALT) is an easily available, low-cost screening tool for detecting hepatocellular disease. Currently, an upper limit of normal (ULN) of ALT has been set at the mean value ± 2SD in a group of healthy individuals. However, the ULN of ALT should also be set at a level that identifies individuals at risk for developing adverse consequences during follow-up.

Research frontiers

Several previous studies, in which the ULN of ALT was defined as the mean value ± 2SD, have demonstrated that the ULN of ALT is lower than the currently accepted thresholds (40 IU/L).

Innovations and breakthroughs

The present study, a long-term outcome study, demonstrated that there was a significant difference in the rate of disease progression between chronic hepatitis C patients with "high-normal" and "low-normal" ALT levels for both genders.

Applications

Because the long-term prognosis significantly differs between patients with "high-normal" ALT values and patients with "low-normal" ALT values, these findings strongly suggest that the currently used normal ALT range warrants further stratification.

Terminology

"High-normal" and "low-normal" refers to ALT levels that are associated with disease progression for each gender within the currently accepted normal ALT level range (< 40 IU/L).

Peer review

The hepatic enzymes is indicator of liver damage, its changes must have personal specificity, individual variations is fact, regardless the gender, however, findings in this study is important.

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BRIEF ARTICLE

Hepatitis B virus induces expression of cholesterol metabolism-related genes *via* TLR2 in HepG2 cells

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Abstract

AIM: To investigate whether hepatitis B virus (HBV) exacerbates hepatic cholesterol accumulation, and explore the underlying mechanisms.

METHODS: HepG2 cells were infected with adenovirus (Ad) containing 1.3-fold overlength HBV genome. Real-time polymerase chain reaction and Western blotting were used to measure mRNA and protein expression of target genes. Cholesterol accumulation was measured by fluorescence microscopy. Cell toxicity due to Ad-HBV treatment was determined by the mitochondrial tetrazolium assay. The protein levels of toll-like receptors (TLRs) were determined by Western blotting.

RESULTS: Ad-HBV increased hepatic cholesterol accumulation and enhanced the mRNA and protein levels of low-density lipoprotein receptor (LDLR) and 3-hydroxy-3-methylglutharyl-coenzyme A reductase (HMGCoAr) mRNA and protein expression in HepG2 cells. In addition, these inductive effects were partly offset by suppressing TLR2 expression levels by small interfering RNA in HepG2 cells.

CONCLUSION: Ad-HBV increases LDLR and HMGCoAr expression, resulting in exacerbated cholesterol accumulation in HepG2 cells, which was mediated *via* the TLR2 pathway.

Key words: Hepatitis B virus; Toll-like receptors; Low-density lipoprotein receptor; 3-hydroxy-3-methylglutharyl-coenzyme A reductase

Core tip: This study investigated whether hepatitis B virus (HBV) exacerbates hepatic cholesterol accumulation and explored the underlying mechanisms. The authors found that adenovirus HBV increased low-density lipoprotein receptor and 3-hydroxy-3-methylglutharyl-coenzyme A reductase expression, resulting in exacerbated cholesterol accumulation in HepG2 cells, which was mediated *via* the toll-like receptor 2 pathway. These results may also have implications in the treatment of atherosclerosis.

Li YJ, Zhu P, Liang Y, Yin WG, Xiao JH. Hepatitis B virus induces expression of cholesterol metabolism-related genes *via* TLR2 in HepG2 cells. *World J Gastroenterol* 2013; 19(14): 2262-2269 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i14/2262.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i14.2262

INTRODUCTION

Hepatitis B virus (HBV) infection is a major public

health problem worldwide[1]. A possible role for infections in atherosclerosis has been deeply scrutinized since the demonstration that herpes virus induced atherosclerosis in chickens in 1978[2]. It has been shown that the incidence of hepatic steatosis in HBeAg-negative chronic hepatitis B patients is about 32%[3]. A published study from a health-screening test cohort showed that there was a strong association between hepatitis virus carriers and carotid atherosclerosis[4,5].

It is still controversial as to whether HBV-induced inflammation correlates with disease in organs other than the liver. To date, there are few data available to prove the association between HBV infection and atherosclerosis. Kiechl *et al*[6] found no significant association between chronic hepatitis and the development of new carotid atheromatous plaques, although they did not specify the type of hepatitis virus. However, another study in Japan demonstrated an increased prevalence of carotid atherosclerosis in HBV carriers[7].

Research has revealed that HBV-induced inflammation correlates with disease in organs other than the liver[8]. A previous report indicated that inflammation plays an important role in atherosclerosis[9]. In addition, this adverse impact of virus infection is partly, if not all, mediated by toll-like receptors (TLRs)[10-13]. For instance, Zhang *et al*[14] found that TLR2/4 signaling involved in the adaptive immune response plays a role in chronic HBV infection.

However, the role of HBV infection in hepatic cholesterol accumulation is still unclear. Therefore, the aims of the present investigation were to test: (1) whether HBV affects the expression of genes related to cholesterol metabolism such as low-density lipoprotein receptor (LDLR) and 3-hydroxy-3-methylglutharyl-coenzyme A reductase (HMGCoAr) in hepatocytes; and (2) whether TLRs are involved in lipid metabolism disorders caused by HBV.

MATERIALS AND METHODS

Cell culture

HepG2 and AD293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% (v/v) penicillin-streptomycin at 37 ℃ in a humid atmosphere of 5% CO_2. HepG2 cells were switched to serum-free medium 24 h before treatment.

Amplification and quantification of adenoviral vector-hepatitis B virus

In this study, adenoviral vectors were designed, which initiated HBV replication from a 1.3-fold overlength HBV genome. A control adenoviral vector (Ad) was also included. AD293 cells were infected with 1×10^5 to 1×10^7 copies of Ad-HBV for 72 h. Cells were harvested and counted under the microscope. An equal number of cells were maintained in phosphate buffered saline (PBS) and underwent three freeze-thaw cycles. Lysates were cleared by centrifugation at 14 000 × *g*, divided into equal volumes and used for real-time polymerase chain reaction (PCR) and infecting HepG2 cells. The HBV DNA was quantified using the BIO-RAD iCycler real-time PCR system and the qPCR Master Mix (Da An Gene Co., Ltd, Guangzhou, China). The copy of viral genome equivalents was determined using a calibration curve containing known amounts of HBV DNA. Ad was amplified in AD293 cells. To quantify Ad, the infected efficiency of Ad in AD293 cells was measured and normalized to the infected efficiency of Ad-HBV.

Table 1 Primers for real-time polymerase chain reaction

Gene	Primers for real-time polymerase chain reaction
LDLR	Sense: 5′-TCAACACACAACAGCAGATGGCAC-3′
	Antisense: 5′-AAGGCTAACCTGGCTGTCTAGCAA-3′
HMGCoAr	Sense: 5′-TATGTGCTGCTTTGGCTGCATGTC-3′
	Antisense: 5′-ATACCAAGGACACACAAGCTGGGA-3′
TLR2	Sense: 5′-ACCTGTCCAACAACAGGATCACCT-3′
	Antisense: 5′-TGTTCAAGACTGCCCAGGGAAGAA-3′
TLR4	Sense: 5′-GCCGAAAGGTGATTGTTGTGGTGT-3′
	Antisense: 5′-ACTGCCAGGTCTGAGCAATCTCAT-3′
GAPDH	Sense: 5′-AGGAGTAAGAAACCCTGGAC-3′
	Antisense: 5′-CTGGGATGGAATTGTGAG-3′

LDLR: Low-density lipoprotein receptor; *TLR2*: Toll-like receptor 2; *TLR4*: Toll-like receptor 4. *HMGCoAr*: 3-hydroxy-3-methylglutharyl-coenzyme A reductase; *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase.

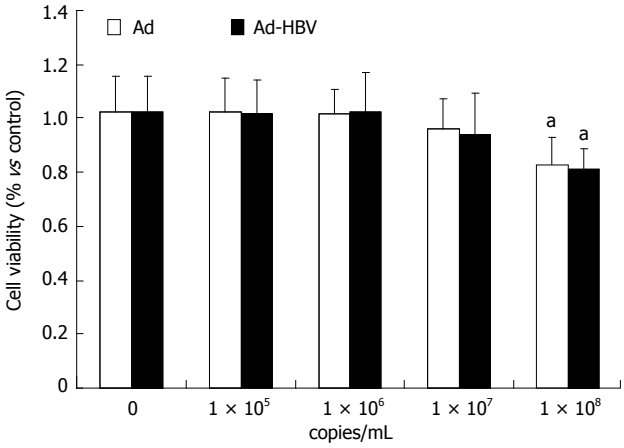

Figure 1 Cell toxicity by adenovirus hepatitis B virus. The data are presented as means ± SE. $^aP < 0.05$ *vs* control group, $n = 6$. Ad: Adenovirus; HBV: Hepatitis B virus.

Cytotoxicity assay

Cell toxicity due to Ad-HBV treatment was determined by the mitochondrial tetrazolium assay (MTT). HepG2 cells were grown in media with Ad-HBV at various concentrations for 4 d before addition of the MTT agent. Optical density was read at 570 nm using the BiotekElx-800 plate reader. Cells treated with vehicle served as controls, and the cell viability of the Ad-HBV treated group was normalized to that of the control group.

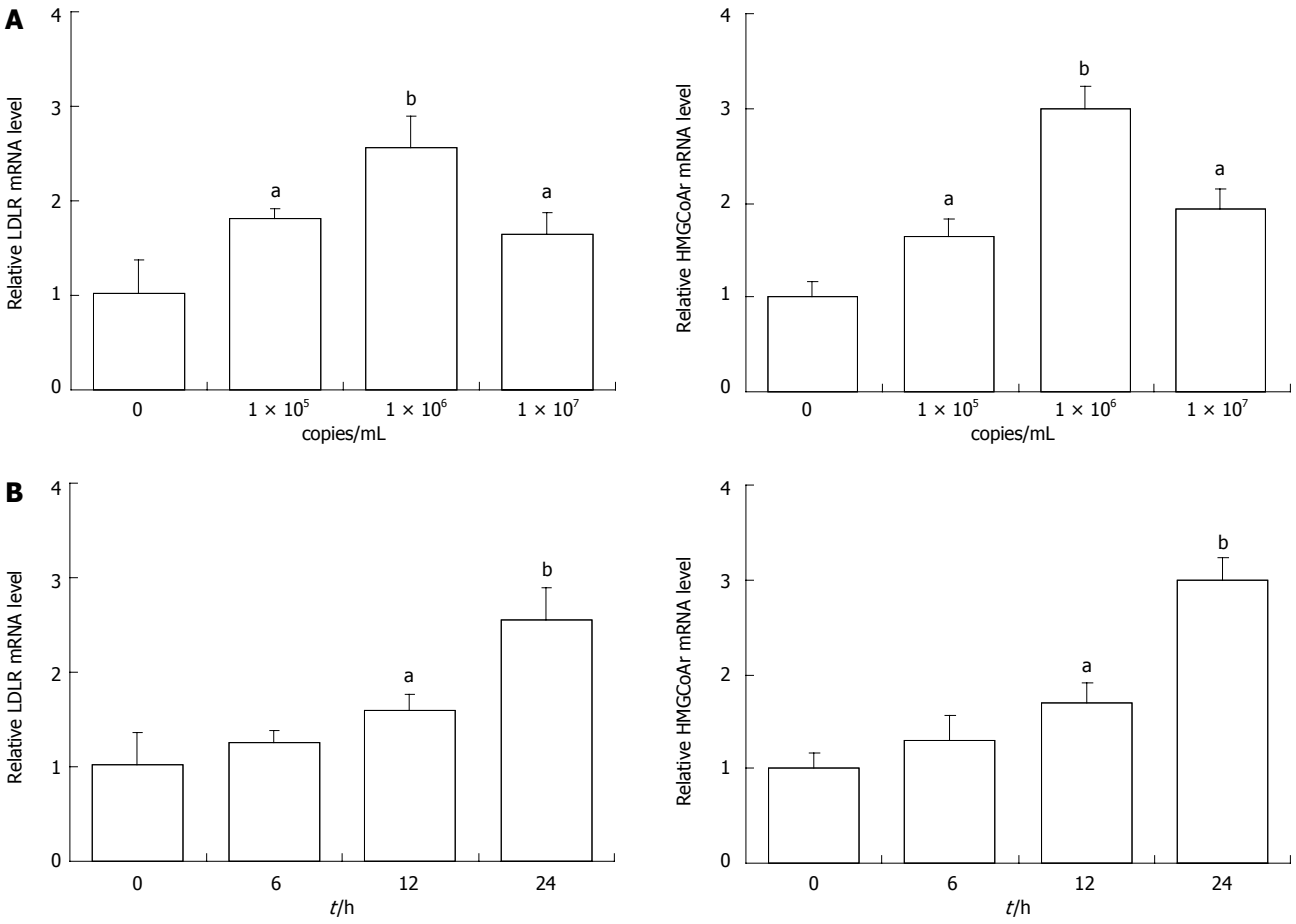

Figure 2 Effects of adenovirus hepatitis B virus treatment on the mRNA levels of genes related to cholesterol metabolism in HepG2 cells. A: HepG2 cells were treated with different concentration of adenovirus-hepatitis B virus (Ad-HBV) (1×10^5, 1×10^6, 1×10^7 copies) for 24 h; B: HepG2 cells were incubated for 0-24 h with 1×10^6 copies Ad-HBV. $^aP < 0.05$, $^bP < 0.01$ *vs* control group. LDLR: Low-density lipoprotein receptor.

Quantitative RT-PCR

Total RNA was isolated from cultured HepG2 cells treated with different concentration of Ad-HBV (1×10^5, 1×10^6, 1×10^7 copies) for 24 h as well as HepG2 cells incubated for 0-24 h with 1×10^6 copies Ad-HBV using the guanidinium-phenol-choloroform method[15]. Total RNA (500 ng) was used as a template for RT-PCR. The RT reaction was set up using a kit from TaKaRa (PrimeScriptTM RT reagent Kit, Dalian, China). Following synthesis, cDNA was split for the separate amplification of target genes using specific primers as shown in Table 1. All primers were designed using the following website (www.idtdna.com/Primerquest/). Real-time PCR was performed in a BIROD using SYBR Premix Ex TaqTM (TaKaRa, Dalian, China) according to the manufacturer's protocol. After PCR, a dissociation curve (melting curve) was constructed at the temperature ranging from 60 ℃ to 95 ℃. Ct values were averaged and normalized to GAPDH. Relative expression was determined by the ΔΔCt comparative threshold method.

TLRs-siRNA

Sequences of TLR2/TLR4 siRNA were purchased from Santa Cruz (United States. The transfection of siRNA was performed using a Lipofectamine kit (Invitrogen, United States) according to the manufacturer's instructions. The medium was changed 6 h after transfection, and the cells were incubated with Ad-HBV or Ad for a further 24 h. The cells were then harvested and the protein levels of TLRs were determined by Western blotting. Cholesterol uptake measurements were then carried out.

Western blotting

Cells were washed with PBS, scraped into lysis buffer (Tris-EDTA + Complete protease inhibitor; Roche, United States) and mechanically homogenized. Total protein samples (40 μg per well) were electrophoresed on 8% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes at 100V for 100 min. Membranes were incubated overnight with anti-LDLR (1:2000, Millipore), anti-HMGCoAr (1:1000, Millipore), anti-TLR2 (1:1000, Millipore), anti-TLR4 (1:1000, Santa Cruz), or anti-GAPDH (1:10 000, Sigma). Anti-mouse secondary antibody conjugated with horseradish peroxidase (Promega, United States) and Super-Signal West Pico Chemiluminescent Substrate (Pierce, United States) were used for detection.

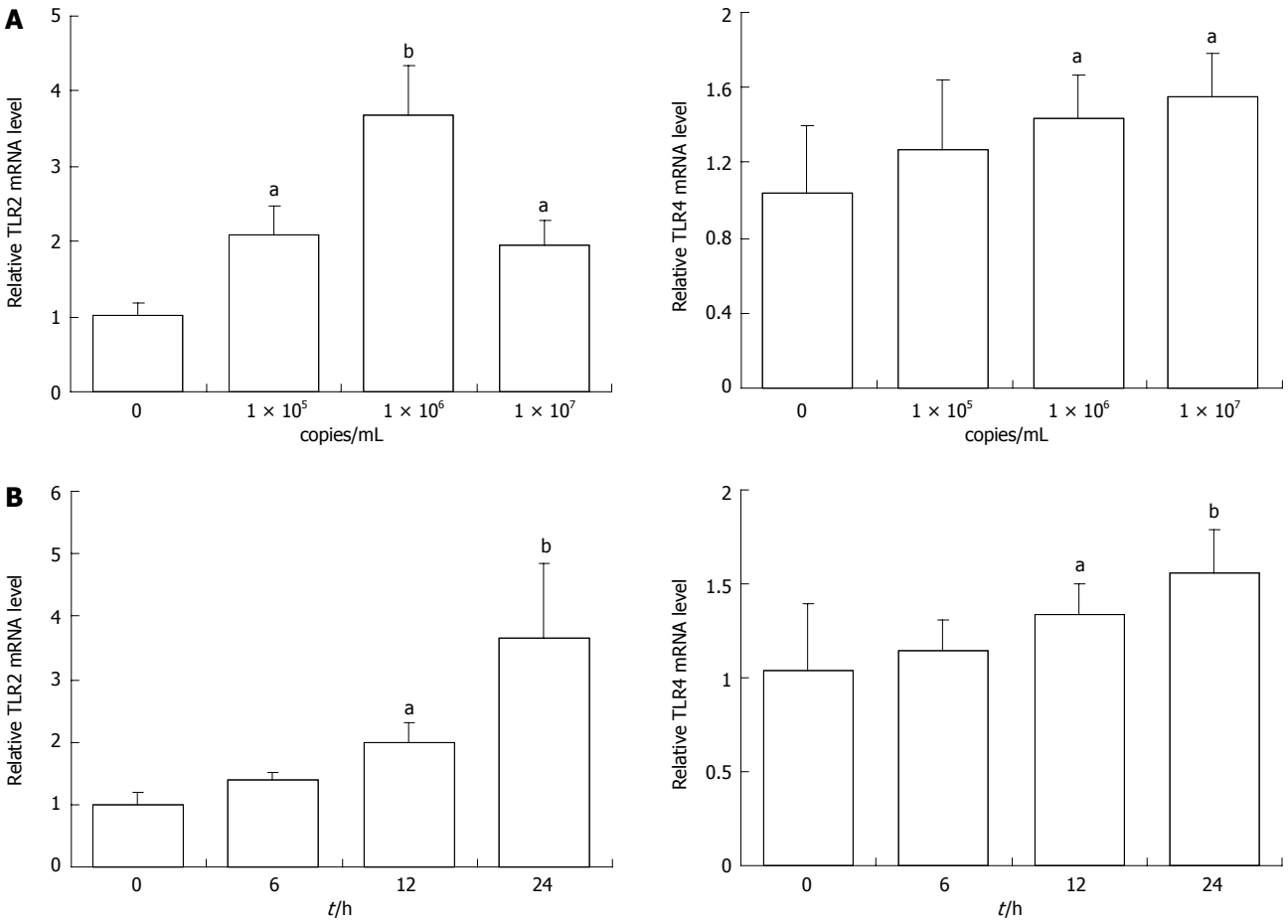

Figure 3 Effects of adenovirus hepatitis B virus treatment on toll-like receptors mRNA levels in HepG2 cells. A: Adenovirus-hepatitis B virus (Ad-HBV) at 10^5 and 10^6 copies profoundly augmented the mRNA levels of toll-like receptor 2 (TLR2). While the concentration of Ad-HBV up to 10^7 copies, the mRNA expression were suppressed instead. B: The mRNA of TLR4 were increased in HepG2 cells treated with Ad-HBV, which was in a dose-dependent manner. Results were representative of three similar experiments. $^aP < 0.05$, $^bP < 0.01$ *vs* control group.

Modified LDL uptake measurements

HepG2 cells were incubated with human 1'-dioctadecyl-1-3,3,3',3'-tetramethyling-docarbocyanine perchlorate (Dil)-labeled acetylated low density lipoprotein (Dil-acl-LDL) (10 μg/mL) for 4 h, DAPI staining was used to detect the nucleus. The two color images were visualized under a fluorescence microscope. ZEN 2008 and Image software were used to analyze the quantity of Dil-acl-LDL.

Statistical analysis

Results were shown as mean ± SE, and all experiments were run in triplicate. The statistical significance of differences between groups was determined using the Student *t* test. $^aP < 0.05$, $^bP < 0.01$, *vs* the control group.

RESULTS

Cell toxicity of Ad-HBV

The toxicity of Ad-HBV against HepG2 cells used for propagation of viral infections was measured. Both Ad and Ad-HBV below 10^7 copies/mL did not exhibit either toxic or proliferative effects on HepG2 cells, while 1×10^8 copies/mL of the virus reduced HepG2 cell survival rates (Figure 1).

Ad-HBV changes the mRNA levels of genes related to cholesterol metabolism in HepG2 cells

Whether HBV leads to cholesterol metabolism disorders in the liver is still controversial. Therefore, we determined the effects of Ad-HBV on the mRNA levels of genes related to cholesterol metabolism in HepG2 cells. As shown in Figure 2A, Ad-HBV at 1×10^5 and 1×10^6 copies/mL significantly augmented the mRNA levels of LDLR and HMGCoAr. When the concentration of Ad-HBV reached 10^7 copies/mL, the mRNA expression was suppressed. Ad-HBV at 1×10^6 copies/mL significantly induced mRNA expression of LDLR (2.56 ± 0.33 *vs* 1.03 ± 0.25, $P < 0.01$, $n = 3$) and HMGCoAr (2.98 ± 0.25 *vs* 1.01 ± 0.18, $P < 0.01$, $n = 3$). Ad-HBV upregulated the mRNA levels of LDLR and HMGCoAr in a time-dependent manner. HepG2 cells were maintained with 1×10^6 copies/mL of Ad-HBV for different time periods and values were expressed as fold changes relative to the controls. After 24 h of incubation the mRNA expression of LDLR (2.56 ± 0.33 *vs* 1.03 ± 0.34, $P < 0.01$, $n =$

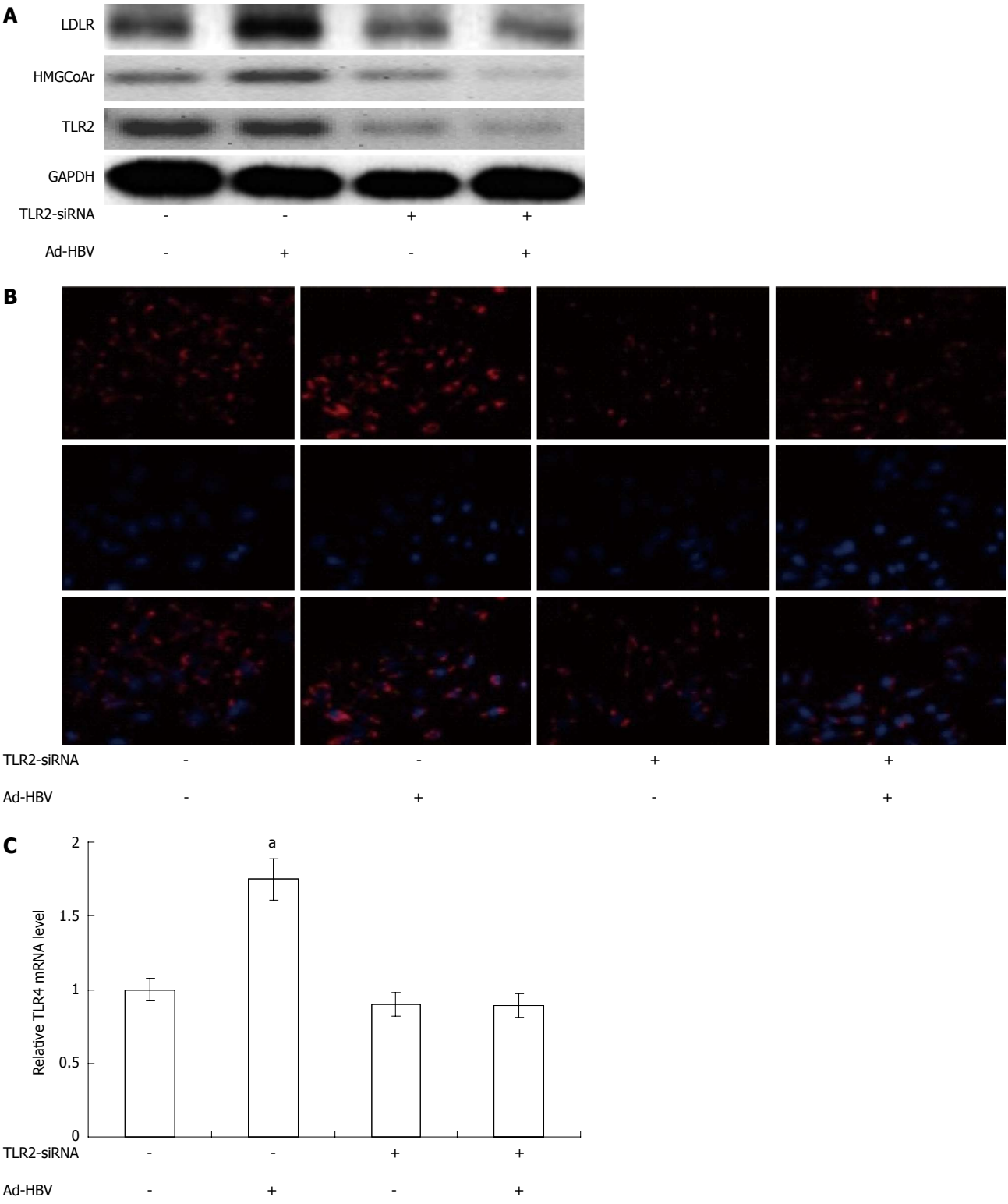

Figure 4 Effects of toll-like receptor 2-SiRNA on the expression of proteins related to cholesterol metabolism and intake of cholesterol by HepG2 cells treated with adenovirus hepatitis B virus. A: The protein expression of low-density lipoprotein receptor (LDLR) and 3-hydroxy-3-methylglutharyl-coenzyme A reductase (HMGCoAr) were assayed; B: HepG2 Cells were exposed for 4 h to Dil-acl-LDL (10 μg/mL), after thorough washing with phosphate buffered solution, fluorescence of Dil-acl-LDL was detected in cytoplasm of cells by fluorescence microscopy; C: Relative quantity of Dil-acl-LDL in HepG2 cells were analyzed by software. Results were representative of three similar experiments. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group. TLR2: Toll-like receptor 2; LDLR: Low-density lipoprotein receptor; LDL: Low density lipoprotein; Ad-HBV: Adenovirus hepatitis B virus.

3) and HMGCoAr (2.99 ± 0.25 *vs* 1.01 ± 0.18, $P < 0.01$, $n = 3$) reached a peak (Figure 2B).

Ad-HBV changes the mRNA levels of TLRs in HepG2 cells

Researchers recently showed that TLRs were involved in viral infection and its downstream effects. To investigate whether Ad-HBV upregulated the mRNA levels of genes related to cholesterol metabolism in HepG2 cells, TLRs mRNA expression was determined. Ad-HBV at 1×10^5 copies/mL and 1×10^6 copies/mL significantly induced the mRNA levels of TLR2. Values were expressed as fold changes relative to the controls. When the concentration of Ad-HBV was increased to 1×10^7 copies/mL, mRNA expression was suppressed (Figure 3A). These changes were consistent with the changes in cholesterol metabolism-related genes. The mRNA levels of TLR4 were increased in HepG2 cells treated with Ad-HBV in a dose-dependent manner (Figure 3B).

TLR2 mediated the effects of Ad-HBV on the expression of proteins related to cholesterol metabolism and intake of cholesterol in HepG2 cells

To clarify the mechanism of Ad-HBV-upregulated mRNA levels of proteins related to cholesterol metabolism in HepG2 cells, we used siRNA-mediated downregulation of TLR2/TLR4 to confirm our hypothesis that TLR2/TLR4 may participate in this process. To evaluate the involvement of TLR2/TLR4 in the effects of Ad-HBV, we attenuated the expression of TLR2 using human TLR2-SiRNA, which suppressed TLR2 protein level by up to 80% (Figure 4). Transient transfection of HepG2 cells with TLR2-siRNA substantially abolished Ad-HBV-mediated upregulation of LDLR and HMGCoAr and the uptake of Dil-acl-LDL (Figure 4). TLR4-SiRNA did not change the expression of proteins related to cholesterol metabolism during Ad-HBV infection (data not shown).

DISCUSSION

There is increasing evidence to indicate that hepatic lipid accumulation is related to hepatic fibrosis and inflammation, resulting in cell apoptosis and cancer[16,17]. In particular, it is assumed that lipid accumulation is a prerequisite for subsequent events leading to liver injury in nonalcoholic fatty liver disease[18]. In addition, it was recently shown that hepatic steatosis may be a factor in HCV-induced liver pathogenesis and may impair the response to interferon-based therapy[19,20]. Due to the importance of lipid accumulation, the mechanism by which nonalcoholic fatty liver disease and HCV infection cause hepatic steatosis has been studied intensively[4]. However, the molecular mechanisms by which HBV infection causes hepatic steatosis have been poorly investigated.

According to current knowledge, liver LDLR is the most important receptor of binding and internalization of plasma-derived LDL-cholesterol and regulates plasma LDL concentration. Changes in receptor activity alter the rates of LDL uptake by the liver with a corresponding increase or decrease in plasma LDL levels[21,22]. Our results showed that the mRNA and protein levels of LDLR were increased following infection of HepG2 cells with Ad-HBV.

3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAr) is an enzyme which catalyzes the conversion of HMGCoA to mevalonate, the rate limiting step in cholesterol biosynthesis. Normally, in mammalian cells this enzyme is suppressed by cholesterol and its derivatives from the LDLR mediated-internalization of LDL as well as oxidized species of cholesterol. Competitive inhibition of the reductase upregulates the expression of LDLR in the liver, which in turn increases the catabolism of plasma LDL and lowers the plasma concentration of cholesterol, an important determinant of atherosclerosis[23-25].

HBV, like many other microorganisms that contribute to the pathogenesis of atherosclerosis, can colonize the vascular tissues[26], induce vasculitis[27], and stimulate inflammatory and immune responses that may lead to vascular damage and precipitate atherosclerosis. It was reported in one Japanese study that HBV infection can be atherogenic in otherwise healthy subjects with preserved liver function[7]. We reasoned that HBV would be a rational candidate pathogen among the stimuli that contribute to atherosclerosis. The mechanism behind this association is still unclear, and additional studies are required.

TLRs have been reported to play an important role in liver damage after infection with HBV, and the mechanisms may involve virus-induced immune modulation[14,28,29]. TLRs are also involved in other immune diseases mediated by HBV. TLR4 had been reported to not only inhibit HBV replication, but also to induce immune injury in cells[30]. Consistent with previous studies, we found that Ad-HBV increased the expression of TLR2/4. Most importantly, we observed that the upregulation of LDLR and HMGCoAr *via* Ad-HBV can be partially blocked by silencing TLR2, but not TLR4. Taken together, these results suggest that Ad-HBV infection-induced cholesterol accumulation in hepatocytes is mediated by TLR2.

In conclusion, our data indicate that HBV is able to induce the gene expression of TLR2, thereby causing hepatic lipid accumulation by increasing genes related to cholesterol absorption and metabolism. Because increased lipid deposition is involved in the progression of severe liver injury such as hepatitis and hepatocellular carcinoma, our results provide important information in understanding the development and progression of HBV-induced pathogenesis.

COMMENTS

Background

Cholesterol accumulation plays an important role in the progression of atherosclerosis. This study was undertaken to investigate whether hepatitis B

virus (HBV) exacerbates hepatic cholesterol accumulation and the underlying mechanisms were examined.

Research frontiers

Previous studies have mainly focused on the potential effect of HBV in the progression of atherosclerosis. The authors hypothesized that HBV increases low-density lipoprotein receptor and 3-hydroxy-3-methylglutharyl-coenzyme A reductase expression resulting in exacerbated cholesterol accumulation in HepG2 cells, which was mediated *via* the toll-like receptor 2 (TLR2) pathway.

Innovations and breakthroughs

To further clarify the potential effect of HBV in the progression of atherosclerosis, the authors examined the effects of adenovirus hepatitis B virus (Ad-HBV) in the progression of atherosclerosis which is partly mediated *via* the TLR2 pathway.

Applications

The results show that Ad-HBV up-regulates the expression of genes related to cholesterol metabolism *via* the TLR2 pathway. Further studies are required to evaluate the mechanism by which HBV regulates the TLR2 pathway.

Terminology

There are some associations between hepatitis virus and carotid atherosclerosis. Hepatitis virus causes liver and even systemic inflammatory reactions, and inflammation is one the pathophysiological changes in atherosclerosis.

Peer review

This manuscript presented that Ad-HBV up-regulated the expression of genes related to cholesterol metabolism in HepG2 cells. Furthermore, the atherosclerosis effect of Ad-HBV is *via* TLR2 pathway. The experiment seems to be correct. The study appears well conducted and the results discussed with honesty, and caution.

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BRIEF ARTICLE

Habitual rapid food intake and ineffective esophageal motility

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Abstract

AIM: To study non-cardiac chest pain (NCCP) in relation to ineffective esophageal motility (IEM) and rapid food intake.

METHODS: NCCP patients with a self-reported habit of fast eating underwent esophageal manometry for the diagnosis of IEM. Telephone interviews identified eating habits of additional IEM patients. Comparison of manometric features was done among IEM patients with and without the habit of rapid food intake and healthy controls. A case study investigated the effect of 6-mo gum chewing on restoration of esophageal motility in an IEM patient. The Valsalva maneuver was performed in IEM patients and healthy controls to assess the compliance of the esophagus in response to abdominal pressure increase.

RESULTS: Although most patients diagnosed with NCCP do not exhibit IEM, remarkably, all 12 NCCP patients who were self-reporting fast eaters with a main complaint of chest pain (75.0%) had contraction amplitudes in the mid and distal esophagus that were significantly lower compared with healthy controls [(23.45 mmHg (95%CI: 14.06-32.85) *vs* 58.80 mmHg (95%CI: 42.56-75.04), $P < 0.01$ and 28.29 mmHg (95%CI: 21.77-34.81) *vs* 50.75 mmHg (95%CI: 38.44-63.05), $P < 0.01$, respectively)]. In 7 normal-eating IEM patients with a main complaint of sensation of obstruction (42.9%), the mid amplitude was smaller than in the controls [30.09 mmHg (95%CI: 19.48-40.70) *vs* 58.80 mmHg (95%CI: 42.56-75.04), $P < 0.05$]. There was no statistically significant difference in manometric features between the fast-eating and normal-eating groups. One NCCP patient who self-reported fast eating and was subsequently diagnosed with IEM did not improve with proton-pump inhibition but restored swallow-induced contractions upon 6-mo gum-chewing. The Valsalva maneuver caused a markedly reduced pressure rise in the mid and proximal esophagus in the IEM patients.

CONCLUSION: Habitual rapid food intake may lead to IEM. A prospective study is needed to validate this hypothesis. Gum-chewing might strengthen weakened esophageal muscles.

Key words: Esophageal manometry; Ineffective esophageal motility; Non-cardiac chest pain; Rapid food intake; Valsalva maneuver

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INTRODUCTION

Non-cardiac chest pain (NCCP) and ineffective esophageal motility (IEM) are often associated with gastroesophageal reflux disease (GERD). Although esophageal dysmotility is considered an uncommon cause of non-GERD-related NCCP[1,2], in our practice it is not infrequent. In recent years, we noted that some patients with a primary complaint of chest pain or discomfort had a life long habit of rapid food intake. Their esophageal manometry exhibited low esophageal contraction amplitudes during wet swallows. This initiated the current investigation into a possible relationship between habitual rapid food intake, symptoms and motility dysfunction.

In a recent study, a possible association was investigated between self-reported eating behavior and metabolic risk factors (overweight, hypertension, hyperglycemia, hypertriacylglycerolemia, low levels of high-density lipoprotein (HDL) cholesterol, hyperuricemia and fatty liver)[3]. The conclusion was that rapid eating increases metabolic risk factors although the mechanism was not investigated. In the present study, we analyzed the clinical and manometric characteristics of IEM patients with and without a habit of rapid eating. Our main objective was to investigate a possible correlation between rapid food intake and IEM. Our hypothesis was that rapid eating is associated with less swallow-induced contractions, contributing to IEM through disuse of the esophageal musculature; hence we predicted that patients with IEM and rapid eating should have more severe ineffective esophageal motility compared to IEM patients without the habit of rapid eating. We also report a case-study of an IEM patient whose symptoms were improved by 6 mo of gum-chewing exercise.

MATERIALS AND METHODS

Subjects

Data were collected from patients in our department with various symptoms including chest pain or discomfort, dysphagia, heartburn that lasted from 1 mo to 30 years who underwent esophageal manometry. Some patients volunteered information about their fast eating habits as a cause of their symptoms. We collected information about the manometry tests of all patients whose eating habits were recorded at the first visit, and in addition, we obtained information about eating habits by telephone interview of 9 additional patients.

Two groups of volunteers participated in the study: Group V1 as healthy controls in the manometric analysis; Group V2 recruited to record healthy Chinese people's daily meal duration. Group V1 were without any digestive or systemic symptoms and the volunteers underwent the same manometric procedures as the patients. Group V2 were sent out to canteens, fast-food restaurants, Chinese restaurants and local families to time the duration of the meal intake.

Written informed consent was provided by all the participants. This study was approved by the Ethics Committee of Renmin Hospital of Wuhan University.

Study protocol

The patients' current symptoms, medical history and basic information including age, gender, body mass index (BMI) was obtained and standard esophageal manometry was performed. During the manometry testing, the patients were instructed to perform the Valsalva maneuver. Volunteers in Group V1 underwent the same manometric procedure after their basic information was obtained. Each of them performed the Valsalva maneuver. Group V2 was assigned to the above-mentioned dining locations to record the meal duration.

Esophageal manometry

Following an overnight fast and 48-h discontinuation of any medication that may interfere with esophageal motility, conventional stationary esophageal manometry was performed using a 3.5 mm diameter, eight-lumen, sleeve sensor catheter assembly (Mui Scientific, Mississauga, Ontario, Canada) with eight side-holes arranged in radial form and located 2-7 cm apart. Manometric data were recorded and analyzed by means of the Polygram 98 and the Polygram Net Esophageal Manometry Testing Application Software (Medtronic A/S, Tonsbakken, Skovlunde, Denmark). The catheter was inserted transnasally into the stomach and intragastric pressure (GP) was obtained in a supine position. The lower esophageal sphincter (LES) resting pressure was defined as the mid-respiratory LES pressure compared with GP. Patients or healthy volunteers were then instructed to perform ten wet swallows (10 mL water each, separated by an interval of 30 s) to measure and calculate the contraction amplitude, duration and velocity in the proximal, mid and distal esophagus. When calculating the velocity, we did not incorporate data indicative of simultaneous (*i.e.*, velocity > 8 cm/s) contractions. The existence of double-peaked or multi ($\geqslant$ 3) -peaked waves was also noted.

The manometric criteria for the diagnosis of IEM were no fewer than 30% of the wet swallows featuring one or more of the following characteristics: (1) contraction amplitude < 30 mmHg at either or both of the distal points 5 and 10 cm above the LES; (2) simultaneous contraction (distal velocity between 5 and 10 cm above the LES > 8 cm/s) with amplitude < 30 mmHg; and (3) absent or non-transmitted peristalsis[4,5].

The valsalva maneuver

After wet swallows, 12 patients and all the volunteers in Group V1 were instructed to perform the Valsalva maneuvers in the supine position, exhaling forcibly with the mouth closed and the nose pinched shut[6,7]. Data on the pressure changes in the esophageal body and the LES

Table 1 Esophageal manometry results, expressed as mean (95%CI) in ineffective esophageal motility patients and healthy

	IEM patients with the habit of fast eating (n = 12)	IEM patients without the habit of fast eating (n = 7)	Healthy controls (n = 10)
LES pressure (mmHg)	12.71 (6.80-18.62)	11.08 (-0.59-22.76)	14.94 (10.38-19.49)
Distal esophagus			
Amplitude (mmHg)	28.29 (21.77-34.81)[b]	33.78 (19.56-48.00)	50.75 (38.44-63.05)
Duration (s)	3.04 (2.44-3.65)	2.74 (1.32-4.15)	3.09 (2.30-3.88)
Velocity (cm/s)[1]	1.42 (1.14-1.70)	3.33 (0.79-5.86)	1.57 (0.89-2.24)
Mid esophagus			
Amplitude (mmHg)	23.45 (14.06-32.85)[b]	30.09 (19.48-40.70)[a]	58.80 (42.56-75.04)
Duration (s)	3.12 (2.32-3.91)	3.18 (1.81-4.55)	2.45 (2.13-2.79)
Velocity (cm/s)[2]	2.18 (1.23-3.12)	3.76	2.35 (1.38-3.33)
Proximal esophagus			
Amplitude (mmHg)	36.75 (22.93-50.57)	41.47 (8.79-74.15)	49.96 (36.28-63.64)
Duration (s)	2.42 (1.84-3.00)	3.13 (1.48-4.77)	2.25 (1.80-2.71)
Velocity (cm/s)[3]	3.66 (1.71-5.60)	2.56	2.42 (1.75-3.09)

[1]Velocity in the distal esophagus of 3 fast-eating and 3 normal-eating IEM patients could not be calculated due to simultaneous contractions; [2,3]Velocity in the mid (proximal) esophagus of 6 fast-eating and 5 normal-eating IEM patients and 2 healthy controls could not be calculated due to simultaneous contractions. Contraction data were in response to wet swallows. Data indicative of simultaneous (*i.e.*, velocity > 8 cm/s) or other non-propulsive contraction were excluded when calculating the mean velocity. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control. IEM: Ineffective esophageal motility; LES: Lower esophageal sphincter.

were collected.

Rapid food intake measurement

We used the length of time it took for a patient to finish an average meal as the indicator of the speed of eating. Meal lengths of the healthy population were recorded when they had regular Chinese meals with or without water. None of them took alcohol or had chat time included.

Statistical analysis

Except for age which was presented as median and range, the other data were expressed as means and 95%CI:. Kolmogorov-Smirnov analysis was applied to determine data distribution. Student's t test was employed for the comparison of data. Statistical significance was acknowledged if $P < 0.05$.

RESULTS

Meal lengths in IEM patients with or without the habit of rapid food intake

Ten NCCP patients mentioned their eating habits specifically during initial evaluation, six of whom reported a habit of rapid eating and were all diagnosed with IEM according the manometric criteria. We managed to obtain information from 9 other IEM patients by telephone calls. Among these 19 patients, 12 (63.2%) (7 males and 5 females, median age 44.5 years, range 18-57 years, mean BMI 22.52; 95%CI: 20.45-24.59) volunteered the fact that they had been eating much faster than normal speed for a long time from 5 to 31 years. The other seven (36.8%) patients (2 males and 5 females, median age 52 years, range 45-74 years, mean BMI 22.48 (95%CI: 20.56-24.39) reported no habit of rapid eating.

Ten [4 males and 6 females, median age 22 years, range 20-33 years, mean BMI 20.69 (95%CI: 19.39-21.87)] healthy volunteers were recruited into Group V1. Group V2 consisted of 91 (50 males and 41 females) healthy volunteers.

For the self-reporting fast-eaters, meals all lasted no more than 8 min (3 min in one patient; 4 min in one; 5 min in five; 6 min in three; 7 min in one and 8 min in one). Their average meal duration was significantly shorter than that of the healthy volunteers (5.42 min, 95%CI: 4.58-6.25 *vs* 16.58 min, 95%CI: 14.21-18.94, $P < 0.01$). The meal lengths in the IEM patients with normal eating habits ranged from 10 to 30 min and their mean meal length was not statistically different from healthy volunteers (18.86 min, 95%CI: 12.31-25.41 *vs* 16.58 min, 95%CI: 14.21-18.94, $P > 0.05$). We found that the meal lengths of all IEM patients with normal eating habits were longer than those of the self-reporting rapidly eating patients.

Some fast-eating patients reported that while eating fast they spent shorter time chewing. They also swallowed more rapidly and frequently though they did not quantify it.

Clinical characteristics

The predominant clinical manifestation in the fast-eating group was chest pain or discomfort (9/12, 75.0%), followed by sensation of obstruction (5/12, 41.7%), heartburn (2/12, 16.7%), acid reflux (1/12, 8.3%), dysphagia (1/12, 8.3%), chest tightness (1/12, 8.3%), food regurgitation (1/12, 8.3%), abdominal discomfort (1/12, 8.3%), nausea (1/12, 8.3%) and eructation (1/12, 8.3%). In the normal-eating group, sensation of obstruction was the most common (3/7, 42.9%), followed by heartburn (2/7, 28.6%), acid reflux (2/7, 28.6%) and chest pain or discomfort (1/7, 14.3%).

Manometric features

Table 1 shows the IEM patients' manometric features. The contraction amplitudes in the distal and mid esophagus of the fast-eating IEM patients were significantly

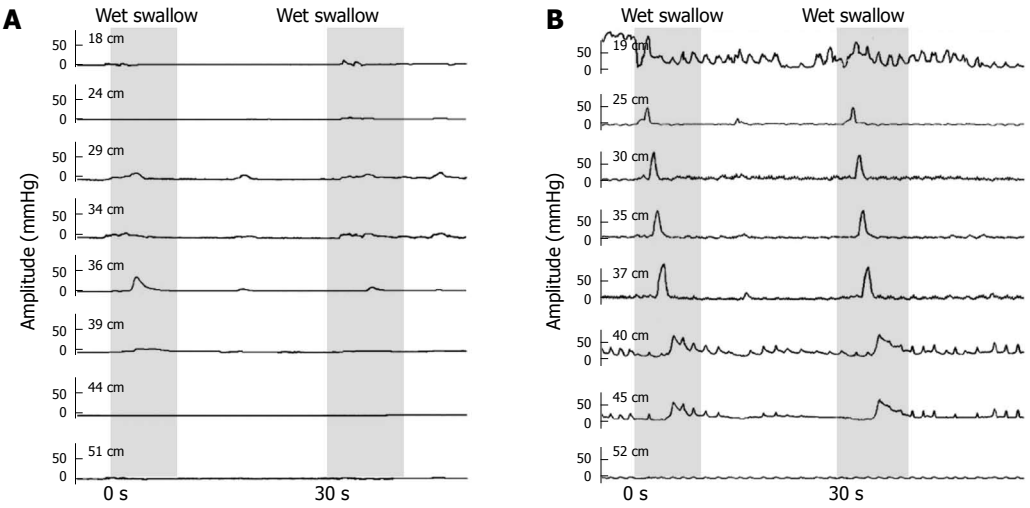

Figure 1 Manometric tracings of a fast-eating ineffective esophageal motility patient (A) and a healthy control (B). The numbers (in cm) on the left indicate the distances from the nose to each side-hole on the catheter.

lower ($P < 0.01$) than in the control group. The amplitude in the mid esophagus of the normal-eating IEM patients was also significantly lower ($P < 0.05$) than in the controls. There was no statistically significant difference in manometric features between the IEM patients with and without the habit of rapid food intake.

Simultaneous contractions were observed in 11 fast-eating and 6 normal-eating IEM patients (91.7% and 85.7% respectively *vs* 30% in healthy controls) and non-propulsive (but not simultaneous) contractions in 1 fast-eating patient (8.3% *vs* 0% in controls). Seven fast-eating and 6 normal-eating patients (58.3% and 85.7% *vs* 20% in controls) exhibited double-peaked waves and 3 fast-eating and 3 normal-eating patients (25.0% and 42.9% *vs* 20% in controls) had multi-peaked waves during certain wet swallows. A typical manometric tracing from one of the fast-eating IEM patients is shown in Figure 1.

Short swallowing interval caused prevention of peristalsis

According to our protocol, wet swallows should be separated by an interval of 30 s. However, in some patients and healthy controls, the interval between certain swallows happened to be shorter than 10 s or even near zero. We observed that in pairs of short-interval swallows, only one peristalsis appeared in response to the first or the second swallow while the response to the other swallows was only contraction in the proximal esophagus, and the contraction in the distal part was prevented, as shown in Figure 2.

Response of the esophageal musculature to the Valsalva maneuver

Pressure alterations in the LES and distal, mid and proximal esophagus during the Valsalva maneuver between IEM patients and healthy controls were compared (Table 2), and the manometry tracings are illustrated in Figure 3. IEM patients showed a much lower increase in esophageal pressure due to the Valsalva maneuver compared with controls. Mean changes in LES pressure of IEM patients were not statistically different from that of healthy volunteers.

Esophageal motility improved by gum-chewing exercise: a case report

A 57-year-old male with a history of rapid food intake for more than 30 years, with each meal lasting less than 5 min, presented to our outpatient department with 2 years of moderate retrosternal chest pain, sensation of obstruction and occasional dysphagia. The initial esophageal manometry revealed that his swallow-induced esophageal contraction amplitude was extremely low (distal amplitude 10.42 mmHg on average). He was advised to slow down his speed of eating and to take proton-pump inhibitor (PPI) for 4 mo, but resulting in no benefit. The drug was discontinued. Then a gum-chewing exercise (about 10 times a day, 15 min each time, for 6 mo) was recommended. The patient returned to the hospital 6 mo later, reporting that his symptoms had been relieved. The contraction amplitude of his repeat manometry was improved (distal amplitude 58.03 mmHg on average). His manometric tracings before and after the gum-chewing exercise are shown in Figure 4. A repeat manometry after another 6 mo revealed continued normalized esophageal motility (distal amplitude 60.07 mmHg on average), though he had reduced the frequency of gum-chewing exercise since the previous manometry. During the manometry this time, the patient was also asked to perform 10 pairs of wet swallows at the interval of 2 s, 8 of which failed to initiate any peristalsis and only 2 of which were observed with peristaltic contraction at the end of the second pair of wet swallows.

DISCUSSION

Of the 19 IEM patients whose eating habits were investi-

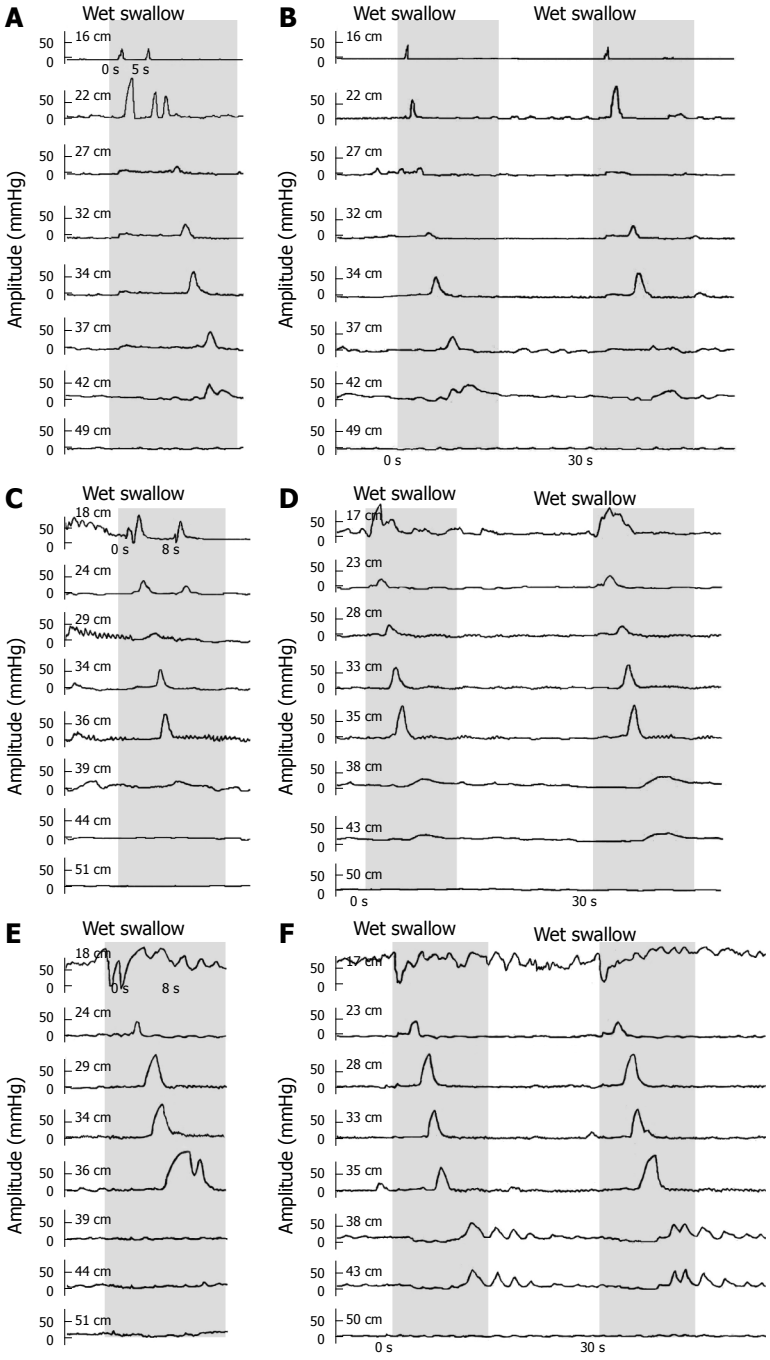

Figure 2 Manometric tracings of swallowing at an interval < 10 s (A, C, E) in comparison with swallowing at the interval of 30 s (B, D, F). The numbers (in cm) on the left indicate the distances from the nose to each side-hole on the catheter. In an ineffective esophageal motility patient who was habitually rapidly eating (A, B), only one peristaltic contraction appeared in response to the second of a pair of wet swallows at the interval of 5 s (A). Similar finding was observed in one healthy control (E, F) whose two wet swallows were almost continuous (E). In another control (C, D), peristalsis was only seen in response to the first of a pair of wet swallows at the interval of 8 s (C).

gated, 12 were fast eaters. The main presenting symptom of the fast eaters was chest pain or discomfort; the main symptom of the normal-eating patients was sense of obstruction. Although the average values of all swallow-induced contraction amplitudes were lower in the fast-eating group, there was no statistically significant difference compared with the normal-eating IEM patients. There are two possible explanations for this result. One is that factors other than fast eating were the dominant cause of weakened esophageal muscle in both groups. The other is that the weakened esophageal muscle could be due to fast eating (disuse of musculature) in fast-eating patients while other causes may contribute to the similar weakening in normal-eating patients. The other causes likely include acid reflux since 57% of the patients in this group reported heartburn or acid reflux, whereas only 25% of the fast eating group reported this symptom. The present study cannot distinguish between these two possibilities although it is very striking that all fast eaters showed dramatic weakening of the esophageal muscle. When

Table 2 Effects of Valsalva maneuver on esophageal pressure, expressed as mean (95%CI) in ineffective esophageal motility patients and controls

	Increase in LES pressure (mmHg)	Increase in distal pressure (mmHg)	Increase in mid pressure (mmHg)	Increase in proximal pressure (mmHg)
IEM patients (n = 12)	11.56 (0.57-22.54)	21.73 (15.46-27.99)	21.18 (12.28-30.08)[a]	19.07 (11.41-26.74)[a]
Control (n = 10)	7.81 (-0.86-16.48)	39.43 (15.37-63.49)	43.44 (22.85-64.03)	34.18 (23.41-44.95)

[a]$P < 0.05$ *vs* control. IEM: Ineffective esophageal motility; LES: Lower esophageal sphincter.

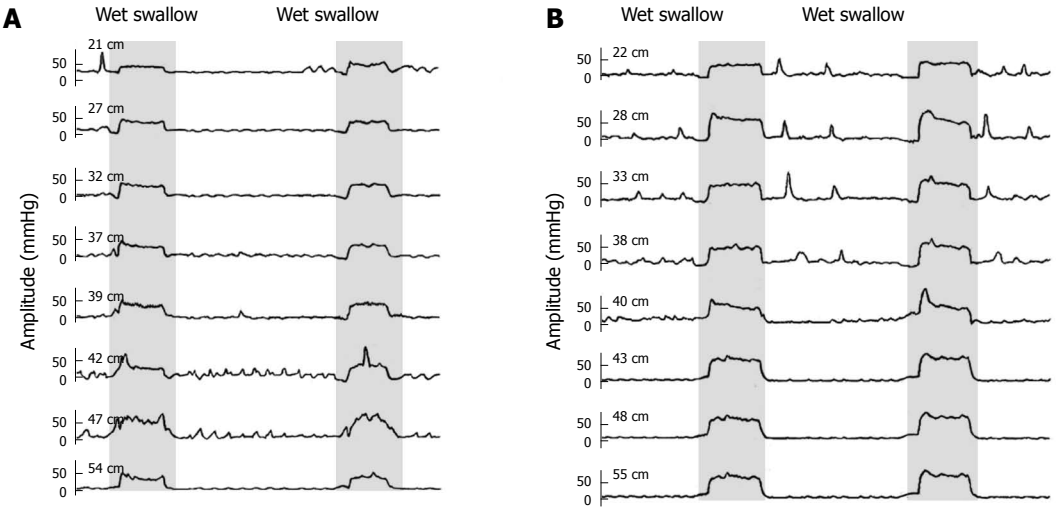

Figure 3 Effects of the Valsalva maneuver on the esophageal manometric tracings in a patient with ineffective esophageal motility (A) and a healthy control (B). The numbers (in cm) on the left indicate the distances from the nose to each side-hole on the catheter.

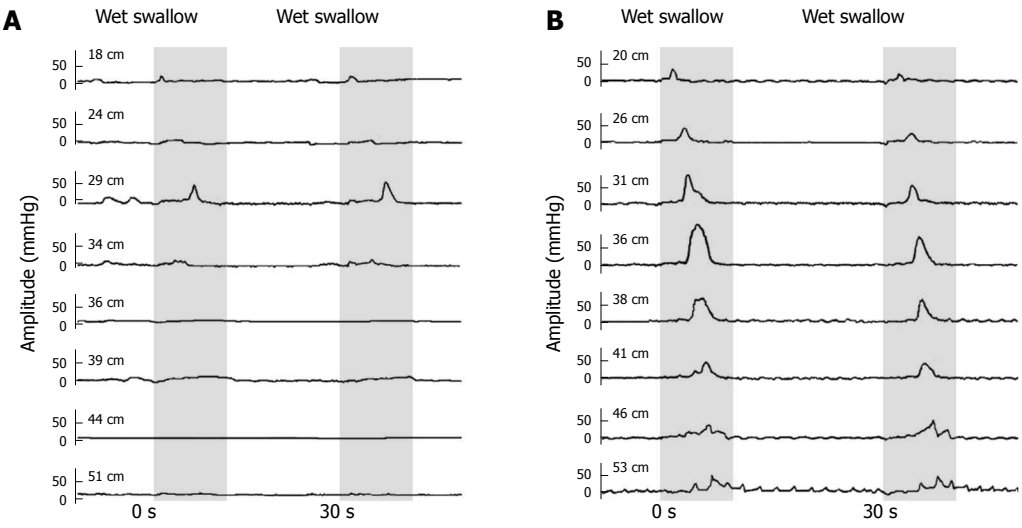

Figure 4 Manometric tracings of a fast-eating non-cardiac chest pain patient diagnosed with ineffective esophageal motility before (A) and after (B) 6-mo gum-chewing exercise. The numbers (in cm) on the left indicate the distances from the nose to each side-hole on the catheter.

NCCP patients are evaluated for esophageal dysmotility, only a few are subsequently diagnosed with IEM. The fact that all NCCP patients who self-reported fast eating were diagnosed with IEM suggests but does not prove a causal relationship. The case study suggests, but does not yet prove, that gum-chewing strengthens the esophageal muscle and it is consistent with the hypothesis that the weakening of the musculature was due to non-use of the musculature because of reduced swallow-induced contractions, although the weakened musculature may have been caused by other factors. In summary, although it is possible that fast eating is associated with weakening of the musculature, the present study does not provide direct evidence for it.

Habitual fast eating associated with rapid swallowing may limit the number of swallow-induced contractions

since only the first or the last bolus are associated with a propulsive contraction. We have observed this phenomenon in this study, which is consistent with previous reports[8-11]. The contractions may become weaker with time. Another feature of rapid eating is insufficient mastication. Reduced duration of chewing prevents the optimization of the size, the softness and the lubrication of food boluses ready for swallowing[12]. Vagus nerve activity, which plays a vital role in the regulation of salivation[13] and esophageal peristalsis[14] and is enhanced by mastication[13], may also be less activated by inadequate chewing. To provide further evidence for or against the hypothesis that fast eating contributes to IEM, a prospective study is needed where the meal composition is standardized and the actual timing of swallows is measured.

The case report suggests that gum-chewing may strengthen the esophageal musculature. It would be important to find out if this is true independent of the cause of IEM. In this patient, PPI treatment did not relieve symptoms, and regular daily gum-chewing restored muscle contractile activity. Chewing gum on a regular basis is a stimulus that induces mastication-associated vagal activation[15] and swallow-associated propulsive contractions.

In the distal and mid esophagus, the contraction amplitudes in the fast-eating IEM patients were significantly reduced. However, their proximal manometric features were not statistically different from controls. This was probably due to the special musculature of the human esophagus, whose upper one-third is composed of striated muscle whereas the lower one-third is made up of smooth muscle and in between both types exist. Peristalsis in the striated muscle portion is induced by the sequential activation of neurons in the ambiguous nucleus which is solely a central mechanism; while in the smooth muscle portion, the peripheral intramural and central mechanisms cooperate to control peristalsis[14]. Considering the different manometric presentation of the distal and proximal esophagus in IEM, it is probable that a disorder in the peripheral neural control of esophageal smooth muscle contributes to the development of IEM in these patients.

Consensus on a causal relationship between NCCP and IEM has not hitherto been reached. Heartburn, dysphagia and regurgitation, reported by our patients, are possible risk factors for NCCP, in addition to psychological factors such as anxiety and depression[16] which often haunt our patients and aggravate their symptoms. Hence, NCCP in IEM is a result of many complex interactions and evidence is insufficient to assert that NCCP is caused by IEM. NCCP is often associated with GERD and IEM is the most common form of dysmotility in GERD and is correlated with more GERD episodes and prolonged acid clearance in a posture-dependent manner[17]. Although 24 h pH monitoring was not carried out, most of our patients did not suffer from GERD and the LES pressure was normal in our patients. Nevertheless, a contribution of gastroesophageal reflux to the symptoms of our IEM patients cannot be excluded.

The habit of rapid eating is a common phenomenon in China and may originate from periods in China when food supply was limited and collective dining was the main form of meal, so to ensure that sufficient food could be secured, many people developed the habit of rapid eating that eventually persisted for years. In addition, certain occupations in China, such as waiters/waitresses in restaurants and sales assistants in shops may not get sufficient free time to eat meals relaxed and hence quick eating may become a habit. We now investigate eating habits routinely in association with IEM and recommend changes in life style and exercise to alleviate their symptoms by strengthening their esophageal musculature.

The Valsalva maneuver increases the intrathoracic[18] and intra-abdominal pressure and leads to the activation of the diaphragm muscle[19]. Both the LES musculature and the crural diaphragm can contribute to the increase in LES pressure in response to an increased intra-abdominal pressure although evidence suggests that no active contraction of the smooth muscle is involved[20,21]. Most of our patients did not show decreased LES, but those who did might benefit from the Valsalva maneuver since it does increase the pressure of the esophageal junction. Previous studies in humans and animals showed that adjusted respiration could increase the pressure around the LES[22-25]. The effect of the Valsalva maneuver on esophageal muscle contraction is rarely mentioned. Our IEM patients showed a dramatic reduction in the proximal and mid esophageal response to the Valsalva, suggesting a weakened adaptive response of the esophageal musculature, at least the skeletal muscle.

In summary, inquiry into eating behavior is an important part of examination of patients with NCCP. Eating fast increases metabolic risk and should be discouraged. Eating fast may lead to ineffective esophageal motility, but more studies are needed to prove a direct causal relationship.

COMMENTS

Background

Esophageal dysmotility is considered an uncommon cause of non-cardiac chest pain (NCCP), but in our practice it is not infrequent. Previous studies have reported the correlation between eating behaviors and development of diseases, but the role of rapid eating in ineffective esophageal motility (IEM) and related symptoms has not been investigated.

Research frontiers

Both IEM and NCCP are often associated with gastroesophageal reflux disease, but the pathophysiological mechanisms underlying IEM and NCCP are still poorly understood.

Innovations and breakthroughs

This study raises the possibility that rapid eating leads to IEM and attaches importance to inquiry into eating behavior as part of the examination of patients with NCCP.

Applications

Clinicians can take into account rapid eating as a potential cause of IEM, and the test in esophageal function. Further studies are needed to prove a direct causal relationship between rapid food intake and IEM.

Terminology

IEM: IEM is defined manometrically as esophageal body contractions with ≥ 30% of wet swallows at an amplitude < 30 mmHg in the distal esophagus.

Peer review

The concept is interesting, as the next step the authors should approach it prospectively, applying an objective definition of eating patterns rather than self-reporting.

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CASE REPORT

Esophageal lichen planus: A case report and review of the literature

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Abstract

Esophageal involvement by lichen planus (ELP), previously thought to be quite rare, is a disease much more common in women and frequently the initial manifestation of mucocutaneous lichen planus (LP). Considering that the symptoms of ELP do not present in a predictable manner, ELP is perhaps more under-recognized than rare. To date, four cases of squamous cell carcinoma in association with ELP have been reported, suggesting that timely and accurate diagnosis of ELP is of importance for appropriate follow-up. In this case report, a 69-year-old female presented with dysphagia and odynophagia. She reported a history of oral LP but had no active oral or skin lesions. Endoscopic examination revealed severe strictures and web-like areas in the esophagus. Histologic examination demonstrated extensive denudation of the squamous epithelium, scattered intraepithelial lymphocytes, rare eosinophils and dyskeratotic cells. Direct immunofluorescence showed rare cytoid bodies and was used to exclude other primary immunobullous disorders. By using clinical, endoscopic, and histologic data, a broad list of differential diagnoses can be narrowed, and the accurate diagnosis of ELP can be made, which is essential for proper treatment and subsequent follow-up.

Key words: Esophageal lichen planus; Esophagus; Immunofluorescence; Immunobullous disorders; Diagnostic accuracy

Core tip: Lichen planus is an idiopathic disorder that generally affects middle-aged patients with clinical manifestations in the skin, mucous membranes, genitalia, hair, and nails. It is fairly common as a skin disease, affecting 0.5% to 2% of the population, the mouth being the most common site of involvement. We present one such case, diagnosed using clinical, endoscopic, and histologic data, and distinguished from primary immunobullous disorders by immunofluorescence.

Nielsen JA, Law RM, Fiman KH, Roberts CA. Esophageal lichen planus: A case report and review of the literature. *World J Gastroenterol* 2013; 19(14): 2278-2281 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i14/2278.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i14.2278

INTRODUCTION

Lichen planus (LP) is an idiopathic disorder that generally affects middle-aged patients with clinical manifestations in the skin, mucous membranes, genitalia, hair, and nails[1]. Proposed etiologies include reaction to medica-

tion, Hepatitis C or other viral infections, bacteria such as *Helicobacter pylori*, or autoimmune processes; however, the exact etiology and pathogenesis are still unknown[1,2]. It is fairly common as a skin disease, affecting 0.5% to 2% of the population[3], the mouth being the most common site of involvement[1]. Conversely, esophageal involvement by LP (ELP) has previously been considered quite rare, with fewer than 50 cases reported in the literature before 2008 and a predilection for women[4]. In 2010 the Mayo Clinic published a series of 27 cases within a 10-year period, suggesting that it is perhaps more under-recognized than rare and often the initial manifestation of mucocutaneous LP[1]. Subsequently, there is often a significant delay between onset of symptoms, dysphagia being the most common, and diagnosis[3]. Considering that four cases of squamous cell carcinoma (SCC) in association with ELP have been confirmed to date[5-7], the seriousness of this diagnostic delay should move physicians to take greater precautions to rule out ELP. We present one such case, diagnosed using clinical, endoscopic, and histologic data, and distinguished from primary immunobullous disorders by immunofluorescence.

CASE REPORT

A 69-year-old female presented with dysphagia and odynophagia that had been ongoing for years. She reported a history of oral LP, but had no active oral or skin lesions, and a previously normal upper gastrointestinal series X-ray. The patient initially declined endoscopy and took proton-pump inhibitors without benefit. Later endoscopic examination revealed severe strictures and rings throughout the length of the esophagus with web-like areas; however, the gastroesophageal junction was spared and appeared essentially normal. The mucosa showed severe, diffuse sloughing with passage of the endoscope (Figure 1). Esophageal biopsy was obtained for routine histology and submitted in 10% buffered formalin. Esophageal dilation was not performed.

Histologically, the esophageal tissue demonstrated extensive denudation of the surface epithelium. The mucosa was detached from the subepithelial tissue in several areas without preservation of the basal layer (Figure 2A). Where attached, the squamous (esophageal) epithelium was somewhat atrophic with diffuse spongiotic change, scattered intraepithelial lymphocytes, rare eosinophils, and dyskeratotic cells (Civatte bodies) (Figure 2B, black circle). The subepithelial tissue showed edema and a diffuse lichenoid infiltrate including lymphocytes, eosinophils, and occasional mast cells (Figure 2C). There was no evidence of *Candida* by virtue of a negative alcian blue/periodic acid-Schiff stain. The absence of significant intraepithelial acute inflammation and/or viral cytopathic effect in conjunction with the lichenoid infiltrate and Civatte bodies excluded a viral infection. However, while a definitive diagnosis of LP could not be made on routine histology alone, it was suggested. The patient was promptly re-biopsied a month later from the middle and upper esophagus. The biopsies were submitted in Zeus transport media for immunofluorescence.

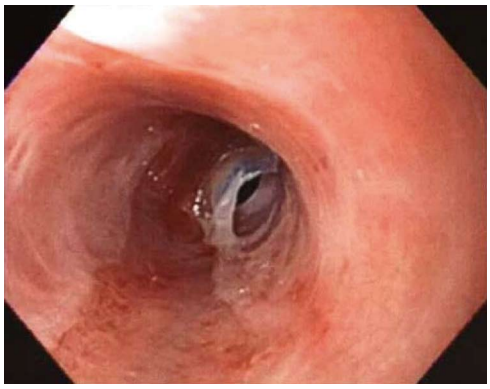

Figure 1 Endoscopy showing webs.

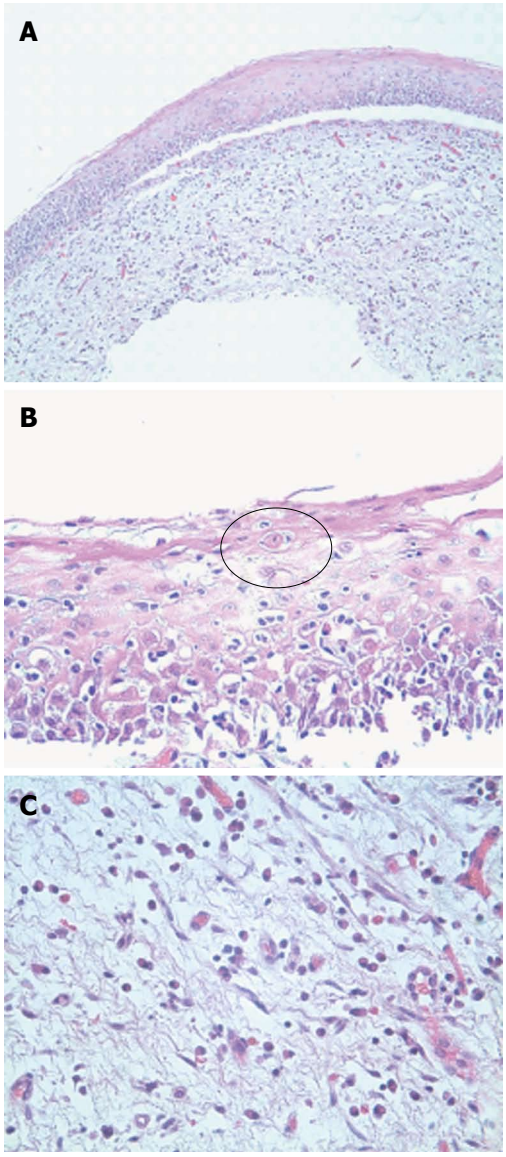

Figure 2 Histologically, the esophageal tissue demonstrated extensive denudation of the surface epithelium. A: Subepithelial separation, HE stain, × 100; B: Civatte bodies (black circle), HE stain, × 400; C: Subepithelial edema and inflammation, HE stain, × 400.

Direct immunofluorescence revealed fibrillar deposi-

Figure 3 Immunofluorescence, fibrinogen.

tion of fibrinogen along the basement membrane zone (Figure 3), characteristic of but not specific for LP. IgG, IgA, IgM, and C3 showed rare cytoid bodies in the same areas without any evidence of a primary immunobullous disorder such as pemphigus or pemphigoid. The basic histomorphology in conjunction with the clinical history of oral lichen planus and the negative immunofluorescence excluded immunobullous disorders, such as esophageal pemphigus vulgaris.

DISCUSSION

First described by Al-Shihabi *et al*[8] and Lefer[9] simultaneously but separately, over 80 cases of ELP have been reported in both English and foreign-language literature to date, only 8 of which are male[3]. Multiple retrospective studies have shown that ELP is under-recognized[1,3,10], since the esophageal symptoms can present before, concurrently, or develop after the diagnosis of extra-ELP[1,3]. In his review of 79 patients that developed ELP, Fox noted that 14 patients developed ELP as the first and only manifestation of LP. Oral LP has been long known to predispose 2%-3% of cases to the development of oral SCC[10]; however, with documentation of 4 cases of ELP progressing to esophageal SCC, early diagnosis and accurate therapy for ELP patients has become a more serious issue[2]. One of these esophageal SCC cases was reported in a series of 8 patients, the mean delay between symptom onset and diagnosis of which was 27 mo[6]. Additionally, Katzka *et al*[1] found in his review of 27 patients with ELP that this delay in diagnosis not only resulted in increased length of time with symptoms (range: 0.33-30 years, mean: 4.72 years) but also increased the number of failed treatments before diagnosis (range: 0-15, mean: 2.5), including prior dilatations, medications such as proton-pump inhibitors, and fundoplication.

Because the symptoms of ELP are not distinctive, many clinicians recommend physicians maintain a low threshold for performing endoscopies to rule out ELP in patients experiencing dysphagia with a history of mucocutaneous LP[3,10]. Esophageal sloughing and refractory strictures in a middle-aged or older female even in the absence of extra-ELP should raise ELP as a diagnostic consideration, as less than half of those with mucosal LP will exhibit concomitant skin lesions[2,10]. Additionally, easy peeling of the esophageal mucosa with minimal contact and formation of "tissue paper-like membranes" is a frequently observed characteristic[11]. Suspecting a more common culprit such as gastroesophageal reflux disease (GERD), endoscopists oftentimes focus on the lower esophagus and could potentially miss proximal lesions caused by ELP[3]. In general, GERD can be distinguished from ELP by the sparing of the gastroesophageal junction in ELP[3]. In a study using magnification chromoendoscopy on 24 consenting patients with cutaneous and/or oral LP, the University Medical Center Utrecht (Netherlands) found that 5 (21%) had ELP, 5 (21%) had GERD, and 7 (29%) had both, with no differences in symptoms amongst the groups[10]. Early diagnosis may be improved by new diagnostic modalities such as chromoendoscopy or magnification endoscopy[2].

The final diagnosis can be reached by combining the historic, endoscopic, and histologic data; whereas the routine light microscopy, while unusual, is not pathognomonic for ELP. The most indicative characteristics of ELP are a lymphohistiocytic interface inflammatory infiltrate and dyskeratotic cells (Civatte bodies)[10]. Other common disorders that affect both esophagus and skin are bullous disorders, such as pemphigus vulgaris, paraneoplastic pemphigus, epidermolysis bullosa aquistia, mucous membrane pemphigoid, bullous pemphigoid, and Hailey-Hailey disease[3]. The lack of specific immunofluorescent staining in conjunction with the subepithelial as opposed to suprabasal separation and history of oral LP clearly excluded the bullous disorders in this case[3,11]. While pityriasis lichenoides chronica (PLC) shows similar histology in cutaneous biopsies, there are no published reports of PLC occurring on mucosal surfaces such as the esophagus[12]. Even more importantly, the patient had no cutaneous lesions or history to support this diagnosis. A viral cause was excluded due to the lack of erosion/ulceration, viral cytopathic effect, and acute inflammation. Further, Civatte bodies are not typically seen in viral infections. More remote possibilities, such as graft-versus-host disease (GVHD) and toxin-associated damage, are characterized by apoptosis, which is absent in this case. Furthermore, Civatte bodies, while characteristic of ELP, are not seen in GVHD or toxic-injury, such as caused by the drug mycophenolate which essentially mimics GVHD. Finally, historical data showing a lack of transplant history or mycophenolate use serves to remove GVHD and/or mycophenolate from a diagnostic consideration here[12]. By taking into consideration the various diagnostic methods and suggestions, other possible diagnoses can be ruled out, and the diagnostic delay of ELP can be decreased, which is essential for appropriate treatment and clinical follow-up, potentially preventing more serious sequelae, including SCC[2].

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CASE REPORT

Sarcina ventriculi of the stomach: A case report

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Abstract

Sarcina ventriculi is a Gram positive organism, which has been reported to be found rarely, in the gastric specimens of patients with gastroparesis. Only eight cases of *Sarcina*, isolated from gastric specimens have been reported so far. *Sarcina* has been implicated in the development of gastric ulcers, emphysematous gastritis and gastric perforation. We report a case of 73-year-old male, with history of prior Billroth Ⅱ surgery and truncal vagotomy, who presented for further evaluation of iron deficiency anemia. An upper endoscopy revealed diffuse gastric erythema, along with retained food. Biopsies revealed marked inflammation with ulcer bed formation and presence of *Sarcina* organisms. The patient was treated with ciprofloxacin and metronidazole for 1 wk, and a repeat endoscopy showed improvement of erythema, along with clearance of *Sarcina* organisms. Review of reported cases including ours suggests that *Sarcina* is more frequently an innocent bystander rather than a pathogenic organism. However, given its association with life threatening illness in two reported cases, it may be prudent to treat with antibiotics and anti-ulcer therapy, until further understanding is achieved.

Key words: *Sarcina ventriculi*; Gram negative; Emphysematous gastritis; Gastric perforation; Bacterial overgrowth

Core tip: *Sarcina ventriculi* is a rare bacterium, seen in gastric biopsies of patients with gastroparesis. Only eight cases have been reported so far, where in it has been implicated in the development of gastric ulcers, emphysematous gastritis and gastric perforation. In our case, gastric erythema improved with antibiotic treatment. Given its association with life threatening illness in two reported cases, it may be prudent to treat with antibiotics and anti-ulcer therapy, until further understanding is achieved.

Ratuapli SK, Lam-Himlin DM, Heigh RI. *Sarcina ventriculi* of the stomach: A case report. *World J Gastroenterol* 2013; 19(14): 2282-2285 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i14/2282.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i14.2282

INTRODUCTION

Sarcina ventriculi is a Gram positive anaerobic bacterium, with carbohydrate fermentative metabolism as its sole energy source[1], and is able survive in very low pH environment[2]. Even though it is similar in appearance to *Micrococcus* species, certain morphological features (*i.e.*, larger size, non-cluster forming pattern) help differentiate it from the latter organism[3].

Various reports in veterinary literature have implicated *Sarcina* in the development of gastric dilatation[4] and death of livestock, cats and horses[5,6]. *Sarcina* has also been reported to be found in feces of healthy humans consuming a predominantly vegetarian diet[7]. Recently, several reports have shown an association between *Sarcina* in the stomach and chronic nausea, dyspepsia, abdominal pain, gastric ulcers[3], and rarely emphysematous gastritis[8] and

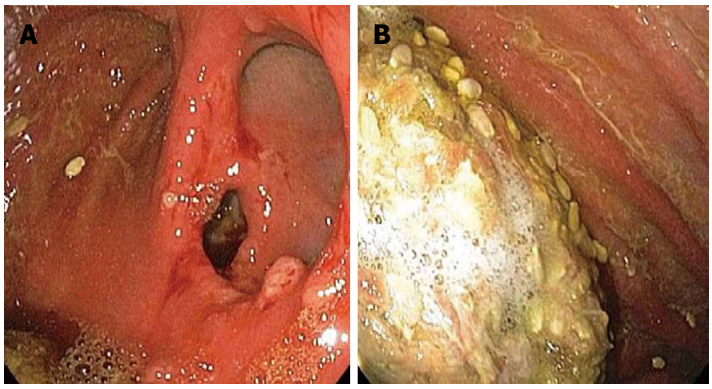

Figure 1 Esophagogastroduodenoscopy. A: Polyps at the anastamosis; B: Gastric erythema and food bezoar.

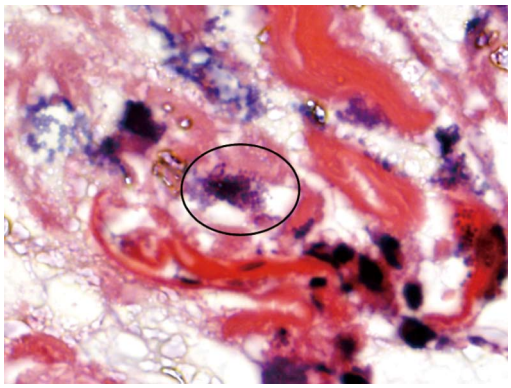

Figure 2 Characteristic 8-10 micron tetrads of *Sarcina* organisms (circled) were identified on endoscopic biopsy. The background shows abundant bacterial overgrowth and debris from retained food. Separate fragments of ulcer bed were present (not pictured) (hematoxylin and eosin; original magnification × 1000 oil lens).

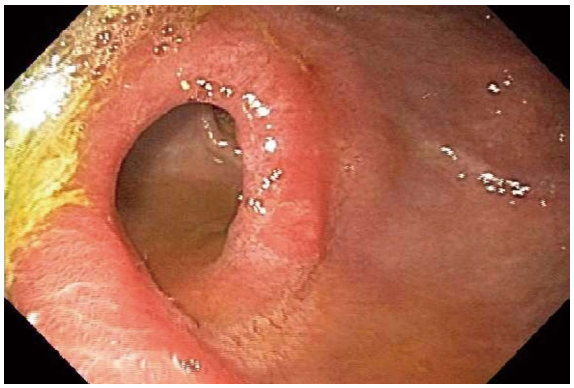

Figure 3 Repeat esophagogastroduodenoscopy showing improvement of gastric erythema.

gastric perforation[9]. However *Sarcina* has also been found in gastric specimens without any other pathologic changes[3], suggesting that it may be a bystander rather than a pathogenic organism. To date, only eight cases of *Sarcina ventriculi* isolated from gastric biopsy specimens have been reported. We now report a case of *Sarcina ventriculi* of the stomach, associated with iron deficiency anemia and gastroparesis.

CASE REPORT

A 73-year-old male presented to the clinic for further evaluation of iron deficiency anemia. The patient had a history of medically refractory gastric ulcers in his 20 s, for which he underwent antrectomy and gastrojejunostomy (Billroth Ⅱ) along with truncal vagotomy in 1985. He continued to be anemic since the surgery, with intermittent intake of oral iron replacement.

On initial evaluation for an incidentally detected anemia prior to unrelated urologic surgery, he did not have any gastrointestinal symptoms. The patient specifically denied nausea, vomiting, abdominal pain or weight loss. His complete blood count revealed decreased hemoglobin of 8.5 g/dL, decreased mean corpuscular volume of 63.2 fL, normal white cell count of 9.8×10^9 L (normal 4.2×10^9-10.2×10^9 L) and elevated platelet count 415×10^9 L (normal 151×10^9-355×10^9 L). Iron studies showed markedly reduced iron level of 12 mg/dL (normal 50-150 mg/dL), with an elevated total iron binding capacity of 490 mg/dL (normal 250-400 mg/dL) and reduced iron % saturation of 2% (normal 14%-50%).

Three years prior, an esophagogastroduodenoscopy (EGD) had revealed an anastomotic ulcer and polyps at the anastomotic site, the biopsies of which showed acute inflammation, but were otherwise unremarkable. A colonoscopy at that time was unremarkable except for diverticulosis. An EGD done during the current evaluation demonstrated diffuse gastric erythema, along with two 4mm polyps at the anastomosis (Figure 1). There was also a large amount of retained food in the stomach.

Tissue biopsies of the erythematous stomach revealed marked inflammation with ulcer bed formation, along with abundant bacterial overgrowth including the presence of *Sarcina* organisms (Figure 2). The *Sarcina* organisms were identified on routine hematoxylin and eosin (HE) stain, and no additional special stains or immunolabeling was performed. Based on prior studies[3], the tetrad morphology and size are characteristic enough to establish a diagnosis without further ancillary testing. Biopsies were negative for *Helicobacter pylori,* both by routine HE staining and by immunohistochemical staining. Aspirates from the small bowel also came back positive for small intestinal bacterial overgrowth, with > 100 000 cfu/mL of mixed Gram-positive and Gram-negative flora.

The patient was treated with metronidazole 250 mg three times a day and ciprofloxacin 250 mg twice daily

Table 1 Clinical, endoscopic and histological features of the eight reported cases of Sarcina ventriculi in the literature

Case No.	Age	Sex	Symptoms/clinical findings	Endoscopic findings	Histologic findings	Treatment	Follow-up
1	14	Male	Abdominal pain CT showed pneumoperitoneum. Intraoperatively there was necrotic stomach and gastric perforation and peritonitis	Not performed	Diffuse acute hemorrhagic gastritis and Sarcina organisms	Gentamicin and metronidazole	Symptoms improved after 5 d and patient discharged
2	50	Male	Chronic nausea, vomiting	Esophagitis, duodenal lesion	Chronic superficial gastritis and ulcer with Sarcina organisms	Not available	Not available
3	3	Female	Vomiting, hematemesis Abdominal X-ray showed dilated stomach with intramural air	Gastric inflammation, blackening of mucosa, cobblestone appearance	Polymorphic inflammatory infiltrate with Sarcina organisms and gas bubbles	Imipene, fluconazole and omeprazole	Repeat endoscopy 6 mo later showed complete normalization
4	58	Female	Nausea and vomiting	Gastritis, food bezoar, inflammatory mass in duodenum	Active chronic gastritis with Sarcina organisms	Partial gastrectomy for obstruction	Treated for adenocarcinoma of pylorus
5	44	Female	Dyspepsia and substernal burning	Gastric ulcer and retained food	Non malignant gastric ulcer with Sarcina organisms	Omeprazole, ranitidine, metoclopramide	Symptoms improved
6	36	Male	Nausea, vomiting, epigastric pain in the setting of narcotic use	Retained food	Sarcina organisms without other histologic abnormalities	Received jejunostomy for malnutrition	Repeat biopsy negative for Sarcina organisms
7	12	Female	Dysphagia in the setting of esophageal atresia status post gastric pull through	Retained food, anastamotic stricture	Reflux esophagitis, Sarcina organisms	Information unavailable	Information unavailable
8	46	Female	Epigastric pain in the setting of pancreatic adenocarcinoma status post pancreatico-duodenectomy	Retained food and bile	Active chronic duodenitis with Sarcina organisms	No treatment	Continues spasms after 1 mo

CT: Computed tomography.

for 1 wk, along with daily sucralfate. He also received intravenous (IV) iron 300 mg × 2 doses followed by oral iron and achieved normal iron stores and hemoglobin levels. Subsequent follow up with a repeat EGD 3 mo later showed improvement of gastric erythema, and absence of food bezoar (Figure 3). Aspirates from the small bowel continued to suggest small intestinal bacterial overgrowth, with > 100 000 cfu/mL. However, repeat biopsies from the stomach were negative for Sarcina organisms, and showed features of chronic gastritis. Clinically, the patient's perception of overall health improved with the above treatment, and he continued to be free of gastrointestinal symptoms.

DISCUSSION

While the pathogenic role of *Sarcina* in the veterinary literature is well established, its role in human disease is not entirely clear. Since the initial description in 1842, the pathogenic role in humans has been questioned, as it has been found in the blood[10] and feces[7] of healthy humans.

Over the last three years, 8 cases[3,8,9] of Sarcina associated with endoscopic biopsies have been reported. While all these patients presented with various gastrointestinal symptoms (nausea, vomiting, epigastric pain, dyspepsia) only two patients had associated life threatening complications of emphysematous gastritis[8] and gastric perforation[9] (Table 1). Our patient did not have any gastrointestinal symptoms, and *Sarcina* was found incidentally, when gastric biopsies were performed for erythematous mucosa.

Another interesting feature is the presence of delayed gastric emptying in five of the eight reported cases. All of these patients had retained food in stomach during endoscopic examination. Similarly, our patient had a Billroth II with truncal vagotomy, which predisposed him to have delayed gastric emptying, as was evident by the gastric bezoar seen during endoscopic examination. Hence impaired emptying of stomach could potentially lead to the growth of *Sarcina* in the stomach.

The need for antibiotic treatment, when *Sarcina* is found in endoscopic biopsies of clinically stable patients is unknown. Of the reported cases, two patients with associated life threatening disease (*i.e.*, emphysematous gastritis and gastric perforation) received intravenous antibiotics and recovered. One patient with non-life threatening disease was treated with combination of proton pump inhibitors and prokinetics, with good relief of symptoms. Some authors suggest that an underlying mucosal defect, such as erosion or ulceration, may predispose patients to more serious sequelae, from this otherwise ubiquitous organism[3]. We elected to treat our patient with antibiotics, as there was significant gastric erythema and ulceration, as well as small intestinal bacterial overgrowth.

In summary, *Sarcina ventriculi* is a rare bacterium, seen

predominantly in patients with delayed gastric emptying. Review of the published cases along with our case suggests that it is more frequently an innocent bystander rather than a pathogenic organism. Given its association with life threatening illness in two reported cases, it may be prudent to treat with antibiotics and anti-ulcer therapy, until further understanding is achieved.

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CASE REPORT

Aggressive juvenile polyposis in children with chromosome 10q23 deletion

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Abstract

Juvenile polyps are relatively common findings in children, while juvenile polyposis syndrome (JPS) is a rare hereditary syndrome entailing an increased risk of colorectal cancer. Mutations in *BMPR1A* or *SMAD4* are found in roughly half of patients diagnosed with JPS. Mutations in *PTEN* gene are also found in patients with juvenile polyps and in Bannayan-Riley-Ruvalcaba syndrome and Cowden syndrome. Several previous reports have described microdeletions in chromosome 10q23 encompassing both *PTEN* and *BMPR1A* causing aggressive polyposis and malignancy in childhood. These reports have also described extra-intestinal findings in most cases including cardiac anomalies, developmental delay and macrocephaly. In this report we describe a boy with a 5.75 Mb deletion of chromosome 10q23 and a 1.03 Mb deletion within chromosome band 1p31.3 who displayed aggressive juvenile polyposis and multiple extra-intestinal anomalies including macrocephaly, developmental delay, short stature, hypothyroidism, atrial septal defect, ventricular septal defect and hypospadias. He required colectomy at six years of age, and early colectomy was a common outcome in other children with similar deletions. Due to the aggressive polyposis and reports of dysplasia and even malignancy at a young age, we propose aggressive gastrointestinal surveillance in children with 10q23 microdeletions encompassing the *BMPR1A* and *PTEN* genes to include both the upper and lower gastrointestinal tracts, and also include a flowchart for an effective genetic testing strategy in children with juvenile polyposis.

Key words: Polyposis; Genetics; Cancer; Endoscopy pediatric

Core tip: Children with aggressive juvenile polyposis related to microdeletions of chromosome 10q23 and involving *PTEN* and *BMPR1A* are rare, however this deletion conveys significant gastrointestinal and extraintestinal risks. Children with this gene mutation are at significant risk for extensive polyposis, early colectomy and gastrointestinal malignancy. This work describes the clinical manifestations associated with these deletions. We also suggest genetic testing strategies for those with juvenile polyps and also propose gastrointestinal surveillance for patients with chromosome 10q23 deletions encompassing *PTEN* and *BMPR1A*.

Septer S, Zhang L, Lawson CE, Cocjin J, Attard T, Ardinger HH. Aggressive juvenile polyposis in children with chromosome 10q23 deletion. *World J Gastroenterol* 2013; 19(14): 2286-2292 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i14/2286.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i14.2286

INTRODUCTION

Classification of polyps in children and subsequent attempts to diagnose hereditary polyposis syndromes begin with histologic sub-typing. The juvenile polyp was first described by Diamond[1] and is characterized histologically by an edematous lamina propria with inflammatory cells and cystically dilated glands which are lined by cuboidal to columnar epithelium[2]. While sporadic juvenile polyps are fairly common in the first decade of life and may be found in up to 2% of the pediatric population[3,4], juvenile polyposis syndrome (JPS) is a rare hereditary polyposis syndrome occurring in 1:100 000[5] and entails an increased risk of colorectal cancer and to a lesser degree gastric cancer. Juvenile polyposis syndrome is defined (Jass Criteria) by the presence of five or more juvenile polyps in the colorectum, any number of juvenile polyps proximal to the colorectum or any number of juvenile polyps with a positive family history of juvenile polyposis[3,4,6]. JPS typically presents in adolescence or adulthood. Treatment consists of surveillance endoscopy with polypectomy. Endoscopy is typically performed on a regular basis after polyps are found. Prophylactic surgery is indicated if polyp burden is unmanageable endoscopically, when juvenile polyps display dysplasia or in the case of severe gastrointestinal bleeding. A severe form of JPS called juvenile polyposis of infancy (JPI) has also been described and is characterized by its early manifestations of generalized polyposis, diarrhea, gastrointestinal bleeding and protein losing enteropathy in the first two years of life resulting in death in infancy in some patients[7].

Three genes have been associated with juvenile polyps. In 45%-60% of patients with typical JPS a mutation can be found in either the *SMAD4* or *BMPR1A* genes[8-12]. *SMAD4* is a tumor suppressor gene located on chromosome 18q21 and is associated with hereditary hemorrhagic telangiectasia in some individuals in addition to JPS. *BMPR1A* is located on chromosome 10q22-23[12] and encodes for a receptor important in the BMP/growth factor signaling pathways. Additionally, a third gene, *PTEN*, also located on chromosome 10q22-23, has been associated with juvenile polyps, in association with Cowden syndrome, a familial cancer syndrome, and the lesser known Bannayan-Riley-Ruvalcaba syndrome[13] associated with macrocephaly, developmental delay and some minor dysmorphia. These conditions, now grouped together as the "*PTEN*-hamartoma syndrome" (PHTS)[14], may present with juvenile or hamartomatous polyps. In addition to an increased risk for breast, thyroid, colorectal and endometrial cancers in adulthood, skin lesions such as lipomas, trichelommomas and papillomatous lesions, and penile macules are common findings.

Of considerable interest are a small group of patients who have been reported to have a chromosome 10q23 deletion involving both the *BMPR1A* and *PTEN* genes and also developed juvenile polyposis. Less than twenty patients have been described with these mutations[8,15-26]. Many of these patients were originally tested by chromosome analysis or more recently by chromosome microarray due to congenital anomalies, macrocephaly and/or developmental delay. Many of them also developed aggressive juvenile polyposis and in some cases required colectomy. The most common physical finding in these children was macrocephaly and the majority also had developmental delay. Other findings seen in multiple patients were atrial septal defect and/or ventricular septal defect, hemangioma, club foot, hypotonia and speckling of the penis.

Herein, we describe a patient who presented with a microdeletion of chromosome 10q23 which resulted in the deletion of both *BMPR1A* and *PTEN* genes and an additional microdeletion involving chromosome 1p31.3 of uncertain significance. His polyposis history is compared to that of others with similar 10q23 deletions and contrasted with those with mutations in either *BMPR1A*, *PTEN* or *SMAD4* alone. We have developed an algorithm for genetic testing for patients presenting with juvenile polyposis since those with microdeletions are subject to significant health risks, including malignancy. We will also focus on the optimal long term gastrointestinal surveillance for these patients.

CASE REPORT

The patient was delivered at 37 wk gestation by Caesarean delivery due to macrocephaly and weighed 9 lbs, 12 oz. Head circumference at birth was 38.7 cm (90th percentile), and at 11 mo of age he was significantly macrocephalic (+4 SD). At a few days of age, a heart murmur was noted and echocardiogram revealed a large ventricular septal defect (VSD), atrial septal defects (ASD) and several smaller VSDs. Repair was performed at 10 d of age. His postoperative course was complicated by ectopic atrial tachycardia which required short term amiodarone therapy. Tracheostomy was performed at 11 mo of age due to multiple episodes of respiratory distress, multiple pneumonias and a diagnosis of tracheobronchomalacia. Other phenotypic characteristics in this child were hypospadias requiring repair, sagittal craniosynostosis requiring surgical correction, exotropia, midface hypoplasia with large cheeks, a prominent Cupid's bow of the upper lip, and deep palmar creases. Additionally, his medical history included adenoidal hypertrophy necessitating adenoidectomy and fundoplication for medically refractory gastroesophageal reflux exacerbating respiratory compromise. Developmental delay was also present with delayed speech and gross motor delays. Endocrine issues included short stature (< 3rd percentile), obesity (body mass index, BMI > 97 kg/m^2), growth hormone deficiency and primary hypothyroidism. The patient was treated with growth hormone and *L*-thyroxine and his height eventually reached the 10th percentile.

Due to the multiple anomalies found in this patient, genetic consultation had been obtained at 4 years of age. He was reported to have had a normal 46, XY karyotype on previous testing. Microarray comparative genomic

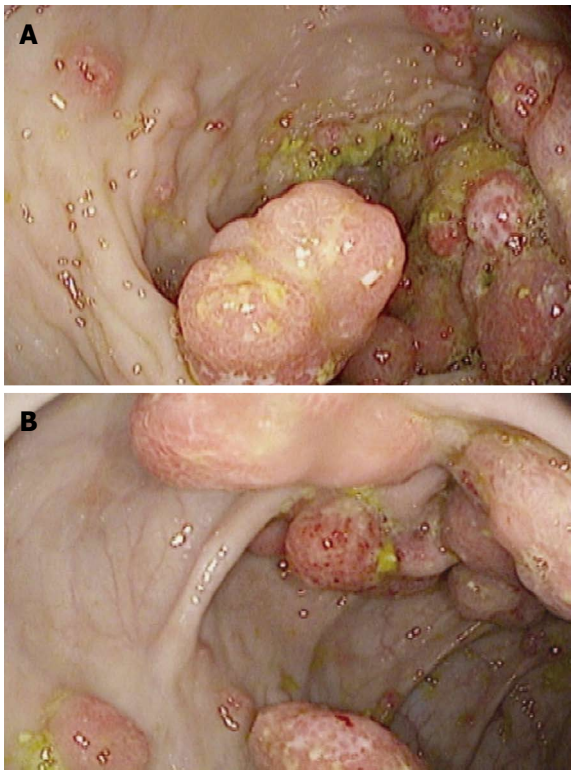

Figure 1 Endoscopic view. A: Polyps noted during colonoscopy, prior to colectomy; B: Endoscopic view of colonic polyps in the patient described.

hybridization (aCGH) analysis was performed (Agilent 244k platform) and two genomic deletions were found in this patient. One is a 1.03 Mb deletion within chromosome band 1p31.3 involving seven annotated genes and transcripts: *CACHD1, RAVER2, JAK1, AK3L1, DNAJC6, LEPR, LEPROT* [chr1:64870449-65897852 (hg18)]. The other one is a 5.75 Mb deletion of chromosome 10q23.1q23.31 involving 26 annotated genes and transcripts including *BMPR1A* and *PTEN* [chr10: 84311235-90064565 (hg18)]. Parental analyses of these two deletions showed normal results indicating these deletions are *de novo* in origin.

At 4 years of age the patient was seen in our Pediatric Gastroenterology Clinic for consultation due to the concern regarding polyposis with the 10q23 deletion and also for his symptoms of abdominal distension of uncertain etiology. At age 5 years he underwent esophagogastroduodenoscopy (EGD) and colonoscopy with significant findings of five small (4-5 mm) duodenal polyps and approximately 30 polyps in the colon, from rectum to cecum. Histopathology revealed juvenile polyps in all cases, without any adenomatous transformation. Growth hormone was stopped at this point due to a concern for increased polyp growth.

Four months later blood was noted in the stool and repeat endoscopy was performed. The polyp burden had increased to approximately 50 small polyps (4-6 mm) in the duodenum and 75-100 polyps in the colon. The majority of these colonic polyps were less than 6 mm, however there were five to six larger polyps 1-2 cm in size. Histology of all polyps was consistent with juvenile polyps.

A third endoscopy was performed six months later and again 50 polyps were noted in the duodenum, with several of them increased in size to 8 mm. Colonoscopy revealed 50-100 polyps from sigmoid to cecum (Figure 1). Approximately half of these polyps were now > 1.5 cm with several larger than 3 cm in diameter. Subsequently, as a result of the polyp burden which precluded endoscopic removal, the child was referred for laparoscopic subtotal colectomy with ileorectal anastamosis. The resected colon contained greater than 50 polyps, ranging in size from 0.6-3.1 cm in diameter. The polyps were juvenile in all cases and there was no dysplasia found. Postoperatively the patient struggled with frequent stooling and skin breakdown but is improved with use of fiber and loperamide.

DISCUSSION

The 10q23 deletion encompassing both *PTEN* and *BMPR1A* is rare, but conveys significant multisystem health problems and is known to have a variable phenotype with many individuals harboring juvenile polyps. Some individuals with this deletion fit the description of JPI with aggressive and early onset gastrointestinal polyposis. Our patient did not meet the criteria for JPI, as there was not diarrhea, bloody stools or hypoalbuminemia in the first two years of life. However, he did have extensive juvenile polyposis at a young age which led to colectomy. This aggressive gastrointestinal phenotype is not expected in generalized JPS, which is typically diagnosed in adolescence or adulthood. Additional clinical features in our patient included cardiac defects, macrocephaly, developmental delay, tracheobronchomalacia necessitating tracheostomy, medically refractory gastroesophageal reflux requiring fundoplication, thyroid and growth hormone deficiency and hypospadias. Whether these additional features represent the variability of the 10q23 deletion syndrome or whether they are associated with the additional 1p31.3 deletion is unknown at this time.

This is the first report of co-existing deletions of 10q23 and 1p31.3. The 1.03 Mb deletion of 1p31.3 has not been reported before. There are no known benign copy number variants in the region (http://projects.tcag.ca/variation/). Petti *et al*[27] reported a 15-year-old boy who carried a heterozygous 3.2 Mb deletion, which covers and extends beyond the deletion in our patient and had obesity, behavioral problems, mild intellectual impairment and facial dysmorphism. Vauthier *et al*[28] reported a three-year-old boy with an 80 kb homozygous deletion of 1p31.3 which included part of *DNAJC6* and *LEPR* genes. This patient showed early onset obesity, mild dysmorphic features, intellectual disability, and epilepsy. Eight additional family members were heterozygous for the 80 kb 1p31.3 deletion. Seven of the eight were either overweight or obese and none had intellectual impairment. Our current patient has a heterozygous deletion

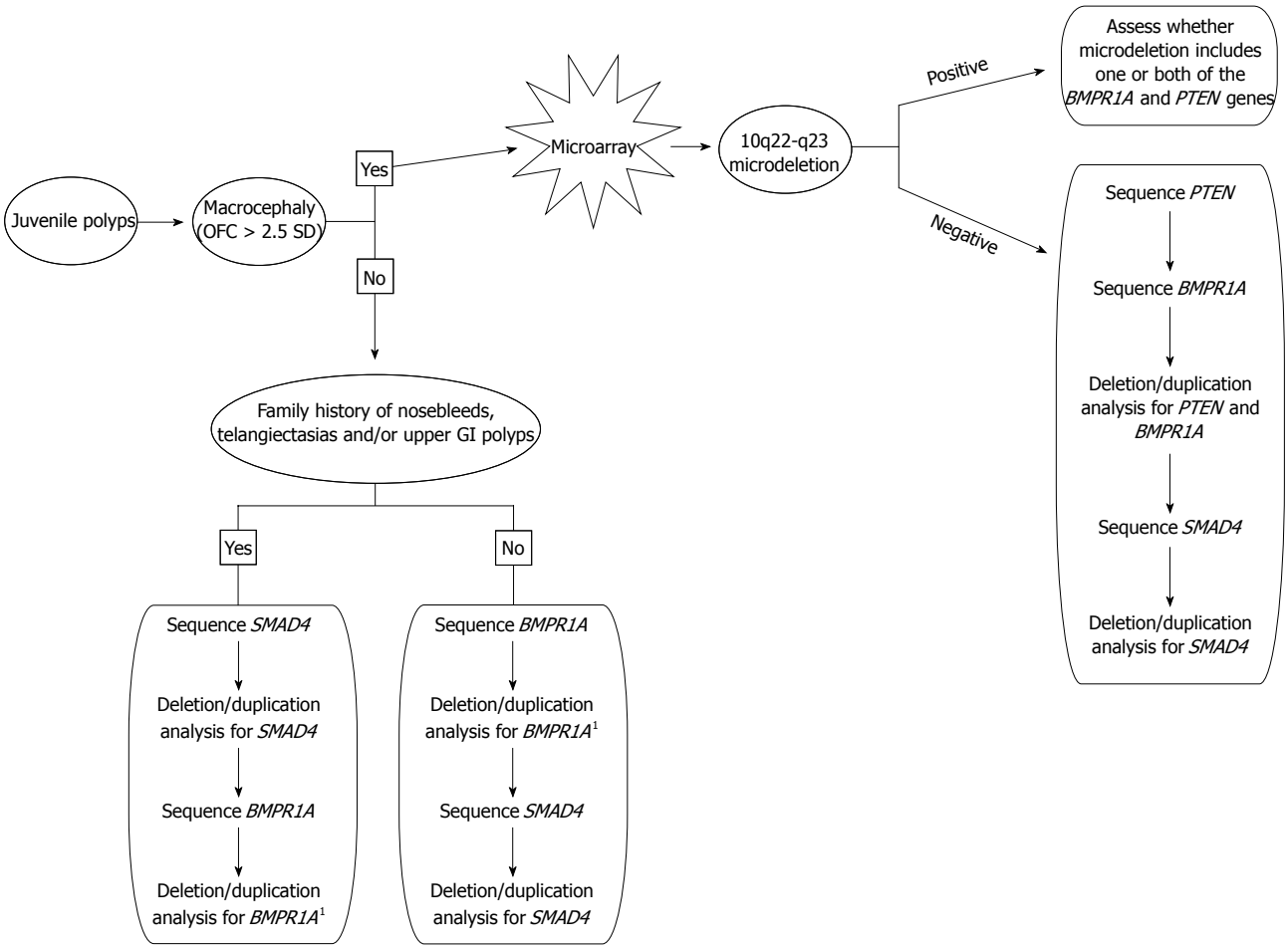

Figure 2 Algorithm for genetic testing and diagnosis of individuals with juvenile polyps. [1]If a deletion in BMPR1A is found, ask the testing laboratory if a microarray is indicated based on the location of the deletion. OFC: Occipital-frontal circumference. GI: Gastrointestinal.

of the *DANJC6* and *LEPR* genes. Since age 2 years, his weight has tracked above the 75th percentile and height at or below the 10th percentile with a BMI at the 99th percentile for age which may reflect the effect of the deleted *LEPR* gene as high BMI is not typically associated with the 10q23 deletion phenotype. Heterozygous loss of the *DNAJC6* gene in the current patient is of unknown significance. A literature review did not reveal any significant clinical associations of other genes deleted in the region within 1p31.3. Therefore, it is unclear how the 1p31.3 deletion may have impacted the phenotype of 10q23 deletion in our patient other than contributing to his elevated BMI.

This patient's most significant medical issues, including polyposis and subsequent colectomy, pertain to his chromosome 10q23 deletion and its disruption of the function of *BMPR1A* and *PTEN*. *PTEN* is an important tumor suppressor gene. Mutations (including sequence changes or deletions) of the *PTEN* gene are associated with PHTS as previously described. Both hamartomatous and juvenile polyps are seen in PHTS. Sequence changes or partial deletions of the *BMPR1A* gene result in loss-of-function of that gene and typically result in juvenile polyposis syndrome. Interestingly, among patients reported to have a deletion of chromosome 10q23 which includes *BMPR1A* but does not include *PTEN*[17,29], none have been reported to have polyposis thus far[30]. A combined and synergistic effect of the deletion of both *BMPR1A* and *PTEN* in 10q23 microdeletion may be involved in this aggressive polyposis. The functions of the PTEN protein include phosphatase activity down-regulating the PI3K/Akt pathway, which helps regulate cell growth, proliferation, and apoptosis[31]. The *BMPR1A* gene encodes a receptor for the BMP pathway binding proteins and this pathway inhibits cell proliferation, especially of the gastrointestinal tract[32,33]. Therefore, the deletion of both of these genes may lead to increased proliferation of gastrointestinal cells predisposing to polyps and potential gastrointestinal malignancies.

Gastrointestinal management of patients with 10q23 microdeletions is determined on an individual basis due to the variability in onset of polyps and severity of progression. In many patients with this deletion, including the patient described in this report, there is an accelerated rate of polyp development that occurs at a very early age and is more aggressive than that seen in PHTS or in *BMPR1A*-associated JPS. In fact, nearly half of the reported patients have been referred for colectomy[16-18,20,23,25,26] in childhood, with several requiring surgery before 2 years of age. When contemplating colectomy, the number of

polyps, size of polyps, associated symptoms and level of concern for malignancy are all considered. Although there is an increased risk of colorectal cancer in adults with PHTS[34] and JPS[35], children are rarely diagnosed with gastrointestinal cancer and do not routinely undergo colectomy. However, in those with 10q23 microdeletions there are reports of early colorectal dysplasia and malignancy. Dysplastic polyps or colonic epithelial dysplasia were noted in the colon in three children[22,23,25] and the duodenum in one[20]. An additional patient developed rectal cancer at age 24 years[23] and is now deceased. These observations suggest children with 10q23 deletions require frequent endoscopic surveillance of not only the lower but also the upper gastrointestinal tracts. We propose yearly EGD and colonoscopy after diagnosis of these mutations. Some patients, such as the one described in this paper, may require more frequent endoscopy if polyps are rapidly increasing in size or number and all polyps cannot be removed during one endoscopy. Small bowel surveillance with capsule endoscopy should also be considered. As in our patient, this risk for early gastrointestinal malignancy should prompt consideration for colectomy when the polyp burden becomes too great to manage through serial polypectomy or when dysplasia develops. Additionally, post-colectomy endoscopic surveillance is also warranted by the presence of upper intestinal polyps in a majority of reported cases, the high recurrence rates of polyps in the remnant rectum and the pouch and the fact that even after colectomy there is continued risk for duodenal or rectal cancer.

Extra-intestinal workup should also be considered due to the frequent non-gastrointestinal manifestations. In the patients' reported, common findings include cardiac (ASD, VSD), developmental delay, hypotonia, lipoma and hemangioma. Extraintestinal malignancies reported in 10q23 microdeletions include thyroid cancer[8] and mucinous cystadenoma of the ovaries[26]. These observations suggest neurodevelopmental assessment, close monitoring of growth parameters, careful dermatologic exam, thyroid exam and/or ultrasound and echocardiography should all be considered in these patients both at time of diagnosis and throughout life.

Genetic testing plays a critical role in establishing the correct diagnosis for patients who have features of JPS, PHTS, or both since this will have an impact on the surveillance and management of the patient. We propose the following algorithm to achieve a genetic diagnosis in the most timely and cost-effective manner (Figure 2). Macrocephaly of greater than 2.5 SD is a common feature seen in PHTS and is not commonly associated with JPS so it is a reasonable starting point in making a diagnosis. Additionally, in patients with JPS, a mutation in *SMAD4* is more likely when there is family history of polyps when compared to *BMPR1A*. A *SMAD4* mutation is also more likely when there is a positive family history of nosebleeds and/or telangiectasias, as *SMAD4* mutations are also associated with hereditary hemorrhagic telangiectasia syndrome, along with features of JPS. Immunohistochemistry for SMAD4 may also be done in some centers, and if positive, guide genetic testing[36]. *BMPR1A* is located more proximal to the centromere on chromosome 10 than *PTEN* and there are several genes located between these two. Therefore, if a deletion is detected in either *BMPR1A* or *PTEN*, it is important to assess the precise location of this deletion in the event it could represent a larger 10q23 microdeletion. For this reason, we recommend a microarray analysis (if one has not already been completed) if a deletion is detected in either *BMPR1A* or *PTEN*. Both PHTS and JPS are inherited in an autosomal dominant pattern and both can either be inherited from a parent or occur as a *de novo* event. Once a genetic diagnosis is established in a presenting patient, parental studies may be critical to assess if either parent is at risk for medical complications that are associated with these conditions.

Our report highlights the phenotypic diversity of deletions including chromosome 10q23 and involving *PTEN* and *BMPR1A*. These patients are at risk for cardiac, endocrine, gastrointestinal and neurodevelopmental abnormalities. They have a heightened risk of accelerated polyposis, in some cases conforming to the traditional definition of JPI and, in addition harboring an increased risk of gastrointestinal malignancy that appears greater than if there is a mutation or deletion in either gene alone. Multidisciplinary assessment of these patients is an early prerequisite for care.

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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