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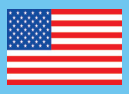

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EDITORIAL

Consensus statement AIGO/SICCR: Diagnosis and treatment of chronic constipation and obstructed defecation (part Ⅰ: Diagnosis)

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Abstract

Chronic constipation is a common and extremely troublesome disorder that significantly reduces the quality of life, and this fact is consistent with the high rate at which health care is sought for this condition. The aim of this project was to develop a consensus for the diagnosis and treatment of chronic constipation and obstructed defecation. The commission presents its results in a "Question-Answer" format, including a set of graded recommendations based on a systematic review of the literature and evidence-based medicine. This section represents the consensus for the diagnosis. The history includes information relating to the onset and duration of symptoms and may reveal secondary causes of constipation. The presence of alarm symptoms and risk factors requires investigation. The physical examination should assess the presence of lesions in the anal and perianal region. The evidence does not support the routine use of blood testing and colonoscopy or barium enema for constipation. Various scoring systems are available to quantify the severity of constipation; the Constipation Severity Instrument for constipation and the obstructed defecation syndrome score for obstructed defecation are the most reliable. The Constipation-Related Quality of Life is an excellent tool for evaluating the patient's quality of life. No single test provides a pathophysiological basis for constipation. Colonic transit and anorectal manometry define the pathophysiologic subtypes. Balloon expulsion is a simple screening test for defecatory disorders, but it does not define the mechanisms. Defecography detects structural abnormalities and assesses functional parameters. Magnetic resonance imaging and/or pelvic floor sonography can further complement defecography by providing information on the movement of the pelvic floor and the organs that it supports. All these investigations are indicated to differentiate

between slow transit constipation and obstructed defecation because the treatments differ between these conditions.

Key words: Slow transit constipation; Dyssynergic defecation; Obstructed defecation; Constipation scoring system; Quality of life; Anorectal manometry; Colon motility; Balloon expulsion test; Defecography

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INTRODUCTION

The mission of the Italian Association of Hospital Gastroenterologists (AIGO) is to advance the knowledge of digestive pathologies, to promote progress in the prevention, diagnosis, care and rehabilitation of gastrointestinal diseases, and to promote research.

The aim of the Italian Society of Colo-Rectal Surgery (SICCR) is to ensure the highest therapeutic standards through the evaluation and introduction into medical practice of the latest advances in the areas of prevention, diagnosis and care of pathologies involving the colon, rectum and anus.

The Joint Committee AIGO/SICCR is made up of members of these two scientific societies, elected on the basis of their experience in treating functional and organic problems of the colon and rectum.

The objective of the committee was to develop a consensus statement on the most important diagnostic and therapeutic aspects of functional constipation and obstructed defecation, including a set of graded recommendations based on a review of the literature and on evidence-based medicine.

LITERATURE SEARCH

A search of the literature was carried out using the online databases of PUBMED, MEDLINE and COCHRANE to identify articles published in English before April 2011 and reporting trials conducted on adult subjects with chronic constipation. The key words used were: Rome criteria, constipation, slow transit constipation, pelvic floor dyssynergia, dyssynergic defecation, dyschezia, colonic inertia, bowel questionnaire, constipation scoring system, quality of life, anorectal manometry, rectal compliance, colonic transit, colon motility, gastrointestinal motility, colonic manometry, balloon expulsion test, pelvic floor imaging, proctography, cystoproctography, dynamic magnetic resonance, anal ultrasound, endosonography, constipation medical therapy, alimentary fibres, laxatives, prokinetics, probiotics, biofeedback, pelvic floor rehabilitation, sacral nerve stimulation, obstructed defecation, outlet obstruction, rectocele, rectal intussusception, rectal prolapse, enterocele, Duhamel operation, Block operation, Sarles operation, stapled transanal resection, Delorme operation, Ripstein operation, colorectal surgery, colectomy, ileorectal anastomosis, segmental colonic resection, laparoscopic colectomy, antiperistaltic cecoproctostomy, cecorectal anastomosis, antegrade colonic enema, Malone's procedure, Malone antegrade continence enema, colostomy, ileostomy, colonic irrigation, pelvic organ prolapse, posterior vaginal prolapse, posterior colporrhaphy, transanal repair, transvaginal repair and mesh.

Table 1 Levels of evidence[1]

Levels of evidence	
I	Randomised clinical trials with $P < 0.05$, adequate sample size, and appropriate methodology
II	Randomised clinical trials with $P < 0.05$, inadequate sample size, and/or inappropriate methodology
III	Non-randomised trials with simultaneous controls
IV	Non-randomised trials with historical controls
V	Case series

LEVELS OF EVIDENCE AND GRADING OF RECOMMENDATIONS

The recommendations of the committee were defined and graded based on the current levels of evidence and in accordance with the criteria adopted by the American College of Gastroenterology's Chronic Constipation Task Force[1].

Five evidence levels were defined (Table 1). The recommendations were graded A, B and C (Table 2).

The committee wishes to underline that insufficient evidence does not automatically imply "evidence against" a statement. Many decisions in daily practice are based on clinical experience. Sometimes, it is difficult to find scientific papers supporting a widespread clinical practice, but this difficulty does not mean that we need to refute or abandon therapies that clinicians have been using for years with their patients. Evidence-based medicine is a useful tool to guide clinical practice, but if applied mechanically and without the application of common sense and personal experience, it can lead to erroneous conclusions[2].

In the development of this consensus statement, the committee identified five key areas (Table 3) and divided

Table 2 Grading of the recommendations[1]

Grading of the recommendations	
A	Recommendation supported by two or more level I trials, without conflicting evidence from other level I trials
B	Recommendation based on evidence from a single level I trial OR, evidence from two or more level I trials with conflicting, evidence from another level I trial OR, evidence from two or more level Ⅱ trials
C	Recommendations based on levels of evidence Ⅲ-V

OR: Odds ratio.

Table 3 Areas defined by the committee for the consensus statement

Area	
1	Clinical evaluation and scoring systems
2	Diagnostic techniques
3	Medical and rehabilitative treatment
4	Surgery for slow transit constipation
5	Surgery for obstructed defecation with or without associated pelvic diseases

them into subsections. Each subsection was researched, and recommendations were prepared by one or more members of the committee in accordance with specific themes defined by the committee.

The process of drafting the consensus statement involved constant communication and evaluation conducted online and during four face-to-face working meetings held at 3-mo intervals. During these meetings, the levels of evidence and the grading of the recommendations were discussed to reach a consensus in all the areas covered in the consensus statement.

The commission presents its results here in a "Question-Answer" format, which will allow clinicians to find concise responses to their specific questions quickly and easily and to peruse the full text at their leisure.

DEFINITION OF CONSTIPATION

Constipation can be either primary or secondary. The commission adopted the definition of primary functional constipation outlined in the Rome Ⅲ criteria[3]. This set of criteria was developed by an international group of experts through a process of consensus, and it has been reviewed and revised more than once since it was first published[3-5].

Stool form was defined using the Bristol stool form score[6]; constipation may involve slow intestinal transit and/or abnormal defecation; the definition of abnormal defecation from the Rome Ⅲ criteria was adopted[7].

CLINICAL EVALUATION AND SCORING SYSTEMS

Clinical evaluation

Is a patient history useful in the evaluation of chronic constipation? A thorough medical history should always be taken in patients with chronic constipation. This process constitutes the first approach to the patient and is designed to detect events that may be directly or indirectly linked to the patient's symptoms[8-11].

The patient history can identify conditions responsible for secondary constipation[11,12], such as the following: (1) alarm symptoms, such as weight loss, bloody stools, anaemia, or a family history of colon cancer; (2) conditions and/or diseases potentially associated with constipation, such as inappropriate diet[13], low physical activity[10], the use of constipating drugs, and metabolic, psychiatric or neurological diseases; and (3) the negative outcome of perineal-pelvic-abdominal or obstetric-gynaecological surgery[14,15].

Can the medical history distinguish among the different subtypes of chronic constipation? No, there are as yet no specific criteria that can distinguish among the subtypes of chronic constipation based on anamnesis[7,16-18]. Level I evidence, Grade A recommendation.

Are there specific symptoms that are present only in patients with functional constipation? No, there are no specific symptoms that distinguish patients with functional constipation from normal subjects[3]. Level I evidence, Grade A recommendation.

The occurrence of two or more symptoms during at least 25% of bowel movements distinguishes patients with chronic constipation from normal subjects[3,19].

Should a physical examination be performed in patients with chronic constipation? A physical examination is essential in the initial workup of a patient with chronic constipation[11]. The examination should include inspection of the anorectal region and exploration of the rectum. This process can detect external signs of anal disease, pelvic organ prolapse, or descending perineum syndrome. A digital rectal examination should detect any signs of organic disease or obstructed defecation. The examination is particularly important if functional alterations in defecation are suspected.

Is blood testing useful in the diagnostic algorithm of functional constipation? Blood testing does not provide useful input. Functional constipation is defined as a primitive condition and is not accompanied by any organic or biochemical alterations, being associated instead with a "functional" pathology of visceral motility. For this reason, there are no laboratory tests for the diagnosis of functional constipation[3,9]. Level I evidence, Grade A recommendation.

Blood tests can, however, be performed to exclude conditions of secondary chronic constipation[12].

Should morphological investigations (colonoscopy, barium enema, or computerised tomographic colonography) be performed in all patients with chronic constipation? Prospective studies on this point are lacking in the literature[20,21]. There is no clear evidence to support the usefulness of colonoscopy in patients with chronic constipation. Level Ⅳ evidence, Grade C recommendation.

However, morphologic investigations should always be performed in patients with alarm symptoms, in patients > 50 years of age, and in patients with a family history of colon cancer.

SCORING SYSTEMS IN CHRONIC CONSTIPATION

Scoring systems to quantify disease severity

Various scoring systems have been developed to quantify the severity of constipation. These systems are particularly important in a subjective, functional disease, such as constipation, to evaluate the results of therapy.

An early scoring system, the chronic idiopathic constipation index (CICI), was published in *Techniques of Coloproctology* in 1996[22]. It is based on seven variables (scored from 0 to 3, with a maximum score of 21) and was designed to detect chronic idiopathic slow transit constipation. The CICI was the first evaluation system that took into consideration signs of autonomic neuropathy. However, it has never been validated in a prospective study.

In 1999, the Patient Assessment of Constipation Symptoms[23] was published. This 12-item, patient-administered questionnaire has been validated and found to be effective, but it is rarely used in clinical studies.

The most widely adopted instrument is the Cleveland Clinic Constipation Score[24]. It is easy to understand and administer and therefore has won broad acceptance, although it has not been formally validated. It consists of 8 items scored from 0 to 4 for a maximum score of 30. It should be noted that one of the items, "duration of symptoms", cannot be modified by therapy.

In 2002, a new, prospectively validated score, the symptom scoring system for constipation[25], consisting of 11 items scored from 0 to 3 or 4 for a maximum possible score of 39, was published, but it is rarely used.

More recently the Constipation Severity Instrument (CSI)[26] was developed. It is a well-designed scoring system consisting of 78 items that can identify and quantify different types of constipation (IBS, slow transit and obstructed defecation).

In 2008, the first instrument specifically designed for obstructed defecation syndrome, the obstructed defecation syndrome (ODS) score, was published in *Colorectal Disease*[27]. It consists of 7 items scored from 0 to 4 with a maximum score of 27, and it has been prospectively validated.

Measuring quality of life in constipation

Three Quality of Life (QoL) questionnaires for constipation have been published. The gastrointestinal QoL questionnaire[28] was designed to address all gastrointestinal symptoms and therefore is not specific for constipation. It includes 36 items with 5 possible answers, and it has a maximum possible score of 180.

In 2005, the first disease-specific questionnaire on constipation appeared, the Patient Assessment of Constipation Quality of Life[29]. It consists of 28 items scored from 0 to 4 with a maximum score of 112.

Recently, a new, statistically validated QoL questionnaire, the Constipation-Related Quality of Life (CRQOL)[30], was published. It includes 4 domains: social impact (11 items), distress (11 items), usual diet (11 items), and defecation features (4 items).

Conclusions

Several scoring systems for constipation can be found in the medical literature. The consensus of the committee is that the most reliable instruments for scoring disease severity are the CSI for constipation in general and the ODS score for obstructed defecation. The CRQOL is an excellent tool for evaluating the effects of constipation on the patient's quality of life. The use of these instruments is recommended for clinical trials.

DIAGNOSIS OF FUNCTIONAL CONSTIPATION

Imaging in chronic constipation and obstructed defecation syndrome

Currently available imaging techniques for chronic constipation and ODS include the following: (1) transit time (TT) studies[31,32]; (2) X-ray videoproctography[33] and colpo-cysto-entero-defecography[34,35]; (3) magnetic resonance (MR)-defecography[36]; and (4) ultrasonography (US) of the pelvic floor[37-40].

Can a TT study differentiate slow transit constipation from obstructed defecation? Depending on the site of accumulation of the radiopaque markers along the large bowel, an initial TT study can differentiate between patients with total or segmental colonic slow transit constipation and patients with outlet obstruction. Unfortunately, lack of standardisation in the procedure makes it difficult to compare results among centres. Level V evidence, Grade C recommendation. In the case of distal obstruction, X-ray defecography is recommended as a second-line examination. The fact that this examination has been universally adopted makes it the benchmark against which to test newer modalities.

When should defecography be performed as opposed to colpo-cysto-entero-defecography? Defecography is indicated to rule out a variety of conditions that could play a role in the aetiology of the presenting symptom(s), such as paradoxical contraction of the puborectalis muscle[41,42], a rectocele, recto-anal intussusception and complete external rectal prolapse. Colpo-cysto-entero-defecography should be performed when multiple compartment defects are suspected, including cystocele, enterocele, or descending perineum syndrome[43].

Because their clinical significance remains a matter of debate, there is general agreement[44-46] that the results of contrast radiography should not be relied on exclusively when making decisions regarding the treatment of a patient (including surgery).

When should MR defecography be considered as an alternative to X-ray examination? Due to the panoramic

view that they provide and the absence of ionising radiation, MR imaging of the pelvic floor and MR defecography are now frequently recommended as a valid alternative to contrast radiography, especially in young patients, female patients of reproductive age, pregnant patients, and those patients at risk for adverse reactions to the contrast medium.

Are the findings commonly observed on defecography captured equally well by MR defecography? Despite the less natural (horizontal) position of the patient during the exam, MR imaging can provide similar, and sometimes better, results than conventional X-rays, with the added advantage (especially in the case of defects affecting multiple compartments) of the superior reproducibility of the results[47,48]. Consequently, while MR defecography is widely recommended as a tool to increase diagnostic confidence in cases of evacuation dysfunctions, MR neurography of the pelvic floor can be extremely useful in detecting pudendal nerve entrapment neuropathy in patients with chronic pelvic pain[49]. Level V evidence, Grade C recommendation.

Can defecographic findings be assessed and measured by perineal, endovaginal and endoanal sonography? There has been a reappraisal of the use of perineal, introital, endoanal and endovaginal US (conventional 2-D and 3-D images recorded using a variety of probes: convex, end-fire, linear and axial 360° rotating models) in the evaluation of the pelvic floor anatomy in patients with evacuation dysfunctions[50-55]. With the exception of rectal evacuation[56], the presence and severity of the most common ODS abnormalities visible on defecography can be equally well documented by any one of these sonography techniques. Level V evidence, Grade C recommendation.

What is the role of endovaginal sonography in chronic constipation? Currently, 2-D and 3-D endovaginal sonography are recommended as alternatives to defecography and MR imaging, respectively, when assessing the overall anatomic configuration and movement of the urogenital hiatus in patients with multiple defects affecting the muscular and fascial components of various compartments (anterior, middle and posterior), which are possibly indicative of descending perineum syndrome or pelvic organ prolapse[53,54]. Level V evidence, Grade C recommendation.

What is the role of endoanal sonography in chronic constipation? Given the inherently static nature of this examination and the presence of a foreign object in the anal canal (i.e., the endocavitary probe), endoanal sonography is of limited value in the diagnosis of chronic constipation. Recently, however, the advent of 3-D reconstruction has significantly increased the diagnostic confidence associated with this technique[55], which can provide detailed imaging of abnormalities, such as the extent of anal sphincter defects, the anatomy of fistulous tracts in complex perianal sepsis, and submucosal invasion in early anorectal cancers.

In summary, general agreement exists among authors that the first-line examination remains TT, followed by X-ray defecography. When the appropriate instruments and trained personnel are available, MRI and/or pelvic floor sonography can further complement defecography by providing information on the movement of the pelvic floor and the organs that it supports.

Anorectal manometry

Anorectal manometry measures anal canal pressures. Perfusion catheters are generally employed, rather than solid-state microtransducers, which are more reliable but too expensive for routine use[57]. Vector volume manometry has been developed to provide a 3-D view of the anal sphincter, but its clinical utility is still under evaluation[58]. Recently, the high-resolution manometry has been shown to provide grater details than water-perfused manometry, but it is still in the experimental stage[59].

The reproducibility of anal manometry is high[60], but its reliability depends on the operator's experience, and its utility is limited by the absence of standardised protocols[61,62] and of data from large numbers of normal subjects[57,63]. Moreover, most of the parameters measured by anorectal manometry (anal canal pressure, sensory thresholds) are influenced by sex and age[64].

Should anorectal manometry always be performed in patients with chronic constipation and/or obstructed defecation? The main indication for anorectal manometry is the presence of obstructed defecation[65,66]. It should also be performed in patients who do not improve with first-line treatments for chronic constipation (a defecation disorder is reported in 51% of such patients)[12,67].

Anorectal manometry, together with other tests, can provide essential information on the rectoanal function defects involved in the physiopathology of obstructed defecation, including increased pressure in the anal canal, rectoanal inhibitory reflex defects, lower rectal sensitivity, and increased rectal compliance[7]. Level II evidence, Grade B recommendation.

Is anorectal manometry sufficient for the diagnosis of obstructed defecation? There is no gold standard for the diagnosis of obstructed defecation, and manometry alone does not provide sufficient grounds for the diagnosis. A comprehensive evaluation of anorectal function is necessary and should include tests to evaluate various aspects of defecation, including the balloon expulsion test, imaging techniques, and perhaps electromyography, in addition to manometry[7]. Defecography can evaluate the morphological and dynamic factors of defecation; anorectal manometry measures anorectal sensitivity and motility; and electromyography can provide information on electrical activity in the external anal sphincter muscle during straining. The balloon expulsion test can confirm the diagnosis of obstructed defecation[68,69]. Level II evidence, Grade B recommendation.

Anorectal manometry consists of several tests; which of them are most useful in the diagnosis of obstructed defecation? At a minimum, the following tests should be performed[70]: resting anal pressure, squeezing pressure,

Table 4 Interpretation of the manometric data

Test	Parameter evaluated	Interpretation
Resting pressure	IAS (70% of resting pressure) and EAS (30% of resting pressure)	*P* increased: Hypertonic sphincters (IAS and/or EAS). Oral nitroglycerin can identify the sphincter involved because it relaxes IAS, but not EAS
Squeeze pressure	EAS	The fatigue rate index can be calculated based on the pressure and duration of the contraction. However, the usefulness of the test in both constipated and incontinent patients is disputed[112,113]
Rectoanal inhibitory reflex	IAS relaxation during rectal inflation	Absent: Possible hirschsprung; If present with elevated volume inflation: Megarectum[57]
Rectal sensitivity	Rectal sensory function at different volumes	Elevated sensory thresholds may be linked to changes in rectal biomechanics (megarectum) or to afferent pathway dysfunction[114,115]
Rectal compliance	Mechanical rectal function	Increased compliance: megarectum[57]
Attempted defecation	Synchronisation between the increase in rectal pressure and the decrease in anal pressure during attempts to defecate	Three types of dysfunction may be detected[65]: Type 1: Adequate rectal *P* increase but associated with anal *P* increase; Type 2: Inadequate rectal *P* increase associated with anal *P* increase or inadequate anal *P* decrease; Type 3: Adequate rectal *P* increase but inadequate anal *P* decrease

IAS: Internal anal sphincter; EAS: External anal sphincter; *P*: Pressure. Modified from Azpiroz *et al*[57].

rectoanal inhibitory reflex, rectal sensations (first sensation, maximum tolerable volume), rectal compliance, and rectal and anal pressure during attempted defecation (straining)[57,71]. The results will vary with age and sex; normal values based on a large cohort of healthy individuals are still lacking[57]. Level Ⅲ evidence, Grade C recommendation.

How should I interpret the results of anorectal manometric tests for obstructed defecation? The interpretation of the manometric data in clinical and physiopathologic terms is summarised in Table 4.

Are there typical manometric abnormalities in chronic constipation and/or obstructed defecation? The main abnormality in obstructed defecation is absent or inadequate relaxation of the anal sphincter, sometimes associated, paradoxically, with contraction during straining (dyssynergia). Obstructed defecation may also be associated with absent or inadequate rectal pressure[65,67]. The "defecation index", or the ratio of maximal rectal pressure to minimal anal residual pressure[65], quantifies recto-anal coordination during attempted defecation. Abnormalities have also been reported in anal canal resting pressure, anal canal squeezing pressure (external anal sphincters exhaustion), rectoanal inhibitory reflex (RAIR), rectal sensitivity, and compliance. Level Ⅲ evidence, Grade C recommendation.

How can anorectal manometry be used to guide choices regarding therapy? Anorectal manometry can shed light on the physiopathologic mechanisms of obstructed defecation and help to develop a pelvic floor rehabilitation program for the patient[72]. It should be included in the pre-operative evaluation when a surgical reduction in rectal capacity is planned[73,74]. If RAIR is absent, Hirschsprung disease should be suspected. Elevated sensory thresholds, increased compliance, and rectal motor dysfunction are frequent in constipated patients[75,76] and can be treated with sensory retraining biofeedback therapy, based on sensory values obtained by means of anorectal manometry[77]. The results of biofeedback and electrical stimulation can be measured with anorectal manometry, and in fact, a reduction in rectal sensory thresholds has been demonstrated[78,79]. Level Ⅲ evidence, Grade C recommendation.

Balloon expulsion test

The balloon expulsion test is a simple, inexpensive test that can identify patients with abnormal defecation.

What is the usefulness of the balloon expulsion test to diagnose dyssynergic defecation? The balloon expulsion test has not yet been standardised; the filling volume of the balloon, the position of the patient, and the expulsion time have differed in various studies.

Trials including healthy controls. Two trials performed the test with the patient seated and the balloon filled with 50 mL of water; 59%[67] and 25%[80] of the constipated patients and 16%[67] of the controls were unable to expel the balloon within 5 min.

In the third trial[81], the expulsion time was not specified, and the test was performed with a balloon filled with different volumes of water; 100% of patients with idiopathic megarectum, 53% of patients with a normal colonic transit time, 36% of patients with a slow transit colonic time, and 7% of controls were unable to expel the balloon.

Other trials. Some trials[82-84] have assessed patients with pelvic floor dyssynergia and have reported positive results in 23% to 57% of patients. However, different methods were used, so the results are not comparable.

In one trial[85], the balloon was filled to the point at which the need to defecate was triggered, and the balloon had to be expelled within one minute. The authors concluded that a negative test is useful "to identify patients who do not have dyssynergia" and resulted in a specificity of 89%, a sensibility of 88%, a positive predictive value of 67%, and a negative predictive value of 97%.

The balloon expulsion test cannot be used as a gold standard for the diagnosis of "dyssynergic defecation" and should be integrated with other anorectal tests. Level

Ⅲ evidence, Grade C recommendation.

Colonic manometry

Slow transit constipation (STC) is characterised by prolonged colonic transit, generally measured in terms of intestinal transit time using radiopaque markers[86]. Colon manometry shows the daily patterns of bowel activity, identifying high amplitude waves, which correspond to mass movement in the intestine, and low amplitude waves[87,88]. Manometric studies[89,90] in STC patients have shown that propagating activity may be altered in frequency, amplitude and duration; segmental activity can be maintained or drastically lost, but there is, above all, a subversion of the periodicity of motor activity in the colon. Recently, a new method of evaluating propagated motor activity or "propagating sequences" has been developed, but it is still in the experimental stage[91].

What are the clinical applications of colonic manometry? In patients with serious STC symptoms, colonic manometry can be helpful in the diagnosis and in decisions regarding therapy (whether conservative or surgical)[61]. Level Ⅳ evidence, Grade C recommendation.

How should colon manometry be performed in patients with slow transit constipation? In the clinical setting, the bisacodyl test should be used. This procedure tests the stimulation of residual colonic propulsive activity, and it can be used to identify the subgroup of patients with severe slow transit constipation or "inertia coli", one incontrovertible indication for total colectomy[92-94]. Thus, colonic manometry may help to diagnose an underlying myopathy or neuropathy and to differentiate slower transit due to neuromuscular function[95]. Level Ⅴ evidence, Grade C recommendation.

Pathologies of the colon

The pathophysiology of slow transit constipation is not known[96], but there is evidence to indicate that certain subtypes of idiopathic constipation are secondary to visceral neuropathy[97-99], such as aberrant regulation of the nervous enteric system or parasympathetic alterations[100].

What STC alterations can be verified on histology? Qualitative and quantitative alterations in the enteric nervous system can be observed on histology, from alterations in the neurotransmitters to the loss of argyrophilic neurons and neurofilaments and myenteric plexus hypoganglionosis[101]. More recently, reductions in the number of cells of Cajal have been described[102,103]. Level Ⅲ evidence, Grade C recommendation.

Is an endoscopic biopsy sufficient, or is a full-wall thickness biopsy necessary? Endoscopic biopsies only provide information on the mucosa and cannot detect other histological alterations; therefore, they are not useful in the pathogenetic evaluation of STC. Given the nature of the alterations, it is necessary to conduct biopsies that reach the muscle layer.

What is the role of the suction biopsy in STC? Suction biopsy is the gold standard for the diagnosis of intestinal neurodysplasia, particularly in children. In the differential diagnosis, four biopsy samples should be taken between 2 cm and 10 cm from the pettinea linea[104]. The histological findings can distinguish STC from Hirschsprung disease and contribute to the diagnosis of intestinal neurodisplasia and other degenerative diseases of the colon (i.e., amyloidosis, desmosis, elastosis)[105]. Level Ⅱ evidence, Grade B recommendation.

What is the role of immunohistochemistry? Immunohistochemistry is the main tool for the histological evaluation of nerves and connective tissues. There are no clinical studies in the literature that focus on this particular examination. Pathologists recommend that immunohistochemical analysis be undertaken in suspected cases of STC[106].

The Consensus Committee therefore recommends that immunohistochemistry be performed to document patterns of slow transit constipation.

Gastrojejunal manometry

There is evidence that slow transit constipation subtends diffuse enteric neurological involvement, probably of the myenteric plexus and, above all, the system of interstitial cells of Cajal[107]. Various studies have highlighted different ileal dysfunctions: in two retrospective analyses, 20.6% of patients with chronic constipation showed gastrojejunal abnormalities[61,108]. Cardiovascular tests for dysautonomia, which are widely used in diabetic neuropathy, are not applicable in the diagnostic workup of slow transit constipation.

The most meaningful test for myopathic or neuropathic involvement (especially in the pre-surgical evaluation) in patients with chronic constipation is gastrojejunal manometry, as stated recently by the American Neurogastroenterology and Motility Society[109-111].

What are the clinical applications of gastrojejunal manometry? Gastrojejunal manometry can be used to analyse antro-duodenal activity and fasting jejunal motility, particularly in patients with autonomic dysfunctions, such as diabetic neuropathy. In a recent study of 61 subjects undergoing gastrojejunal manometry, all STC patients and 94% of those patients with normal transit constipation exhibited alterations in small bowel motility in the postprandial and fasting phases, but there were no significant differences between the two groups[109].

When should gastrojejunal manometry be performed in STC patients? In cases of STC, gastrojejunal manometry is recommended before surgery[93,109]. Level Ⅲ evidence, Grade C recommendation.

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TOPIC HIGHLIGHT

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Adjuvant and neoadjuvant treatment in pancreatic cancer

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Abstract

Pancreatic adenocarcinoma is one of the most aggressive human malignancies, ranking 4th among causes for cancer-related death in the Western world including the United States. Surgical resection offers the only chance of cure, but only 15 to 20 percent of cases are potentially resectable at presentation. Different studies demonstrate and confirm that advanced pancreatic cancer is among the most complex cancers to treat and that these tumors are relatively resistant to chemotherapy and radiotherapy. Currently there is no consensus around the world on what constitutes "standard" adjuvant therapy for pancreatic cancer. This controversy derives from several studies, each fraught with its own limitations. Standards of care also vary somewhat with regard to geography and economy, for instance chemo-radiotherapy followed by chemotherapy or *vice versa* is considered the optimal therapy in North America while chemotherapy alone is the current standard in Europe. Regardless of the efforts in adjuvant and neoadjuvant improved therapy, the major goal to combat pancreatic cancer is to find diagnostic markers, identifying the disease in a pre-metastatic stage and making a curative treatment accessible to more patients. In this review, authors examined the different therapy options for advanced pancreatic patients in recent years and the future directions in adjuvant and neoadjuvant treatments for these patients.

Key words: Pancreatic ductal adenocarcinoma; Adjuvant; Neoadjuvant; Fluorouracil; Gemcitabine

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive human malignancies, ranking 4th among causes of cancer-related death in the Western world[1].

Unlike most of the more frequent causes of cancer mortality (lung, colon, prostate and breast cancers) whose death rates are declining, the death rate for pancreatic cancer is relatively stable.

The poor prognosis is reflected by a median survival of 5-8 mo and a 5-year survival of less than 5% when all stages are combined[1-3].

PDAC is characterized by a rapid disease progression

and absence of specific symptoms, largely precluding an early diagnosis and curative treatment[3,4].

In most cases, PDAC is already locally advanced at time of diagnosis and only approximately 10%-20%[1,5] of patients are considered candidates for curative resection. The majority of patients (50%-60%) present with metastatic disease, and thus palliative chemotherapy remains the only option for almost all of these patients[6]. Owing to the high recurrence rate, surgical PDAC patients require adjuvant chemotherapy with or without radiotherapy providing a 5-year survival rate of 15%-25%[7] (Table 1).

Due to the described overall prognosis for all pancreatic cancer patients, systemic chemotherapy, radiation therapy or a combination of both is used following surgical resection (adjuvant therapy) and also prior to the tumor resection (neoadjuvant therapy) to improve cure rates.

Although the benefit of adjuvant and neoadjuvant therapy has been improved in recent years, the best choice of treatment modality still remains highly controversial.

The objective of this review is to examine therapies received by advanced pancreatic cancer patients in recent years and to examine the principal chemotherapeutic agents or molecular-targeted therapies useful for clinicians.

Table 1 Staging of pancreatic cancer

Stage	TNM classification	Clinical classification	5-year percent survival (mo)
Stage 0	TisN0M0	Resectable	
Stage Ⅰ A	T1N0M0	Initial	31.4
Stage Ⅰ B	T2N0M0		27.2
Stage Ⅱ A	T3N0M0		15.7
Stage Ⅱ B	TXN1M0	Locally advanced	7.7
Stage Ⅲ	T4NXM0		6.8
Stage Ⅳ	TXNXM1	Metastatic	2.8

Tis: Cancer *in situ*: T: Size and/or extent of invasion; N: Extent of lymph node involvement; M: Whether distant metastases are present.

ADJUVANT THERAPY

In an effort to improve the outcome in patients undergoing potentially curative resection, systemic chemotherapy (Table 2), radiotherapy or a combination of both have been applied following surgery.

SYSTEMIC THERAPY

Chemotherapy

The first randomized controlled trial of adjuvant therapy in pancreatic cancer was designed by the Gastrointestinal Tumor Study Group, which concluded that treatment with 5-fluorouracil (5-FU) plus radiation followed by two years of weekly 5-FU maintenance provided better outcomes than surgery alone[8]. Although this trial was criticized for many reasons, it served to establish 5-FU as the only standard adjuvant therapy for many years in pancreatic cancer. Different drugs and combinations have emerged and been incorporated for the best treatment of these patients (Table 2).

5-FU: 5-FU is a thymidylate synthase inhibitor that blocks the synthesis of pyrimidine thymidine, a nucleotide required for DNA replication.

5-FU had been considered the only chemotherapeutic option for about 20 years until the registration of gemcitabine. Several trials conducted in the late 1970s and early 1980s demonstrated that adjuvant chemotherapy using bolus 5-FU therapy conferred a survival benefit in patients with resected pancreatic cancer[8].

Different studies in the last years have demonstrated a survival benefit from six months of postoperative leucovorin-modulated 5-FU in patients with resected pancreatic cancer, compared to those receiving no adjuvant chemotherapy (median overall survival 19.7 mo *vs* 14 mo respectively, statistically significant)[9-11].

Although for locally advanced and metastatic patients this drug leads to an improved survival compared to the best supportive care[12,13], the combination of 5-FU with other drugs such as doxorubicin or mitomycin did not prove superior to the antimetabolite alone. Similar results were obtained comparing single agent 5-FU to 5-FU plus cyclophosphamide, methotrexate and vincristine[14] as the combination did not offer a survival advantage over 5-FU alone.

Only the combination of 5-FU/irinotecan/oxaliplatin (FOLFIRINOX) has been associated with a high objective response rate based on imaging study, and this finally is the preferred regimen for patients who have good performance status and a normal serum bilirubin level[15].

In the last years, new fluoropyrimidines that mimic the effect of a continuous infusion of 5-FU have been approved. One of the most common but not available in all countries is S-1, an orally active fluoropyrimidine, with favorable antitumor activity in gemcitabine-refractory disease[16,17].

Capecitabine: Capecitabine is an orally administered fluoropyrimidine that is absorbed intact through the intestinal wall and then converted to 5-FU in three sequential enzymatic reactions: carboxylesterases, cytidine deaminase and thymidine phosphorylase. The last enzyme is present at consistently higher levels in tumor rather than normal tissue, thereby providing the basis for enhanced selectivity and better tolerability[18]. The efficacy of capecitabine in monotherapy was shown with high clinical response rate (24%) but low objective response (7%)[19]; however, no advantage using capecitabine in monotherapy over gemcitabine alone has been demonstrated.

Gemcitabine: The development of gemcitabine may be considered a major advance in the treatment of pancreatic cancer. This drug is a difluorinated analog of deoxycytidine. As a prodrug, gemcitabine must be phosphorylated by cytoplasmic and mitochondrial enzymes to its active metabolites, gemcitabine diphosphate and gemcitabine triphosphate. The cytotoxic effect of this drug is attributed to a combination of two actions of the

Table 2 Mode of action of principal drugs used in pancreatic cancer

Agent	Mode of action
5-FU	5-FU is a folate antimetabolite that forms a ternary complex involving 5-fluoro-2-deoxyuridine-5-monophosphate, thymidylate synthase, and 5,10-methylene THF. The formation of this complex thereby inhibits thymidylate synthase activity, which subsequently depletes intracellular thymidylate levels and ultimately suppresses DNA synthesis Also, two metabolites of 5-FU, 5-fluoro-2-deoxyuridine-5-triphosphate and 5-fluorouridine-5-triphosphate, can be incorporated into DNA and RNA, respectively, resulting in DNA instability and interfering with RNA processing and function
Gemcitabine (Gemzar®)	Gemcitabine is an S-phase nucleoside analogue (diflourodeoxycytidine) that is phosphorylated to difluorodeoxycytidine triphosphate by deoxycytidine kinase. Gemcitabine also stimulates deoxycytidine kinase and inhibits both ribonucleotide reductase and deoxycytidine monophosphate deaminase. Gemcitabine triphosphate is incorporated into nascent DNA to inhibit DNA synthesis
Capecitabine (Xeloda®)	Capecitabine an oral, tumor-selective fluoropyrimidine carbamate that is sequentially converted to 5-FU by three enzymes located in the liver and in tumors. The final step is the conversion of 5'-deoxy-5-fluorouridine to 5-FU by thymidine phosphorylase in tumors
Platinum analogues	Platinum forms adducts with DNA inhibiting transcription and replication causing cell death. Oxaliplatin is a third-generation platinum analogue (a diaminocyclohexane platinum derivative) that may have activity in tumors resistant to cisplatin or carboplatin and may have an additive/synergistic activity in doublet or triplet therapy
Taxanes	The taxanes include paclitaxel and docetaxel (Taxotere®) and are semi-synthetic microtubule inhibitors with a different mechanism of action from the vinca alkaloids. Taxanes bind to β-tubulin, promoting microtubule assembly and preventing depolymerisation thus forming stable non-functional complexes and inhibiting the function of the mitotic spindle; This results in cell cycle arrest and increased sensitivity to radiation
Irinotecan (CPT11, Camptosar®)	Irinotecan is a topoisomerase I inhibitor that impedes the DNA helix tortional stress-relieving activity of DNA topoisomerases and also prevents their release from the DNA thus prompting apoptosis

5-FU: 5-fluorouracil.

diphosphate and triphosphate nucleosides, which leads to inhibition of DNA synthesis[20,21].

The first pivotal trial found that gemcitabine is more effective than 5-FU in alleviation of some disease-related symptoms in patients with advanced, symptomatic pancreatic cancer, conferring a modest survival advantage over treatment with 5-FU. As the treatment with gemcitabine was associated with significant clinical response and better survival, this drug was approved for first-line therapy of metastatic pancreatic cancer. This pivotal phase Ⅲ trial demonstrated improvement in median overall and 1-year survival compared to 5-FU (5.7 mo *vs* 4.4 mo and 18% *vs* 2%, respectively)[22].

Many phase Ⅱ studies have demonstrated the efficacy of gemcitabine combination treatments, but not all of the phase Ⅲ trials confirmed the improvement in overall survival (OS) of gemcitabine-based regimens compared to gemcitabine alone. However, an improvement in six-month survival was seen by combining gemcitabine-fluoropyrimidine analogues and gemcitabine-platinum analogues, as demonstrated in the meta-analysis of Heinemann and colleagues[2].

Due to the results obtained in monotherapy, gemcitabine has been combined with many other active cytotoxic agents including 5-FU, cisplatin, docetaxel, oxaliplatin and irinotecan, in an attempt to improve the response in pancreatic cancer patients and each will be discussed here separately.

Gemcitabine and 5-fluorouracil: Based on the complementary pharmacology of their mechanisms of action, the combination of 5-FU and gemcitabine has been evaluated in phase Ⅰ, Ⅱ and Ⅲ trials. Finally, phase Ⅲ trials showed that there is no significant improvement in median OS and median progression-free survival when evaluating the combined regimen compared to that of gemcitabine alone[23-26].

Gemcitabine and capecitabine: Different phase Ⅲ trials have shown that patients who received gemcitabine and capecitabine compared to gemcitabine alone have a significant improvement in survival[27,28].

These data and the meta-analysis performed by these authors suggest that the combination of gemcitabine plus capecitabine should be considered a standard first-line option for locally advanced and metastatic pancreatic cancer.

Gemcitabine and platinum combinations: Since gemcitabine enhances the formation of cisplatin-DNA adducts, an effect that may be due to suppression of nuclear excision repair by gemcitabine, and the platinum may augment the incorporation of gemcitabine triphosphate into DNA[29], the gemcitabine and platinum combination has been assessed in different trials.

Although in preclinical studies the combination of gemcitabine and cisplatin is synergistic, at least three phase Ⅲ trials comparing gemcitabine to the combination of gemcitabine plus cisplatin showed no significant survival advantage for this approach[30-32]. Furthermore, the combination of gemcitabine and platins has not shown improvement in terms of response and is not a considered option for pancreatic cancer patients.

Gemcitabine and irinotecan: As irinotecan (a topoisomerase inhibitor) had minimal clinical activity in patients with advanced pancreatic cancer, combined therapy with gemcitabine is not recommended[33,34] and in some

cases the combination could lead to major toxicity.

Gemcitabine and taxanes: Antitumoral action of taxanes is due to their mechanism of microtubule stabilization and consequently to cell cycle arrest. The association of gemcitabine with paclitaxel or docetaxel in advanced pancreatic patients was studied in different trials and has shown encouraging response rates[35,36]. A phase Ⅲ trial has not yet been completed. Thus, whether this regimen represents an improvement over gemcitabine alone is unclear.

The available data suggest that if there is a benefit to gemcitabine combination therapy compared to gemcitabine alone, it is modest and best documented for capecitabine plus gemcitabine. Today, only gemcitabine alone and the combination of gemcitabine plus capecitabine represent good options for initial therapy.

In summary, adjuvant fluorouracil has been shown to be of benefit for patients with resected pancreatic cancer but gemcitabine is the most effective agent in advanced disease. Compared with the use of fluorouracil, gemcitabine does not result in improved overall survival in patients with completely resected pancreatic cancer[37].

Combined therapies

As compared with gemcitabine, FOLFIRINOX was associated with a survival advantage and had increased toxicity. FOLFIRINOX is an option for the treatment of patients with metastatic pancreatic cancer and good performance status[38].

Targeted molecular therapy

Based on the biological properties of pancreatic cancer, new systemic therapies have been tried. The most common molecular targets have been epidermal growth factor receptor (EGFR)/KRAS, human epidermal growth factor receptor type 2 (HER2) and vascular endothelial growth factor (VEGF), as these genes are overexpressed or mutated in pancreatic tumors.

Targeting EGFR: Currently, there are two approaches targeting the EGFR system: monoclonal antibodies (i.e., cetuximab/Erbitux®) and small molecule tyrosine kinase inhibitors. In spite of promising preclinical trials, cetuximab as monotherapy, or in combination with other cytotoxic agents such as gemcitabine or with radiotherapy, has failed to improve the outcome of PDAC patients[39,40].

Up to now, the only EGFR targeting demonstrating a clinical benefit is erlotinib (Tarceva®, OSI 774), a tyrosine kinase inhibitor that inhibits ErbB-1 phosphorylation. One phase Ⅲ trial of erlotinib with gemcitabine was able to show at least a small gain in the survival of patients with advanced PDAC[41]. Although erlotinib obtained Food and Drug Administration approval and access in clinical application in 2005, the therapeutic benefit for patients with advanced PDAC remains poor.

Targeting HER2: In several studies, HER-2 overexpression in pancreatic cancer has been reported to vary widely (10%-82%)[42,43] and it does not correlate with poor prognosis[44]. Although studies in a mouse model have shown that combination of anti-HER2 antibodies (i.e., trastuzumab) and other chemotherapy may be effective for HER2-overexpressing pancreatic cancer patients[45], the clinical significance is uncertain. A phase Ⅱ clinical trial of trastuzumab for pancreatic cancer has been conducted and showed only 6% response to combined therapy with trastuzumab and gemcitabine in patients with metastatic pancreatic cancer, which is not superior to therapy with gemcitabine alone[46].

Currently, anti-Her2 therapy is experimental and still under investigation for the treatment of pancreatic cancer.

Targeting VEGF: A phase Ⅲ trial concluded that there is no benefit for the addition of bevacizumab to gemcitabine *vs* gemcitabine alone and *vs* gemcitabine and cetuximab[47].

Also some studies have failed to demonstrate a benefit for adding axitinib (an oral inhibitor of VEGF receptors 1, 2 and 3) to gemcitabine[48,49]. Currently, the anti-VEGF approach is not recommended in pancreatic cancer.

Hormonal therapy

Tamoxifen and octreotide are not indicated in metastatic pancreatic cancer because both of them have failed to demonstrate any survival advantage for treated patients[50,51].

RADIOTHERAPY

The use of adjuvant radiotherapy for pancreatic cancer is controversial and the role of radiation therapy continues to be investigated. Currently, the addition of radiotherapy depends on the country in which a patient is being treated[52].

Chemo-radiotherapy followed by chemotherapy is considered the optimal therapy in North America (Gastrointestinal Tumor Study Group; Radiation Therapy Oncology Group) while chemotherapy alone is the current standard in Europe (European Study Group for Pancreatic Cancer; Charité Onkologie)[10,53,54].

The rationale for adjuvant radiotherapy for pancreatic cancer is to improve loco-regional control. Modern radiation delivery techniques, such as intensity-modulated radiation therapy or image-guided and stereotactic body radiation therapy, permit dose escalation in order to reduce normal tissue toxic effects and simultaneously deliver increased doses of radiation to affected areas[55,56]. It is clear that breakthroughs in the treatment of this devastating disease will come mostly from advances in systemic therapy, so radiotherapy should not be abandoned, but rather, intensified.

Intraoperative radiotherapy has also been considered, since local recurrence rates are very high. In general, intraoperative radiotherapy can slightly increase survival rates among patients with pancreatic cancer in localized

stages. There is no clear evidence to indicate that intraoperative radiotherapy is more effective than other therapies in treating pancreatic cancer in locally advanced and metastatic stages[57].

CHEMORADIOTHERAPY

Some studies demonstrated improved survival when radiotherapy was combined with 5-FU chemotherapy compared with radiotherapy alone, in patients with locally advanced unresectable pancreatic cancer[58]. This combined therapy has been applied to patients undergoing RO resection to improve surgical cure rate.

In locally advanced pancreatic cancer, recent evidence using modern radiotherapy techniques and dosing suggests a continued role for radiotherapy. In both resected and unresected disease, further studies are needed to define optimal radiation dose, field size, and technique, and to assess the effect of radiotherapy not only on survival, but also on local disease control and quality of life[59].

NEOADJUVANT THERAPY

The low rate of resectability and the poor outcomes following pancreaticoduodenectomy have led to the investigation of preoperative and postoperative therapies to identify those patients who are not candidates for surgery and who could benefit from neoadjuvant chemotherapy and/or radiotherapy.

The initial reports using radiation therapy with or without 5-FU did not demonstrate an obvious improvement in either resectability or overall survival[60,61]. Subsequent studies improved the treatment by increasing radiotherapy dose, adding intraoperative radiotherapy and using combined chemotherapy. The drugs tested were mitomycin, 5-FU, 5-FU and cisplatin, and paclitaxel, but their efficacy remains uncertain[62-64].

Subsequent reports used gemcitabine-based chemotherapy which provided an enhanced local effect, although with potentially more toxicity than 5-FU-based regimens. Gemcitabine has also been combined with radiotherapy and cisplatin[65,66].

Currently, neoadjuvant radiation is associated with improved survival in patients with resectable pancreatic cancer[67] but chemotherapy alone without radiotherapy is beginning to be studied and the experience is limited[68,69].

ADJUVANT *VS* NEOADJUVANT THERAPY

Although the median survival times reported from some uncontrolled trials of neoadjuvant therapy compare favorably to those reported with adjuvant therapy approaches[65,70,71], the question as to whether preoperative therapy is better than postoperative therapy is uncertain as there are no randomized trials comparing the two approaches.

One advantage of neoadjuvant therapy is that it avoids the morbidity of pancreaticoduodenectomy in patients who have occult, micrometastatic disease that becomes evident during therapy. A second advantage is that in patients undergoing surgery, prolonged recovery prevents the delivery of postoperative adjuvant chemotherapy in about a quarter of them[72].

Recent studies have shown that neoadjuvant therapy is associated with a lower rate of lymph node positivity and improved overall survival and should be considered an acceptable alternative to the surgery-first paradigm in operable pancreatic cancer[73].

SECOND-LINE THERAPY

There are few trials of second-line therapy in patients who have failed chemotherapy, and there is no widely accepted standard of care.

For patients who retain a good performance status after failing initial gemcitabine therapy, benefit has been suggested from a second-line therapy based on oxaliplatin/fluoropyrimidine combination such as 5-FU and oxaliplatin[65,70,71]. Other oxaliplatin combinations are also acceptable with the agents gemcitabine, irinotecan or capecitabine[74-76].

There are no data for patients who fail initial 5-FU and oxaliplatin, but a reasonable option is gemcitabine as monotherapy.

CONCLUSION

All the treatment options examined in this review demonstrate and confirm that advanced pancreatic cancer is among the most complex cancers to treat.

Currently there is no consensus regarding the optimal management of patients after resection of an exocrine pancreatic cancer, and the approach is different in Europe and in the United States. Most European clinicians use chemotherapy alone after resection of a pancreatic neoplasm. The American approach more often includes chemoradiotherapy as well as adjuvant chemotherapy.

Although it is mainly accepted that a 6-mo course of systemic chemotherapy with gemcitabine or 5-FU should be part of any adjuvant treatment, there is no single adjuvant regimen of chemotherapy or chemoradiotherapy that can claim unequivocal superiority over others. Among these options there are no differences in outcome but fewer side effects occur with gemcitabine, and this is nowadays the preferred regimen.

Based on current data, it is clear that treatment with gemcitabine or 5-FU results in a median survival of just a few months[77,78]. The limitation of this treatment is mainly due to the profound resistance of PDAC cells towards anti-cancer drugs, emerging from the efficient protection against chemotherapeutic drugs by an altered balance of pro- and anti-apoptotic proteins which results in a markedly reduced apoptotic responsiveness[79,80].

Currently there are around 1070 clinical trials focusing on studying new biomarkers, different drug combinations and vaccines designed for pancreatic cancer (www.clinicaltrial.gov).

Regardless of these efforts in adjuvant and neoadjuvant therapy, the major goal to combat PDAC is to find diagnostic markers, identifying the disease in a pre-metastatic stage and making a curative treatment accessible to more patients. Given an earlier diagnosis, surgical interventions together with adjuvant radio/chemotherapy are the most promising options. Considering such evidence, the urgent need for an individualized and more effective adjuvant therapy is evident.

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REVIEW

2011 update on esophageal achalasia

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Abstract

There have been some breakthroughs in the diagnosis and treatment of esophageal achalasia in the past few years. First, the introduction of high-resolution manometry with pressure topography plotting as a new diagnostic tool has made it possible to classify achalasia into three subtypes. The most favorable outcome is predicted for patients receiving treatment for type Ⅱ achalasia (achalasia with compression). Patients with type Ⅰ (classic achalasia) and type Ⅲ achalasia (spastic achalasia) experience a less favorable outcome. Second, the first multicenter randomized controlled trial published by the European Achalasia Trial group reported 2-year follow-up results indicating that laparoscopic Heller myotomy was not superior to endoscopic pneumatic dilation (PD). Although the follow-up period was not long enough to reach a convincing conclusion, it merits the continued use of PD as a generally available technique in gastroenterology. Third, the novel endoscopic technique peroral endoscopic myotomy is a promising option for treating achalasia, but it requires increased experience and cautious evaluation. Despite all this good news, the bottom line is a real breakthrough from the basic studies to identify the actual cause of achalasia that may impede treatment success is still anticipated.

Key words: Esophageal achalasia; High resolution manometry; Endoscopic pneumatic dilations; Minimally invasive surgical procedures; Peroral endoscopic myotomy

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INTRODUCTION

Achalasia is the only primary motor disorder of the esophagus with a well-understood pathophysiology. It affects both sexes and all races equally[1-3]. Achalasia involves the selective loss of inhibitory neurons in the myenteric plexus, leading to the production of vasoactive intestinal polypeptide (VIP), nitric oxide (NO), and inflammatory infiltrate responsible for abnormal lower esophageal sphincter (LES) dysfunction. An unopposed excitation of the LES causes its dysfunction or failure to relax in response to swallowing[3,4].

Clinical presentations include dysphagia for both liquid and solid food, and food regurgitation may be severe enough to produce pulmonary complications such as cough or aspiration pneumonia. Weight loss usually

occurs as a result. The diagnosis of achalasia is made on the basis of the results of barium esophagography, esophageal manometry, and endoscopy. Typical radiological signs are classic "bird-beak" of the gastroesophageal junction, with atonia and a dilated esophageal body observed by barium ingestion and fluoroscopy. Manometry is still the standard diagnostic test for achalasia. The basic criteria required for diagnosis of achalasia include the absence of relaxation of the LES or abnormal swallowing relaxation of the LES and the absence of peristalsis in the esophageal body. However, an endoscopic examination, preferably endoscopic ultrasonography or computed tomography, is always necessary to distinguish primary achalasia from the secondary form[5], in cases of possible malignancy[6]. A greater risk of esophageal squamous cell carcinoma among achalasia patients is well established. Male achalasia patients have substantially greater risks for both squamous cell carcinoma and adenocarcinoma of the esophagus[7]. Recent advances in the diagnosis of esophageal achalasia by using updated high-resolution manometry (HRM) with pressure topography plotting have made it possible to classify achalasia into three subtypes. Furthermore, it is now possible to predict the outcome of each type of achalasia[8-10]. In this paper, the implications of the recent findings in the diagnosis and treatment of esophageal achalasia are reviewed and discussed.

PATHOGENESIS

The mechanisms responsible for the selective loss of inhibitory neurons in the myenteric plexus that produces VIP, NO and inflammatory infiltrate responsible for abnormal LES dysfunction is still not well understood[11]. However, specimens taken from autopsy or myotomy have shown the histological damage of the esophageal myenteric plexus and an inflammatory response consisting of CD3/CD8-positive cytotoxic T lymphocytes, variable numbers of eosinophils and mast cells, loss of ganglion cells, and neurofibrosis[12,13]. These events occur even at the early inflammatory stages of achalasia, and the underlying cause has not yet been identified. Previous studies have implicated hereditary, neurodegenerative, genetic, infectious, and autoimmune mechanisms. The most acceptable hypothesis suggests that achalasia may be caused by viral and autoimmune factors, leading to the inflammatory changes and damage to the myenteric plexus.

Achalasia patients with certain genetic backgrounds are reported to develop an autoimmune reaction and hence the production of autoantibodies that cause chronic inflammation and destruction of inhibitory neurons[12,13]. In addition, infiltration of the myenteric ganglia with CD3/CD8-positive lymphocytes that express activation markers, IgM antibodies, and evidence of complement activation have been observed within the myenteric ganglia[14-16]. Moreover, antibodies against myenteric neurons have been detected in the serum of patients with esophageal achalasia; especially in those with a specific human leukocyte antigen genotype (DQA1 × 0103 and DQB1 × 0603 alleles)[17-19]. The above evidence indicates that an autoimmune mechanism likely plays an important role in achalasia. However, the trigger for the destructive autoimmune events is unknown. So far, viral infection is believed to be the main cause. Some possible causative infections are varicella zoster and measles viruses[16,20-22]. However, some may argue that antineuronal antibodies have also been reported in the serum of gastroesophageal reflux disease patients and even in healthy individuals, suggesting that these antibodies may simply result from tissue damage secondary to inflammation[19]. The other unanswered question is why only neurons in the esophagus and LES are destroyed. The results of some studies have led to the hypothesis that neurotropic viruses, especially those with a predilection for squamous epithelium, could be involved; however, these findings have been inconsistent[16,20-23].

Although there have been many excellent basic studies, the presence of viral antibodies in the serum of patients has been an inconsistent finding. The method to verify the actual cause of achalasia that may impede treatment success is yet to be determined.

DIAGNOSIS

The diagnosis of achalasia is on the basis of the results of gastroscopy, manometry, and timed barium esophagography. Pseudoachalasia is always excluded by either computed tomography or endoscopic ultrasonography. Since the emergence of HRM with pressure topography plotting, esophageal achalasia can be classified into three subtypes[8-10]. In type I achalasia (classic achalasia), impaired LES relaxation but no significant pressurization within the esophageal body is observed. In type Ⅱ achalasia (with compression), swallowing of water causes rapid panesophageal pressurization. This may exceed LES pressure, causing the esophagus to empty. Type Ⅲ achalasia (spastic achalasia) is also associated with rapidly propagated pressurization; however, the pressurization is attributable to an abnormal lumen, obliterating contraction. HRM can be used to predict the outcome of each type of achalasia. Patients in whom HRM shows type Ⅱ achalasia (esophageal pressurization) are more likely to respond to therapies such as pneumatic dilation (PD), heller myotomy (HM), and botulinum toxin (BT) (overall, 70%-100%), compared to those with type I (overall, ⩾ 50%-63.3%) and type Ⅲ (overall, about 30%) achalasia[8,10]. HRM may play an increasingly important role in the diagnosis of esophageal achalasia in the future, especially when the technique becomes more affordable.

CURRENT TREATMENT OPTIONS

Currently, there is no cure for esophageal achalasia. The only available therapeutic options are to loosen the LES and treat the symptoms[24]. However, the advantages of each option must be considered in the patients.

Pharmacological management such as smooth muscle relaxation usually plays a minor role in the treatment of esophageal achalasia[2,24]. Nitrates increase NO concentration in smooth muscle cells, and calcium antagonists block calcium and hence esophageal muscle contractions. By so doing, LES pressure can be reduced, but the efficacy is usually unsatisfactory and incomplete, with intolerable side effects such as headache, dizziness, and pedal edema. This is the same for other drugs such as sildenafil[25].

ENDOSCOPIC TREATMENT

Endoscopic treatment with BT injection at the terminal nerve endings of myoneural junctions prevents the release of acetylcholine from vesicles. This causes chemical denervation, which may last for several months[26]. As a result of its wider safety range and fewer complications, local injection of BT into the LES muscle of patients with achalasia lowers LES tone, and the patient becomes asymptomatic. This treatment yields excellent immediate responses with success rates of > 90%. However, the results last only 6-9 mo on average in most patients, and only half of all the patients benefit for > 1 year[27]. Complications of BT therapy for achalasia are minor because the dosage used is too small to induce serious adverse effects such as generalized paralysis. It is therefore used to treat elderly patients or patients with high surgical risks[28].

The most commonly used endoscopic balloon dilator is the rigiflex dilator. The dilation procedure can be performed under fluoroscopic[29] or endoscopic guidance[30,31]. The number of dilation sessions and the inflation time needed for a successful dilation vary and are operator dependent. Immediate and short-term results have reportedly been good in most series[30-33]. However, large-scale long-term follow-up investigations[28,34,35] have reported unfavorable recurrence in patients who have undergone fluoroscopically guided PD. During a prolonged observation period (median, 13.8 years) in a prospective follow-up investigation study conducted by Eckardt *et al*[29], only 40% of the patients treated using a single PD procedure remained in remission at 5 years. Generally, the response to PD is still determined on the basis of subjective improvement in symptoms, such as dysphagia, regurgitation, and chest pain, by performing structured interviews with validated symptom score methods[29,36]. However, additional radiographic findings could reliably predict clinical remission and strongly suggest the need for further treatment in patients with poor esophageal clearance after each dilation. This could prevent sigmoid-type achalasia[37-39]. It is generally accepted that the predictors of risk factors for relapse after PD include young age (< 40-45 years), male sex, single dilation with a 3.0 cm balloon, post-treatment LES pressure > 10-15 mmHg, poor esophageal emptying after timed barium swallow, and type I and type Ⅲ achalasia pattern on HRM[2,40]. Complications attributable to PD are uncommon. The most severe complication is perforation[41], which was reported to be approximately 2% in a recent analysis by Katzka *et al*[42].

Peroral endoscopic myotomy (POEM) is a novel endoscopic esophagomyotomy for achalasia that was first reported by Pasricha *et al*[43] in porcine models and subsequently by Inoue *et al*[44] in humans. POEM is performed by dissection and division of the inner circular muscle layer of the esophagus through a submucosal tunnel created endoscopically by a small proximal opening in the esophageal mucosa. POEM can be used to perform deeper myotomy incisions in the thoracic esophagus than that performed in surgical myotomy, which is difficult for the surgeon, and is indicated especially for patients with advanced disease and for those with severe fibrosis. Theoretically, injury to the vagus nerve should be less than that with the surgical approach. So far, several centers are using the POEM technique and have achieved good short-term results without serious complications, but long-term follow-up results are required[45]. There is concern that POEM is a sophisticated and demanding technique, even for experienced endoscopists, and serious complications such as purulent mediastinitis may develop. Revisional surgery might be difficult and involve extensive procedures such as esophagectomy because the plane between the submucosal and muscular layers will be inflamed and scarred after the endoluminal approach[46].

A few Chinese studies have reported the utility of self-expanding, 30-mm metallic stents for achalasia at a single center over a 10- to 13-year period, with a long-term clinical success rate > 80%[47-50]. There were no perforations or mortality associated with the treatment, but stent migration occurred in 5% of the patients, reflux in 20%, and chest pain in 38.7%. Overall, the self-expanding, 30-mm metallic stents were associated with better long-term clinical efficacy in the treatment of patients with achalasia than treatment with PD.

SURGICAL TREATMENT

From a surgical point of view, minimally invasive HM has become the gold standard procedure for achalasia in the spectrum of current treatment options[51,52]. Myotomy of the LES is the most direct method used and by far the best treatment modality for satisfactory long-term results with very low mortality. Overall success rates of laparoscopic HM (LHM) were 47%-82% at 10 years[53,54]. Systematic reviews and meta-analysis that have compared existing treatment methods for achalasia have found that surgery is superior to PD[55,56]. However, the major adverse event after surgery is severe reflux. There is much debate on the role of fundoplication with myotomy in the reported literature[57-59]. Intraoperative endoscopy during videoscopic HM is used to guide the extent and adequacy of myotomy by utilizing a focused dissection with preservation of the natural antireflux mechanisms around the gastroesophageal junction and by limiting the extent of myotomy along the cardia. By

so doing, postoperative reflux symptoms are minimized. A concomitant endoscope examination during HM to guide myotomy and routine fundoplication is clinically necessary, despite disagreement about the fundoplication procedure[60,61]. In addition, there is a lot of debate on the choice of laparoscopic cardiomyotomy as the primary treatment for achalasia or as a second-line treatment following the failure of nonsurgical intervention[62]. Some doctors believe that laparoscopic cardiomyotomy can be more technically difficult following PD[63]. However, it has been shown that laparoscopic cardiomyotomy can be as safe and effective as first- or second-line treatment, even after the failure of PD[64]. In general, esophagectomy should be reserved only for those cases in which simpler operations have failed. In summary, as stated in the recent Kagoshima consensus, despite the variations as to the length of the myotomy and the addition of an antireflux procedure, good overall long-term results suggest that these operative variations are not critical[65].

WHICH IS THE BETTER CHOICE: LHM OR PD? THE ONGOING DEBATE

In general, LHM is considered to be superior to PD for treating achalasia. Many experts have regarded LHM as the first choice of treatment for achalasia, at the cost of reflux complications. However, it should be noted that the first randomized controlled multicenter trial published by the European Achalasia Trial group that compared LHM and PD reported that, after 2 years of follow-up, LHM was not associated with superior rates of therapeutic success[66]. The large sample size gathered from 15 European centers and the excellent design of the study gave adequate statistical power for obtaining convincing results for the two treatment groups. Perforation of the esophagus occurred in 4% of the patients during PD, whereas mucosal tears occurred in 12% during LHM. Abnormal exposure to esophageal acid was observed in 15% and 23% of the patients in the PD and LHM groups, respectively. In addition, when considering the cost-effectiveness of treatment strategies for achalasia, laparoscopic myotomy has a higher initial cost, and PD is the most cost-effective treatment option for adults with achalasia. It is unclear how the results of the European Achalasia Trial actual affect the ongoing debate between gastroenterologists and surgeons on the treatment of choice for esophageal achalasia. This study had the following limitations. First, this was only a 2-year cohort study, and the intermediate and long-term remission rates have yet to be proven. Second, the good results for PD may be questioned by the definition of treatment failure in redilation sessions. In fact, all patients in the PD group routinely received at least two sessions of dilation. A maximum of three redilation sessions was allowed. However, this is an ongoing study, and more information will be collected in the future. The debate on which is the better choice between LHM and PD for esophageal achalasia is ongoing; however, it is generally accepted that myotomy is usually suggested for younger patients (age, < 40-45 years), male patients, and those with pulmonary symptoms who failed to respond to one or two initial dilations[2,67].

In summary, despite the ongoing debate and the report of the first randomized control trial, the minimally invasive surgical treatment seems to yield better results than PD with the currently available evidence, despite being less cost-effective and resulting in more reflux symptoms. POEM is a promising technique and is associated with good short-term results without serious complications, but long-term results are not yet available. Despite these advancements, the actual cause of achalasia has not yet been identified, and this knowledge may improve treatment success in the future.

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ORIGINAL ARTICLE

Dual regulatory role for phosphatase and tensin homolog in specification of intestinal endocrine cell subtypes

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Abstract

AIM: To investigate the impact of phosphatase and tensin homolog (Pten) in the specification of intestinal enteroendocrine subpopulations.

METHODS: Using the Cre/loxP system, a mouse with conditional intestinal epithelial *Pten* deficiency was generated. *Pten* mutant mice and controls were sacrificed and small intestines collected for immunofluorescence and quantitative real-time polymerase chain reaction. Blood was collected on 16 h fasted mice by cardiac puncture. Enzyme-linked immunosorbent assay was used to measure blood circulating ghrelin, somatostatin (SST) and glucose-dependent insulinotropic peptide (GIP) levels.

RESULTS: Results show an unexpected dual regulatory role for epithelial Pten signalling in the specification/differentiation of enteroendocrine cell subpopulations in the small intestine. Our data indicate that *Pten* positively regulates chromogranin A (CgA) expressing subpopulations, including cells expressing secretin, ghrelin, gastrin and cholecystokinin (CCK). In contrast, *Pten* negatively regulates the enteroendocrine subtype specification of non-expressing CgA cells such as GIP and SST expressing cells.

CONCLUSION: The present results demonstrate that *Pten* signalling favours the enteroendocrine progenitor to specify into cells expressing CgA including those producing CCK, gastrin and ghrelin.

Key words: Phosphatase and tensin homolog; Enteroendocrine cells; Intestinal epithelial cell specification; Chromogranin A

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INTRODUCTION

The phosphatase and tensin homolog (*PTEN*) tumour

suppressor gene is one of the most frequently mutated/deleted genes in various human cancers[1,2]. PTEN is a lipid and protein phosphatase. Its best-known substrate, the phosphatidylinositol 3,4,5-trisphosphate (PIP3), is a lipid second messenger mainly produced by class IA phosphatidylinositol 3-kinases (PI3Ks)[3]. PTEN dephosphorylates PIP3 to produce phosphatidylinositol 4,5-bisphosphate, which inhibits PI3K-dependent effectors such as the downstream kinases Akt and pyruvate dehydrogenase kinase 1. PI3Ks have been implicated in many signalling pathways that regulate cell survival, growth, proliferation, migration, phagocytosis, and metabolism[4]. PTEN has also been shown to regulate genomic stability[5,6], stem cell renewal[7,8], senescence[9] and cell differentiation[10-12].

The multiple cellular functions of PTEN suggest that this protein plays major roles in overall system homeostasis. Indeed, homozygous deletion of *Pten* in the mouse causes early embryonic lethality by embryonic day (E) 9.5, whereas *Pten* heterozygous mice ($Pten^{+/-}$) develop, over a period of time, various dysplasia and hyperplasia in organs such as the breast, thyroid, prostate and intestine[1,2,13]. As reviewed by Knobbe *et al*[14], *Pten* has also been conditionally deleted in many specific tissues. These models have established the tumour suppressive function of *Pten* but have also unravelled its important role in the maintenance of normal physiological functions in various tissues such as the immune system, skin, lung, liver, pancreas and hypothalamus[14].

In a previous study, we reported that Pten is important for intestinal homeostasis[10]. The villin-Cre system was used to specifically inactivate *Pten* in the mouse intestinal epithelium. *Pten* mutant mice developed an intestinalomegaly associated with an increase in epithelial cell proliferation. Histological analysis also demonstrated significant perturbation of the crypt-villus architecture, a marked increase in goblet cells and a decrease in enteroendocrine cells, suggesting a role for *Pten* in the commitment of the multipotential-secretory precursor cell[10].

Enteroendocrine cells are hormone-secreting epithelial cells that are scattered throughout the gastrointestinal epithelium and although they represent only 1% of the intestinal epithelium, taken together, they constitute the major endocrine organ of the body[15,16]. At least 10 different enteroendocrine cell types have been identified in the small intestine and are classified based on their main hormonal products[16,17]. The various hormones produced by these endocrine cells [ghrelin (GHR), gastrin-releasing peptide (GRP), glucose-dependent insulinotropic peptide (GIP), secretin (SCT), peptide YY (PYY), glucagon-like peptide-1 (GLP-1), glucagon-like peptide-2 (GLP-2), cholecystokinin (CCK), neurotensin, serotonin, substance *P*, somatostatin (SST) and motilin] control important physiological functions, such as gastrointestinal motility, glycaemia, exocrine pancreatic secretion, biliary secretion, digestion, gut epithelial renewal and appetite[16,18,19]. Most enteroendocrine cell types secrete chromogranin A (CgA), a soluble glycoprotein stored with hormones and neuropeptides in secretory granules of endocrine cells. The important role of enteroendocrine cells in whole body homeostasis prompted us to further analyze the effect of intestinal epithelial deletion of *Pten* on the specification of the various enteroendocrine subpopulations. Using our Cre/loxP *Pten* conditional knock out mouse model[10], we report herein an unexpected dual regulatory role for epithelial Pten signalling in the specification of enteroendocrine cells. Our data indicate that Pten positively influences the determination and specification of CgA-expressing cell subpopulations in the small intestine including those expressing secretin, ghrelin, gastrin and CCK. Conversely, Pten limits determination and specification of non-expressing CgA endocrine cell subpopulations, including GIP and SST.

MATERIALS AND METHODS

Animals

BALB/c-$Pten^{fx/fx}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME, United states). The C57BL/6 12.4KbVilCre transgenic line was provided by Dr. Deborah Gumucio (University of Michigan, Ann Arbor, MI, United states)[20]. Genomic DNA was isolated using the Spin Doctor genomic DNA kit from Gerard Biotech according to the manufacturer's protocol. Both mutations were genotyped following protocols already published[20] or as directed by The Jackson Laboratory. For this study, the BALB/c-$Pten^{fx/fx}$ mice mice were first crossed with the C57BL/6 12.4KbVilCre to generate F1-generation heterozygous animals. F1-generation heterozygous animals were then backcrossed with BALB/c-$Pten^{fx/fx}$ mice to produce F2-generation experimental animals. All experiments were conducted in F2-generation experimental animals. All mice were maintained on regular diet in the transgenic mouse facility at the Faculty of Medicine and Health Sciences of the Université de Sherbrooke. All experiments were approved by the animal research committee of the Faculty of Medicine and Health Sciences of the Université de Sherbrooke.

Tissue collection, tissue preparation, RNA extraction and gene expression analysis

Digestive tracts from 120-d-old $Pten^{\Delta IEC}$ mice and control littermates were fixed in 4% paraformaldehyde (PFA) overnight at 4 ℃, then dehydrated and embedded in paraffin. Sections of 5 μm were applied to Probe-On Plus slides (Fisher Scientific, Ottawa, ON, Canada) and kept at room temperature until used[10,21]. Total RNA was isolated and processed using the Totally RNA extraction kit (Ambion, Grand Island, NY, United states). Reverse-transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR were performed as described previously[21]. Quantitative real-time PCR conditions were as follows: one cycle of 15 min at 95 ℃; 50 cycles at 95 ℃ for 15 s; 59 ℃ for 30 s and 72 ℃ for 30 s. The following forward and reverse primers were used: Hairy and enhancer of split 1 (NM_008235), 5'-TTCCAAGCTAGAGAAGGCAGA-3', 5'-GTTGATCTGGGT-

CATGCAGTT-3'; Atonal homolog 1 (NM_007500), 5'-GCTTCCTCTGGGGGTTACTC-3', 5'-ACAACGATCACCACAGACCA-3'; Neurogenin 3 (NM_009719), 5'-CGGATGACGCCAAACTTACAAAG-3' 5'-CACAAGAAGTCTGAGAACAACAG-3'; Growth factor independent 1 (NM_010278), 5'-TCCGAGTTCGAGGACTTTTG-3', 5'-CATGCATAGGGCTTGAAAGG-3'; Neurogenic differentiation 1 (NM_010894), 5'-AGCCACGGATCAATCTTCTCT-3', 5'-GACGTGCCTCTAATCGTGAAA-3'; Pancreatic and duodenal homeobox 1 (NM_008814), 5'-AACCCGAGGAAAACAAGAGG-3', 5'-TTCAACATCACTGCCAGCTC-3'; Forkhead box O1 (NM_019739), 5'-CCGGAGTTTAACCAGTCCAA-3', 5'-TGCTCATAAAGTCGGTGCTG-3'; Forkhead box a1 (NM_008259), 5'-CAAGGATGCCTCTCCACACTT-3', 5'-TGACCATGATGGCTCTCTGAA-3'; Forkhead box a2 (NM_010446), 5'-GAGCACCATTACGCCTTCAAC-3', 5'-GGCCTTGAGGTCCATTTTGT-3'; PDGB (NM_013551), 5'-TGCACGATCCTGAAACTCTG-3', 5'-TGCATGCTATCTGAGCCATC-3'.

Immunofluorescence

Immunofluorescence staining was performed as previously described[21]. The following antibodies were used at the indicated dilutions: FITC-conjugated anti-mouse IgG (1:200, Vector, Burlingame, CA, United states), FITC-conjugated anti-rabbit IgG (1:200, Vector), AlexaFluor 568 donkey anti-goat (1:400, Invitrogen, Grand Island, NY, United states), AlexaFluor 488 donkey anti-goat (1:400, Invitrogen), AlexaFluor 488 donkey anti-rabbit (1:400, Invitrogen), rabbit anti-SP-1 CgA (1:1000, ImmunoStar, Hudson, WI, United states), goat anti-CgA (1:50, SantaCruz, Santa Cruz, CA, United states), rabbit anti-gastrin (1:200, Chemicon, Billerica, MA, United states), goat anti-GIP (1:100, SantaCruz), mouse anti-serotonin (1:200, LabVision, Kalamazoo, MI, United states), rabbit anti-secretin (1:1000, Phoenix pharmaceuticals, Burlingame, CA, United states), goat anti-SST (1:100, SantaCruz), goat anti-ghrelin (1:100, SantaCruz), rabbit anti-CCK (1:100, ab92128 gift from Rehfeld JF)[22].

Measurement of circulating hormone levels

Blood was collected on 16 h fasted mice by cardiac puncture. Serum levels of total ghrelin and GIP were measured using Millipore ELISA kits (EZRGRT-91K, EZRMGIP-55K) (Millipore, Billerica, MA, United states) according to manufacturer's instructions. Serum levels of SST were measured using the Phoenix Pharmaceuticals ELISA kit EK-060-03, according to the manufacturer's instructions.

Statistical analysis

All cell count analyses were performed using continuous serial sections from low-powered fields of well-oriented intestinal cross-sections in a blind manner on an average of 10 independent fields per animal. Three different intestinal sections were evaluated: duodenum, jejunum and ileum. The total number of enteroendocrine cells was counted per crypt-villus axis. Image magnification was calibrated by comparison with a stage micrometer (graticulesTM Ltd., Tonbridge, Kent, England). Statistical analyses were performed using two-way ANOVA. For qRT-PCR, data were analyzed using the Mann Whitney-test for abnormal distribution. Differences were considered significant with a P value of < 0.05. All statistical analyses were carried out using Graph Pad Prism 5 (Graph Pad Inc., San Diego, CA).

RESULTS

CgA is not expressed in all enteroendocrine cell subtypes of the mouse small intestinal epithelium

Mice homozygous for the floxed exon 5 of the *Pten* gene[23] were bred to the *villin*-Cre transgenic line, which directs expression of the transgene in all epithelial cells of the small intestine and colon, including stem cells, but not in the mesenchymal compartment[20]. Conditional knockout mice for *Pten* ($Pten^{\Delta IEC}$) were born at the expected Mendelian ratios, survived for more than 1 year, and grew normally without obvious gross physical abnormalities[10]. In a previous study with these mice, we reported an overall decrease in the number of enteroendocrine cells using a CgA antibody[10]. Over the years, there has been a lingering controversy where a number of studies showed that all endocrine cell subpopulations express CgA[18,24,25] while others reported that some cell subpopulations do not express CgA[17,26]. Therefore, individual analysis of various intestinal endocrine subpopulations was first performed for their co-expression with CgA in the mouse small intestine. Double-labelling with CgA (Figure 1B, E, H and K) and specific antibodies directed against ghrelin (Figure 1A), CCK (Figure 1D), gastrin (Figure 1G) and secretin (Figure 1J) confirmed co-expression of CgA with ghrelin (Figure 1C) as well as with CCK- (Figure 1F), gastrin- (Figure 1I) and secretin- (Figure 1L) producing enteroendocrine cells in the mouse small intestine. On the other hand, double-labelling with CgA antibody (Figure 1N and Q) and specific antibodies directed against GIP (Figure 1M) and SST (Figure 1P) supported the exclusion of co-expression between GIP (Figure 1O), SST (Figure 1R) and CgA in the mouse small intestine. The specificity of our CgA antibodies was confirmed with the use of two different CgA antibodies from two different commercial sources, in which the exact same cells were labeled in consecutive sections from a same specimen with both antibodies.

Loss of intestinal epithelial Pten impairs the specification of CgA expressing enteroendocrine cells

Enteroendocrine subtype specification appears to be regulated by distinct mechanisms[17,26]. Since our previous study only investigated CgA-expressing cells, the impact of *Pten* loss of expression on the specification of the various enteroendocrine cell subpopulations in the small intestine was further analyzed. We first analyzed how the loss of epithelial *Pten* alters specification of CgA-expressing en-

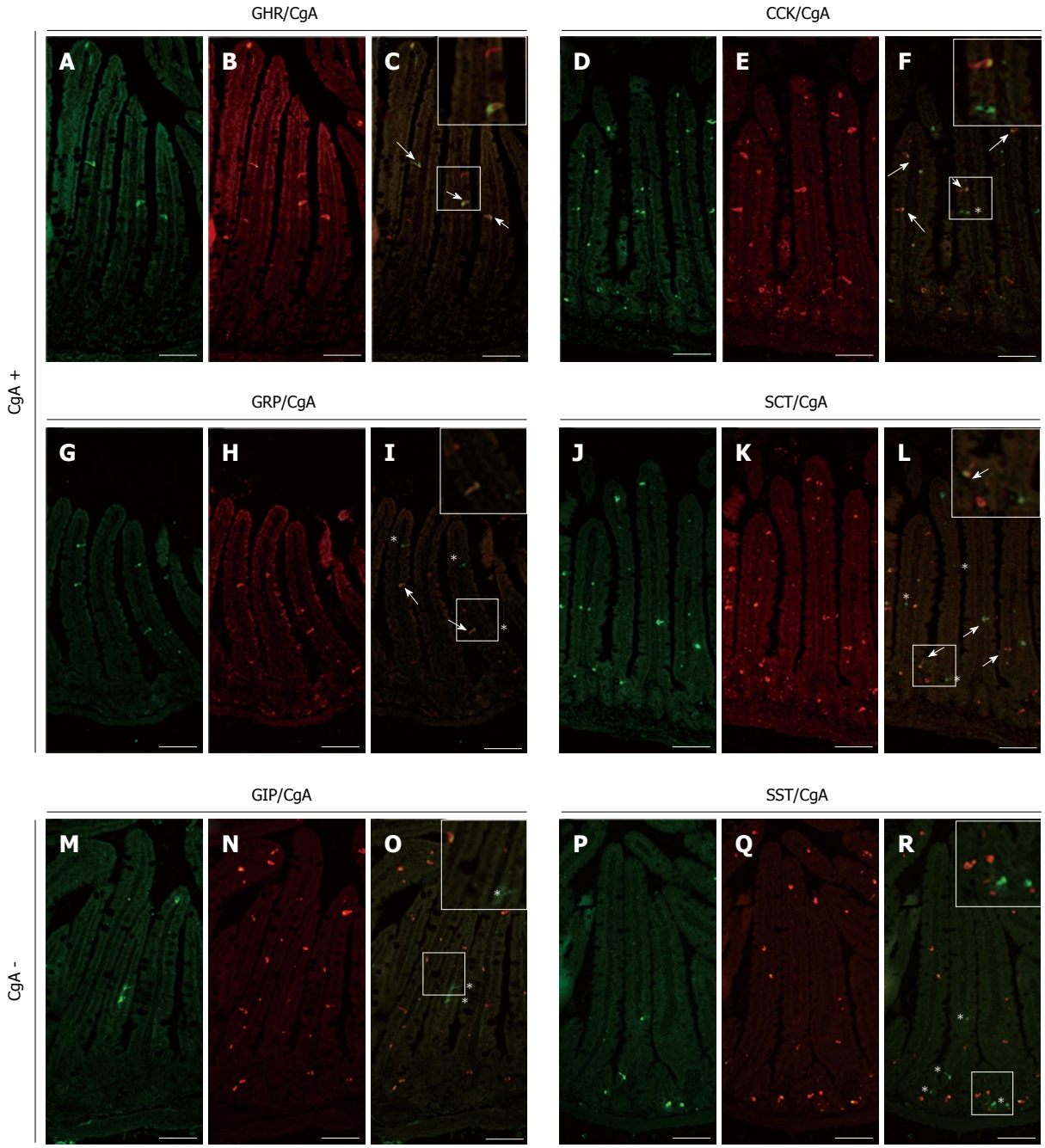

Figure 1 Analysis of chromogranin A co-expression in mouse small intestinal endocrine subpopulations. Small intestine sections of adult control mice were co-immunostained with antibodies directed against ghrelin (GHR) (A), cholecystokinin (CCK) (D), gastrin-releasing peptide (GRP) (G), secretin (SCT) (J) glucose-dependent insulinotropic peptide (GIP) (M) or somatostatin (SST) (P) and against chromogranin A (CgA) (respectively B, E, H, K, N and Q). The arrows in images C, F, I and L show co-expression of CgA respectively with GHR, CCK, GRP and SCT while asterisks in images F, I, L, O and R point to CgA-negative enteroendocrine cells. The number of arrows and asterisks within the crypt-villus axis represents the average proportion of labelled cells per units. Scale bar: 50 μm.

teroendocrine cells along the various sections of the small intestine (duodenum, jejunum and ileum). Although some enteroendocrine cells are restricted to specific regions of the small intestine, each region was analyzed in order to verify the possible delocalization of subpopulations along the rostro-caudal axis of the gut. The intestinal mucosa of $Pten^{\Delta IEC}$ and control mice was stained with specific markers for each enteroendocrine cell subtype and positive cells were counted (Figure 2). A significant decrease of 29% in the jejunum (1.2 positive cells per crypt-villus axis *vs* 1.7) and 51% in the ileum (0.25 cell *vs* 0.52 cell) was observed (Figure 2C) in the ratio of positive ghrelin cells in *Pten* mutant mice (Figure 2B) compared to control littermates (Figure 2A). A modest but significant decrease of 10% (Figure 2F) was also observed in the ratio of positive CCK cells in the duodenum of the mutant mice (4.4 cell *vs* 4.7 cell) (Figure 2E). There was also a significant 23% decrease in gastrin-positive cells in the jejunum (1 cell *vs* 1.3 cell) and a decrease of 29% in the ileum (0.34 cell *vs* 0.44 cell) in $Pten^{\Delta IEC}$ (Figure 2H and I) when compared to

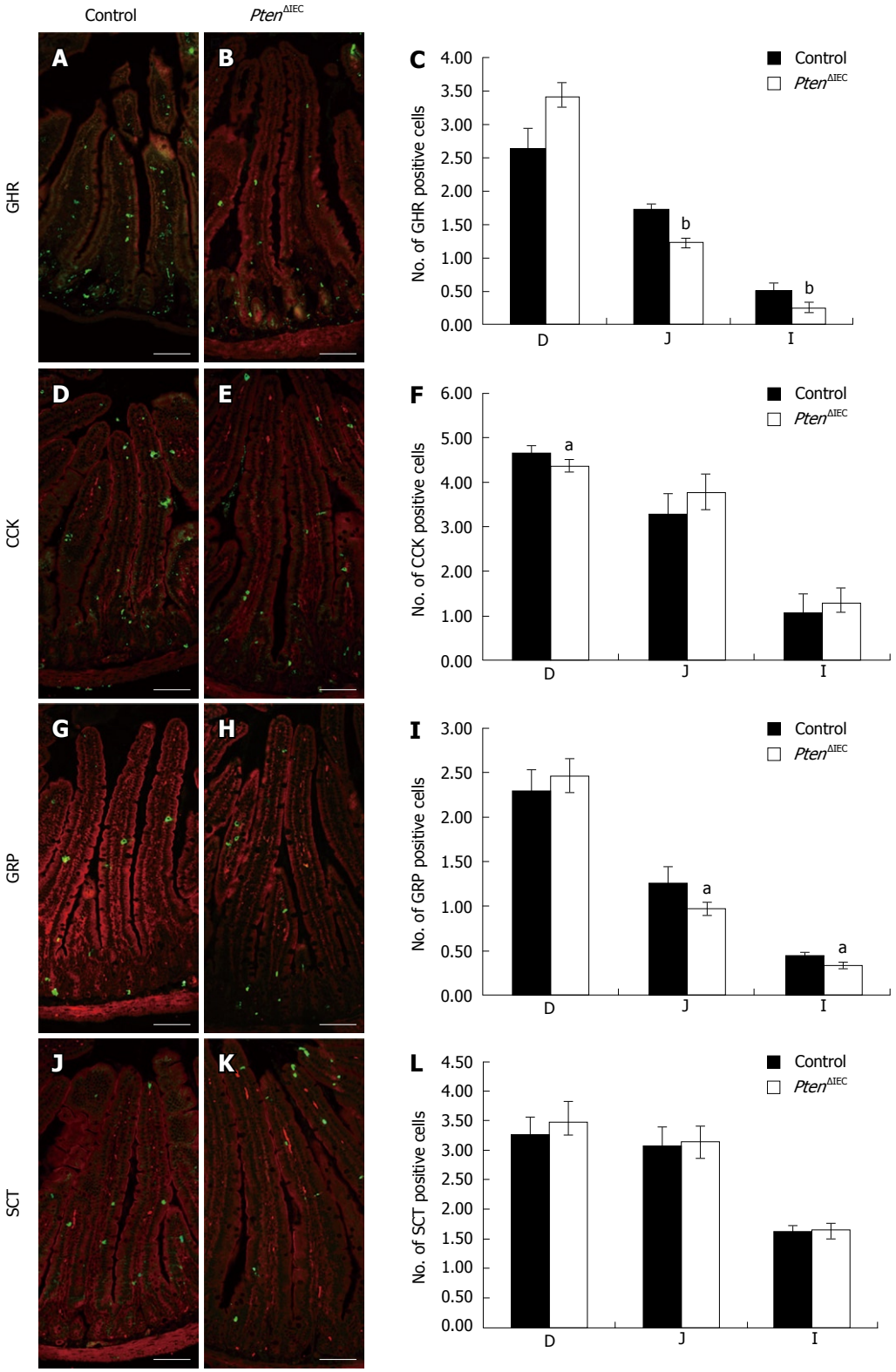

Figure 2 Epithelial *Pten* positively regulates commitment of chromogranin A-positive enteroendocrine subpopulations in the small intestine. Duodenum, jejunum and ileum of adult control and *Pten*$^{\Delta IEC}$ mice were immunostained with antibodies against ghrelin (GHR) (A and B), cholecystokinin (CCK) (D and E), gastrin-releasing peptide (GRP) (G and H) and secretin (SCT) (J and K). Positive cells were counted from intestinal sections of controls (n = 6) and mutants (n = 5). Statistical analysis (C, F, I, L) represents the average number of positive cells per crypt-villus axis in each section of the intestine. Error bars represent SE. Scale bar: 50 μm. D: Duodenum; J: Jejunum; I: Ileum. $^{a}P < 0.05$, $^{b}P < 0.001$.

control mice (Figure 2G and I). Finally, secretin immunostaining showed no modulation in the number of secretin-positive cells in *Pten*$^{\Delta IEC}$ (Figure 2K and L) *vs* control mice (Figure 2J and L). Taken together, these results suggest that *Pten* positively influences production of CgA-expressing enteroendocrine cell subpopulations.

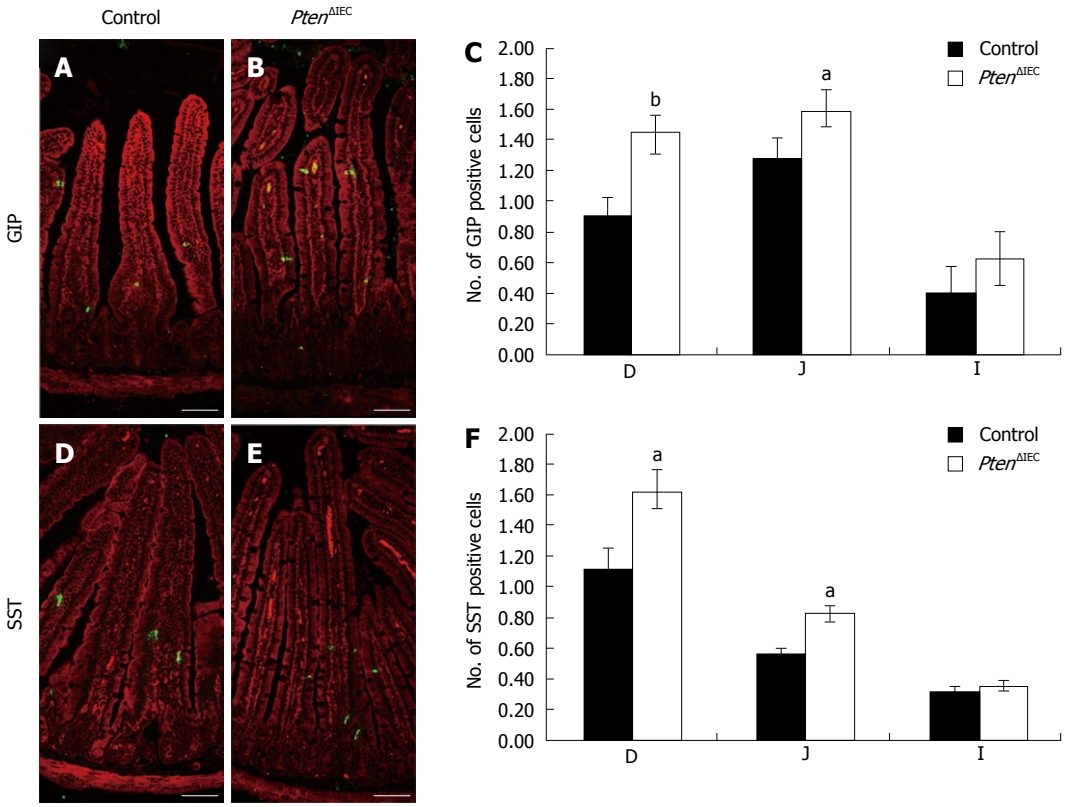

Figure 3 Epithelial *Pten* negatively regulates commitment of chromogranin A-negative enteroendocrine subpopulations in the small intestine. Duodenum, jejunum and ileum of adult control and *Pten*$^{\Delta IEC}$ mice were immunostained with antibodies against glucose-dependent insulinotropic peptide (GIP) (A and B) and somatostatin (SST) (D and E). Positive cells were counted from intestinal sections of controls (n = 6) and mutants (n = 5). Statistical analysis (C and F) represents the average number of positive cells per crypt-villus axis in each section of the intestine. Error bars represent SE. Scale bar: 50 μm. D: Duodenum; J: Jejunum; I: Ileum. [a]$P < 0.05$, [b]$P < 0.01$.

Loss of epithelial intestinal Pten positively influences the specification of CgA negative enteroendocrine cells

We next examined if the specification of CgA-negative cells was affected following the loss of epithelial *Pten*. As illustrated in Figure 3, there was a marked 61% (duodenum) and 25% (jejunum) increase in GIP-positive cells in *Pten*$^{\Delta IEC}$ (Figure 3B and C) when compared to control mice (Figure 3A and C) (respectively 1.45 positive cells *vs* 0.9 in the duodenum and 1.6 cells *vs* 1.28 in the jejunum). SST immunostaining in both duodenum and jejunum revealed an increase of 45% in the number of SST-positive cells in *Pten*$^{\Delta IEC}$ (Figure 3E and F) *vs* control mice (Figure 3D and F) (respectively 1.65 cells *vs* 1.15 cell and 0.85 cell *vs* 0.6 cell). Hence, these data suggest that Pten signalling negatively controls specification of CgA-negative cells in the intestinal epithelium.

Loss of epithelial Pten signalling leads to deregulation of circulating GIP and SST levels

In light of these observations, we next investigated whether deregulation in the number of enteroendocrine cells in the intestinal epithelium of the *Pten*$^{\Delta IEC}$ mice has an impact on their circulating levels. We chose to focus on the enteroendocrine subpopulations where the deregulation was more considerable. Circulating ghrelin, GIP and SST levels were analysed by ELISA assay. A 1.5-fold and 1.3-fold increase in GIP (Figure 4B) and SST (Figure 4C) levels, respectively, were observed in *Pten*$^{\Delta IEC}$ mice when compared to control littermates. No significant difference in ghrelin levels was observed between *Pten*$^{\Delta IEC}$ mice and control mice (Figure 4A).

Pten expression impacts differently on various pro-enteroendocrine specification factors

Comparative analysis of secretory lineage and specific pro-enteroendocrine determination factors was next investigated by quantitative PCR to clarify the role of *Pten* during enteroendocrine subtype specification. The Notch pathway, and more specifically the transcription factors Hairy enhancer of Split (Hes-1) and Math1, is crucial in the determination of the intestinal progenitor cell to absorptive or secretory cell fate (Figure 5)[27,28]. We also investigated if loss of epithelial *Pten* could deregulate the production of secretory precursors. Quantitative PCR analysis of mutant *vs* wild-type littermates revealed no modulation of Math1 or Hes-1 mRNA levels in the mutant animals (Table 1). Modifications downstream of the Notch pathway during enteroendocrine cell determination were also subsequently assessed. The proendocrine bHLH transcription factor Ngn3 has been shown to contribute to the maintenance and specification of enteroendocrine precursors (Figure 5)[29]. Our analysis revealed that the Ngn3 mRNA expression was significantly reduced by 2.07-fold in the mutant animals (Table 1). BETA2/

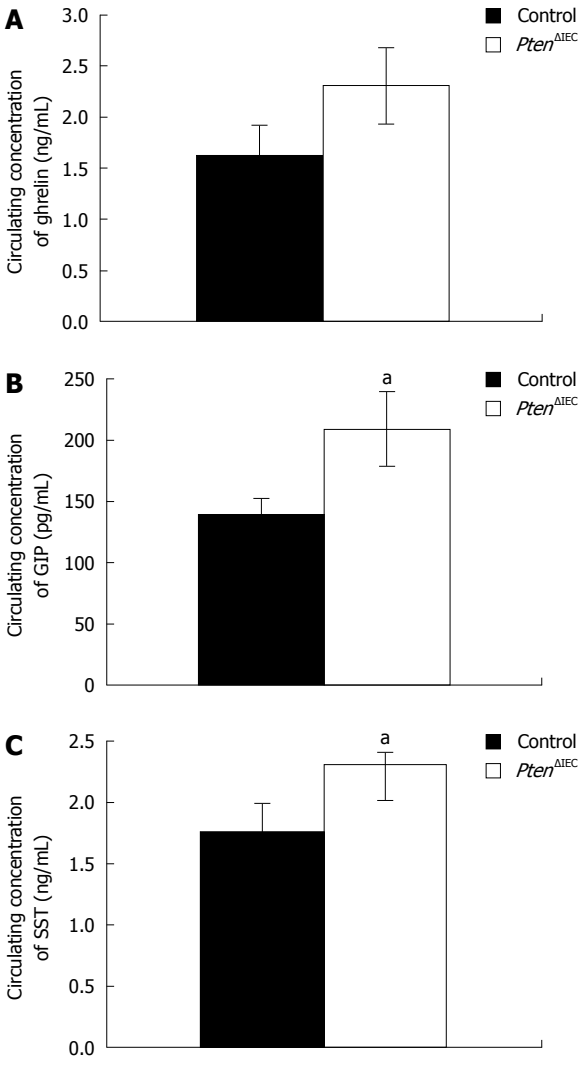

Figure 4 Loss of epithelial *Pten* signalling modulates circulating levels of glucose-dependent insulinotropic peptide and somatostatin. A: Analysis of circulating ghrelin level revealed no significant modulation between adult *Pten*$^{\Delta IEC}$ mice (n = 10) and control littermates (n = 10); B: Analysis of circulating glucose-dependent insulinotropic peptide (GIP) level revealed a 1.5-fold increase in adult *Pten*$^{\Delta IEC}$ mice (n = 10) when compared to control littermates (n = 10); C: Analysis of circulating somatostatin (SST) level revealed a 1.3-fold increase in adult *Pten*$^{\Delta IEC}$ mice (n = 10) when compared to control littermates (n = 10). Error bars represent SE. $^{a}P < 0.05$.

NeuroD1, Pancreatic and duodenal homeobox 1 gene (*Pdx1*), the winged helix Foxa1 and the forkhead box-containing (FoxO1) transcription factors have also been shown to control the determination of specific enteroendocrine subpopulations[16,30-33]. The gene transcript level of BETA2/NeuroD1, linked to specification of secretin and CCK producing cells[34], was reduced by 1.40-fold in mutant animals (Table 1). Pdx1, which regulates serotonin and GIP producing cells (Figure 5)[31,32], was found to be significantly increased by 2.15-fold at the gene transcript level in mutant mice (Table 1). FoxO1 factors are downstream targets of the PI3K/AKT pathway[35] and affect the subcellular localization of Pdx1 in the pancreas and, hence, its transcriptional activity[36]. *FoxO1* gene transcript level was found to be reduced by 1.95-fold in the *Pten*$^{\Delta IEC}$ mice (Table 1). Finally, the winged helix transcription factors Foxa1 is essential for the differentiation of SST-, GLP-1- and PYY-expressing endocrine cells (Figure 5)[33]. Accordingly, we found an increase of 2.64-fold in *Foxa1* gene transcript expression in *Pten*$^{\Delta IEC}$ mice (Table 1).

Table 1 Gene expression changes in the small intestine of *Pten*$^{\Delta IEC}$ mice

Gene description	Gene symbol	Fold	*P* value
Hairy and enhancer of split 1	*Hes1*	-1.08	NS
Atonal homolog 1	*Math 1*	-2.05	NS
Neurogenin 3	*Ngn3*	-2.07	0.033
Growth factor independent 1	*Gfi1*	1.07	NS
Neurogenic differentiation 1	*NeuroD1*	-1.40	0.002
Pancreatic and duodenal homeobox 1	*Pdx1*	2.15	0.048
Forkhead box O1	*FoxO1*	-1.95	0.017
Forkhead box a1	*Foxa1*	2.64	0.017
Forkhead box a2	*Foxa2*	-1.25	NS

Target expression was quantified relatively to PDGB expression. Fold changes represent the ratio of mean expression values (control/mutant). Negative values indicate reduction in *Pten*$^{\Delta IEC}$ intestines. NS: Non significant fold change (Mann-Whitney test).

DISCUSSION

Endocrine cells found scattered in the gastrointestinal epithelium represent the major endocrine organ of the body[15,16]. The various hormones produced by these endocrine cells control numerous physiological functions[16,18,19]. Recently, by using conditional tissue-specific disruption of *Pten* in the epithelium of the gut, we revealed a key role for epithelial Pten in intestinal morphogenesis, in the maintenance of crypt-villus axis architecture, in cell proliferation and in secretory cell commitment[10]. We had also reported an overall decrease in the number of enteroendocrine cells using a CgA antibody. However, the choice of CgA as a pan marker for all enteroendocrine cells has been challenged. Commonly used as a biomarker for endocrine granules, CgA plays a role in the biogenesis of mobile secretory granules and the release of hormones through the regulated secretory pathway[37]. Over the years, there has been a lingering controversy in which some studies showed that all endocrine cell subpopulations express CgA[18,24,25] while others reported that enteroendocrine cell subpopulations producing GIP, GLP-1 or SST do not express CgA[17,26]. Fixation artefacts and different CgA antibodies may account for this controversy. Also, it has been demonstrated that CgA expression in enteroendocrine subpopulations varies from one species to another as well as in pathologies such as colorectal cancer and inflammatory bowel diseases[38-42]. Herein, our analysis of the various intestinal endocrine subpopulations with CgA antibodies confirmed the absence of co-expression between GIP and SST with CgA. Therefore, the important role of enteroendocrine cells in whole body homeostasis prompted us to further analyze the effect of intestinal epithelial deletion of *Pten* on the specification of the various en-

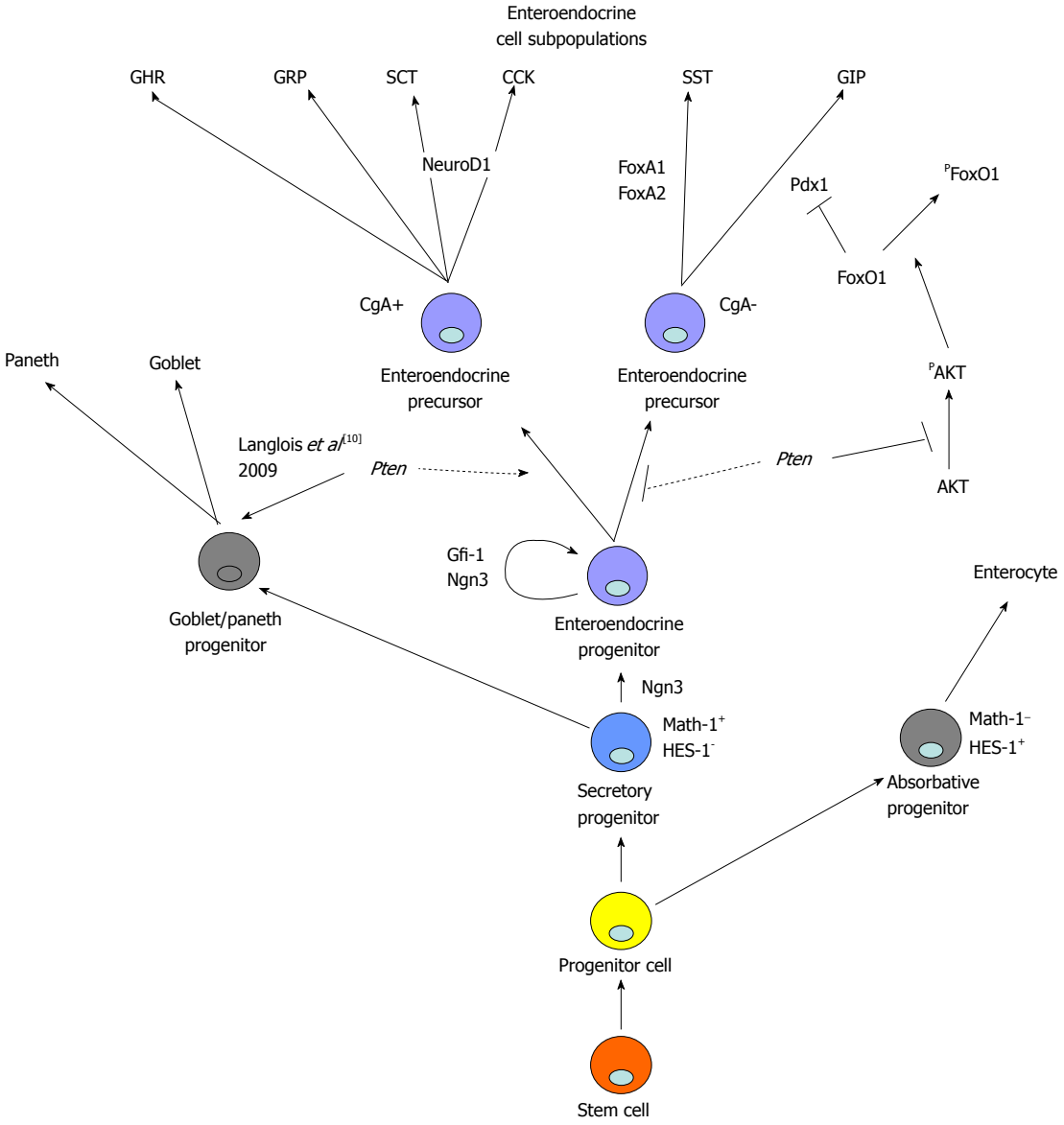

Figure 5 Proposed model for mode of action of epithelial Pten signalling in intestinal epithelial determination and specification of enteroendocrine progenitor cell fate. Epithelial Pten signalling is not essential for maintenance or determination of the secretory precursor. Pten represses specification of glucose-dependent insulinotropic peptide (GIP)-expressing cells by maintaining FoxO1 in the nucleus. GHR: Ghrelin; GRP: Gastrin-releasing peptide; SCT: Secretin; CCK: Cholecystokinin; SST: Somatostatin; GIP: Glucose-dependent insulinotropic peptide; CgA: Chromogranin A.

teroendocrine subpopulations. Since all enteroendocrine subtype cells are still detectable in the mutant mice, our results suggest that Pten is not a direct and indispensable regulator of enteroendocrine cell determination. Nevertheless, our data revealed a dual role for Pten signalling in enteroendocrine cell specification. Indeed, our results indicate that Pten signalling facilitates the specification of CgA-expressing enteroendocrine cell subpopulations while it negatively controls specification of CgA-negative cells in the intestinal epithelium. Furthermore, our results showed that the number of GIP and SST cells as well as their associated circulating hormone levels was increased in mutant mice. Although the number of ghrelin cells was decreased, no significant modulation in ghrelin serum level was observed in the $Pten^{\Delta IEC}$ mice. This may be explained by the fact that ghrelin endocrine cells found in the stomach epithelium are strong contributors for total circulating ghrelin levels[43,44], and are not likely affected by the loss of epithelial *Pten* in the intestine. Nevertheless, the lack of modulation in circulating levels of ghrelin does not imply that the reduction observed in the cell number in the intestine has no local consequences in this tissue. Indeed, such a reduction could influence specific physiological intestinal functions, such as motility, digestion and epithelial renewal[16,18,19]. Finally, analyses of each enteroendocrine cell subtypes along the rostro-caudal axis of the small intestine confirmed that the loss of *Pten* does not influence normal distribution of these endocrine cell subpopulations.

Our data also indicate that Pten affects the expression of key regulators for cell lineages and/or proenteroendocrine determination. Since the Notch/Hes-1

path is required for the specification of progenitor cells into the absorptive lineage and since Math1 is required for specification into the secretory lineage[27,28], we therefore analyzed whether the loss of epithelial *Pten* could alter their expression. Lack of modulation in Math1 and Hes-1 gene transcripts suggest that Pten is not involved in the initial decision steps for lineage determination. Once the initial decision is made between secretory and absorptive cell lineages, the fate of enteroendocrine progenitor cells is defined by proendocrine bHLH transcription factors such as Ngn3 and BETA2/NeuroD1. Ngn3 acts downstream of Math1[27,29] and has been shown to contribute to the maintenance of the enteroendocrine precursors and to the differentiation of all enteroendocrine subpopulations in mice[29,45,46]. Unlike Ngn3, expression of BETA2/NeuroD1 is restricted to a subset of enteroendocrine cells[34]. BETA2/NeuroD1 controls terminal differentiation of secretin and CCK producing cells in the intestine as revealed by the absence of these subpopulations in BETA2/NeuroD1 null mice[34]. In addition, BETA2/NeuroD1 acts downstream of Ngn3[45]. Our analysis revealed that the expression of both bHLH transcription factors was reduced in absence of epithelial Pten, thereby impacting on the production of specific enteroendocrine subpopulations (Figure 5). Over the years, other factors have been shown to be important in the differentiation/specification of several enteroendocrine cell subpopulations[16,30-33]. Such is the case for the winged helix transcription factor Foxa1, previously shown to be essential for the differentiation of SST, GLP-1 and PYY expressing cells[33]. Foxa1 expression was found to be increased in $Pten^{\Delta IEC}$ mice, hence correlating with the increased production of SST-expressing cells in these mice (Figure 5). The same logic can be applied to Pdx-1. Indeed, studies from Pdx1-null mice revealed an increase in the number of serotonin cells and a decrease in the GIP-expressing cell population[31,32]. Herein, *Pdx-1* gene transcript was found to be significantly increased in absence of epithelial *Pten*, thereby matching the deregulation seen in GIP cell specification (Figure 5). In addition, *FoxO1* gene transcript was found to be significantly reduced in the absence of epithelial Pten. FoxO1 competes with FoxA2 for binding to the Pdx1 promoter, resulting in inhibition of Pdx1 transcription[36] (Figure 5). Aside from these observations, one could speculate that phosphorylation of FoxO1 affects its subcellular localisation leading to its exclusion from the nucleus. This nuclear/cytoplasm shuttling phosphorylation of FoxO1 ultimately decreases its transactivation potential[36,47]. Furthermore, PI3K/Akt is a major upstream signalling pathway leading to the phosphorylation of FoxO1 and its exclusion from the nucleus[35]. In a previous study with $Pten^{\Delta IEC}$ mice, we reported that loss of *Pten* resulted in increased phosphorylation levels of Akt[10]. Thus, it is tempting to extrapolate that following the loss of intestinal epithelial Pten and activation of Akt, targeted FoxO1 protein would become more phosphorylated and exported to the cytoplasm allowing expression of Pdx1 and specification of GIP-expressing cells.

In summary, our results reveal a distinctive role for Pten in specification/differentiation of enteroendocrine cell subpopulations. Pten signalling negatively regulates the enteroendocrine subtype specification of non-expressing CgA cells such as GIP and SST expressing cells. In contrast, Pten signalling positively affects CgA-expressing cells such as ghrelin, gastrin and CCK cells. Many of these enteroendocrine cell subtypes are known to play critical roles in whole body physiological functions. Incretin hormones such as GLP-1 and GIP have been shown to potentiate glucose-stimulated insulin secretion[48], while double-mutant mice for GIP and GLP-1 exhibit glucose intolerance[49]. Likewise, the importance of enteroendocrine cells in lipid absorption has recently been shown with the generation of intestinal-conditional Ngn3 null mice[46]. A study with Gip-receptor null mice revealed a crucial role for GIP in promoting the efficient storage of ingested fat suggesting that inhibition of the GIP signal could represent a therapeutic approach against obesity[50]. Further analysis will be needed to better evaluate the impact and possible networking of small intestinal endocrine cell deregulation following the loss of Pten signalling on overall metabolism in the mouse.

ACKNOWLEDGMENTS

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COMMENTS

Background

The phosphatase and tensin homolog (*PTEN*) tumour suppressor gene is a lipid and protein phosphatase frequently mutated/deleted in various human cancers. Its best-known substrate, the phosphatidylinositol 3,4,5-trisphosphate, is a lipid second messenger mainly produced by class IA phosphatidylinositol 3-kinases (PI3Ks). PI3Ks have been implicated in many signalling pathways that regulate cell survival, growth, proliferation, migration, phagocytosis, and metabolism. In previous study, authors reported that *Pten* is important for intestinal homeostasis as well as in the commitment of enteroendocrine cells. The important role of enteroendocrine cells in whole body homeostasis prompted people to further analyze the effect of intestinal epithelial deletion of *Pten* on the specification of the various enteroendocrine subpopulations.

Research frontiers

Enteroendocrine cells located in the gut epithelium are the largest and least understood population of hormone-producing cells in the body. The various hormones and peptides produced by these endocrine cells control important physiological functions, such as gastrointestinal motility, glycaemia, exocrine pancreatic secretion, biliary secretion, digestion, gut epithelial renewal and appetite. In recent years, studies have placed the regulation of these gut hormones as potential targets for novel treatments of metabolic diseases such as type 2 diabetes and obesity.

Innovations and breakthroughs

In the current study, the authors report a distinctive role for Pten in specification/differentiation of enteroendocrine cell subpopulations. Pten signalling negatively regulates the enteroendocrine subtype specification of non-expressing chromogranin A (CgA) cells such as glucose-dependent insulinotropic peptide and somatostatin expressing cells. In contrast, Pten signalling affects positively CgA-expressing cells such as ghrelin, gastrin and cholecystokinin cells.

Applications

Many of these enteroendocrine cell subtypes are known to play critical roles in whole body homeostasis. These experimental data can be used in further studies to better evaluate the impact on general metabolism and possible networking of small intestinal endocrine cell deregulation following the loss of Pten signalling.

Peer review

This is a high quality descriptive study in which authors analyze the impact of the *PTEN* intestinal knockdown in the specification of intestinal enteroendocrine subpopulations.

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ORIGINAL ARTICLE

Overexpression of Dickkopf-3 induces apoptosis through mitochondrial pathway in human colon cancer

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Abstract

AIM: To investigate the mechanisms of the biological roles of Dickkopf-3 (Dkk-3) in cell invasion, survival and apoptosis in colon cancer cells.

METHODS: Three human colon cancer cell lines, i.e., HT-29, LoVo and SW480, were used. Overexpression of Dkk-3 induced by pEGFP-N1-Dkk-3-GFP plasmid in LoVo cells was performed using Lipofectamine 2000 reagent. Reverse transcription polymerase chain reaction and Western blotting were performed to determine the mRNA and protein expression levels of Dkk-3, respectively. Cell proliferation assay, cell cycle analysis, hoechst 33258 assay and Matrigel invasion assay were performed on Dkk-3 overexpressing transfectants.

RESULTS: The mRNA and protein expressions of Dkk-3 in HT-29 (mRNA: 0.06 ± 0.02, protein: 0.06 ± 0.01) and LoVo (mRNA: 0.07 ± 0.02, protein: 0.07 ± 0.02) cells were significantly lower than that in SW480 cells (mRNA: 0.92 ± 0.04, protein: 0.69 ± 0.13; all $P < 0.05$), and the greatest levels of invasiveness was in LoVo cells. Dkk-3 overexpression inhibited the proliferation and invasion of LoVo cells and induced cell cycle arrest at G_0/G_1 phase and subsequent apoptosis, as indicated by increased chromatin condensation and fragments, upregulated Bax and cytochrome c protein, downregulated survivin and Bcl-2 protein, and the activation of caspase-3 and caspase-9. Furthermore, Dkk-3 overexpression reduced the accumulation of cytosolic fraction of β-catenin.

CONCLUSION: Dkk-3 overexpression induced apoptosis in human colon cancer possibly through the mitochondrial pathway. Dkk-3 may be involved in the Wnt/β-catenin signaling pathways in colon cancer.

Key words: Dickkopf-3; Overexpression; Invasion; Apotosis; Colon cancer; Mitochondria

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INTRODUCTION

The prevalence of colorectal cancer is increasing in Asia. Many Asian countries, including China, Japan, South Korea, and Singapore, have experienced a 2-4 folds increase in the incidence of colorectal cancer (CRC) during the past few decades[1]. Even in the United States, colorectal cancer is the third most commonly diagnosed cancer and the second leading cause of cancer deaths among cancers

that affect both men and women[2,3]. However, the cellular mechanisms involved in CRC are not fully described. Recent studies have shown that the Wnt signaling pathway, which is composed of canonical Wnt signaling *via* Wnt/β-catenin and noncanonical Wnt signaling *via* the Wnt/Ca^{2+} pathway and Wnt/c-Jun N-terminal kinase (JNK) (planar cell polarity), regulates proliferation, fate specification, polarity and migration of cells[4,5]. The Wnt signaling pathway can be blocked by two functional classes of Wnt antagonists: the secreted frizzled-related proteins (sFRP) and the Dickkopf (Dkk)[6].

Dkk-3, also known as reduced expression in immortalized cells, is a member of a recently identified gene family encoding secreted proteins that control cell fate during embryonic development[7-9]. Deletion at Dkk-3 locus has been found in many cancers, such as lung cancer[10], gastric cancer[11] and ovarian cancer[12]. In acute lymphoblastic leukaemia[13], prostate cancer[14], bladder cancer[15,16] and renal cell carcinoma[16], Dkk-3 expression is reduced or silenced. Interestingly, Dkk-3 is strongly expressed at the base of the crypts in human colon, which is known to contain proliferating epithelial precursor cells[17]. Therefore, Dkk-3 may be an important component of the gastrointestinal proliferative regulatory net work[17].

However, the relationship between Dkk-3 and colon cancer remains unclear. We hypothesized that: (1) Dkk-3 expression may be inhibited epigenetically in colon cancer cells; (2) Dkk-3 may be a tumor suppressor and plays an important role in mitochondria-mediated apoptosis; and (3) Dkk-3 may be involved in the Wnt/β-catenin signaling pathways in colon cancer cells. In the present study, we investigated the mechanisms of the biological roles of Dkk-3 in cell invasion, survival and apoptosis of human colon cancer cells.

MATERIALS AND METHODS

Construction of expressing plasmids

The pEGFP-N1-Dkk-3-GFP plasmid constructed to target Dkk-3 (RefSeq ID: BC007660) was obtained from Genechem Co., Ltd. (Shanghai, China). pEGFP-N1 plasmid (Genechem Co., Ltd.) was cut with *Xho* I /*Kpn* I and ligated by T4 DNA ligase with gene encoding Dkk-3, making Dkk-3-pEGFP construct. The plasmid construct was confirmed by DNA sequencing.

Cell culture and transfection conditions

The human colon cancer cell lines HT-29, LoVo and SW480 were obtained from the Cell Collection Center of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (GibcoBRL, Grand Island, NY, United States) supplemented with 10% fetal bovine serum and were maintained in a humidified incubator at 37 ℃ with a supply of 5% CO_2/95% air atmosphere. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 48 h of transfection, cells were used for cell cycle analysis, hoechst 33258 assay, Matrigel invasion assay, reverse transcription polymerase chain reaction (RT-PCR) analysis and Western blotting analysis. The transient expression of green fluorescent protein (GFP) was detected under a fluorescence microscope (Olympus; Shinjuku-ku, Tokyo, Japan) at an excitation wavelength of 460-490 nm.

RT-PCR

After 48 h of transfection, total cellular RNA was isolated by Trizol (Invitrogen, Carlsbad, CA) and 2 μg of RNA was treated with DNase and used as a template for the reverse transcription reaction following the manufacturer's instructions (Fermentas, United States). The resultant cDNA was then used in PCRs and analyzed by gel electrophoresis. The following primers were used: Dkk-3 sense 5'-GGGAGACGAAGAAGGCAGAAGG-3' and Dkk-3 antisense 5'-CCAGGTGATGAGGTCCAGAAGC-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense, 5'-AGGTGAAGGTCGGAGTCAAC-3', and GAPDH anti-sense, 5'-CGCTCCTGGAAGATGGTGAT-3'. The PCR conditions were as follows: 94 ℃ for 5 min, followed by 35 cycles of 94 ℃ for 30 s, 60 ℃ for 30 s, and 72 ℃ for 1 min. The final extension was at 72 ℃ for 5 min. PCR products were analyzed on 1.2% agarose gels containing 0.5 g/mL ethidium bromide and were visualized under ultraviolet light. Band density was analyzed and quantified using Genetools software (Syngene, Cambridge, United Kingdom).

Western blotting analysis

Equal amounts of proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Millipore, Bedford, MA, United States) by wet transfer system (Bio-Rad, Hercules, CA, United States). Membranes were blocked with 10% non-fat dry milk in Tris-buffered saline Tween-20 and incubated first with primary antibodies at 4 ℃ overnight and then with horseradish peroxidase-conjugated anti-mouse, anti-rabbit or anti-goat secondary antibody for 2 h at room temperature. The following antibodies were used: Dkk-3 (R and D Systems Inc., Minneapolis, MN, United States) 1.5 μg/mL, β-catenin (Abcam, Cambridge, United Kingdom) 1:5000, survivin, Bax, Bcl-2 and Cyt-c (Santa Cruz Biotechnology, Santa Cruz, CA, United States) 1:1000, Caspase-9 (Abcam, United States) 1:1000, Caspase-3 (Abcam, United States) 1:250 and Actin (Santa Cruz Biotechnology, Santa Cruz, CA, United States) 1:2000. Specific proteins were visualized using an enhanced chemiluminescence system (Millipore, Bedford, MA, United States) and then exposed with Kodak X-ray film. Protein band intensities were determined densitometrically using the video-imaging CMIASWIN system (Bio-Rad, Hercules, CA, United States).

Cell proliferation assay

Cell proliferation was determined by the WST-8 tetrazolium salt assay (Cell Counting Kit-8, Beyotime Inst Bio-

tech, China), which quantifies the amount of formazan dye formed when tetrazolium salt is cleaved by cellular mitochondrial dehydrogenase present in viable cells. Cells were seeded in 96-well plates at a density of 2 × 10^3/well in 0.1 mL of culture medium. Viability of cells 0, 12, 24, 36, 48, 60 and 72 h after transfection was evaluated. Two hours before the end of the specified incubation period, 10 μL WST-8 reagent was added to the cells. At the end of the incubation, cell density was estimated by measuring the absorbance of the colored formazan reaction product at 450 nm using an iMark Microplate Absorbance Reader (Bio-Rad, United States).

Cell cycle analysis

Cell cycle status was determined by measuring cellular DNA content after staining with propidium iodide by flow cytometry. After 48 h transfection, cells were centrifuged, washed twice with ice-cold phosphate buffer saline (PBS), and fixed in 70% ethanol at 4 ℃ for 24 h. Cells were then centrifuged at 1000 r/min for 5 min, and the supernatant was discarded. The pellets were then washed twice with 4 mL PBS and then stained with 0.5 mL RNase A (2 mg/mL) and 0.5 mL propidium iodide (0.1% in 0.6% Triton-X in PBS) for 30 min in the dark. Samples were then analyzed on a FACSCalibur flow cytometer (Beckman Coulter, Inc. Fullerton, CA).

Hoechst 33258 assay for apoptosis

Apoptotic cells were detected by Hoechst 33258 staining following the manufacturer's protocol (Apoptosis Hoechst staining kit, Beyotime Biotechnology, Jiangsu, China) after 48 h transfection. Briefly, cells were first fixed in 0.5 mL methanol for 30 min and then rinsed with PBS twice; 1 mg/mL Hoechst 33258 reagent was used to stain the apoptotic cells in dark at room temperature for 5 min, after which the cells were again washed with PBS twice. The stained cells were examined and immediately photographed under a fluorescence microscope (Olympus; Shinjuku-ku, Tokyo, Japan) at an excitation wavelength of 330-380 nm. Apoptotic cells were identified on the basis of morphologic changes in their nuclear assembly by observing chromatin condensation and fragment staining by Hoechst 33258. In each group, ten microscopic fields were selected randomly and counted.

Invasion assay

Transwell chambers (Corning, New York, NY, United States) were used to examine the ability of cells to invade through a Matrigel-coated filter following the manufacturer's instructions. DMEM was added to the upper chambers and allowed to hydrate for 2 h at 37 ℃ with 5% CO_2. Next, 1 × 10^5 LoVo cells transfected with various plasmids were added to the upper chamber and grown in serum-free medium on 8.0 μm porous polycarbonate membranes, which were coated with diluted Matrigel basement membrane matrix. The lower chambers were filled with DMEM medium containing 10% fetal bovine serum. After 24 h incubation, the cells remaining on the upper surface of the filter were removed using cotton tips, and the cells that migrated to the underside of the membrane were fixed with 4% paraformal dehyde and stained with Giemsa (Sigma). Cells in 10 random fields of view at ×400 magnifications were counted and expressed as the average number of cells/field of view.

Colony formation assay

Cells from the colon cancer cell line LoVo (2 × 10^5 cells per well) were transfected with 0.5 μg Dkk-3-expressing or empty vector (pEGFP-N1) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Transfected cells were selected with antibiotic G-418 Sulfate (0.4 mg/mL) (Merck, Darmstadt, Germany) for 2 wk. Colonies were fixed with methanol/acetone (1:1), stained with Giemsa, and counted. All experiments were performed in triplicate.

Statistical analysis

All continuous values were expressed as mean ± SD. One-way analysis of variance was used for comparisons among groups. Student's *t* test was used for comparison of the values between two groups. SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, United States) was used for statistical analysis. Statistical significance was defined as $P < 0.05$.

RESULTS

Correlation between Dickkopf-3 expression levels and invasion ability in human colon cancer cell lines

To determine the endogenous expression of Dkk-3, we compared the Dkk-3 level in three human colon cancer cell lines (HT-29, LoVo and SW480). As shown in Figure 1A and B, Dkk-3 expression was significantly higher in SW480 cells (mRNA: 0.92 ± 0.04, protein: 0.69 ± 0.13; all $P < 0.05$) as compared with HT-29 (mRNA: 0.06 ± 0.02, protein: 0.06 ± 0.01) and LoVo cells (mRNA: 0.07 ± 0.02, protein: 0.07 ± 0.02). We also examined the ability of these cells to invade Matrigel, which is a well-established *in vitro* model for assessing tumor invasiveness. The result showed that the greatest levels of invasiveness was in LoVo cells (19.25 ± 1.65), which was followed by the SW480 (15.50 ± 2.12) and HT-29 (8.75 ± 2.10, $P < 0.05$ *vs* LoVo or SW480), an order consistent with their known metastatic potentials (Figure 1C). These preliminary findings provoked us to track the question of whether modulation of Dkk-3 could affect colon cancer progression.

Overexpression of Dickkopf-3 by pEGFP-N1-Dkk-3-GFP plasmid in human colon cancer LoVo cells

To study the biological role of Dkk-3 in colon cancer progression, we used pEGFP-N1-Dkk-3-GFP plasmid coding for full-length human Dkk-3 to enhance the Dkk-3 gene expression in the human colon cancer LoVo cells. The expression of the recombinant human Dkk-3 was analyzed by RT-PCR and Western blotting

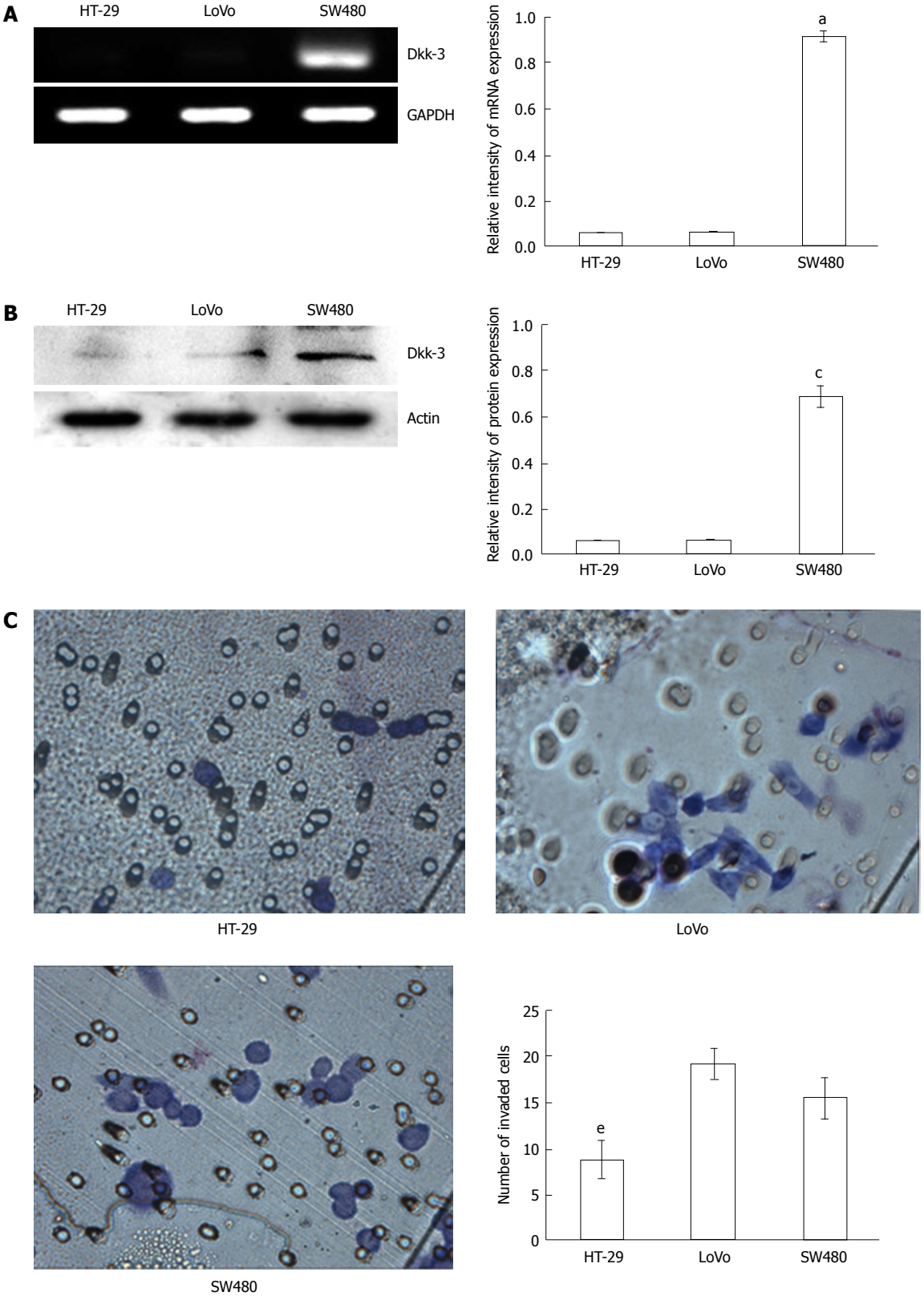

Figure 1 Levels of Dickkopf-3 mRNA and protein expression correlate with invasive potential of human colon cancer cell lines. A: Semi-quantitative reverse transcription polymerase chain reaction of RNA extracted from colon cancer cell lines, HT-29, LoVo and SW480, respectively, and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified as a control. [a]$P < 0.05$ *vs* HT-29 or LoVo using Student's *t* test; B: Endogenous Dickkopf-3 (Dkk-3) protein expression was examined by immunoblot analysis of total cellular protein isolated from three colon cancer cell lines: HT-29, LoVo and SW480, and actin was utilized as a loading control. [c]$P < 0.05$ *vs* HT-29 or LoVo using Student's *t* test; C: Human colon cancer cells, HT-29, LoVo and SW480 invading through the Matrigel were counted under a microscope in ten random fields at × 400 magnification. [e]$P < 0.05$ *vs* LoVo or SW480 using Student's *t* test.

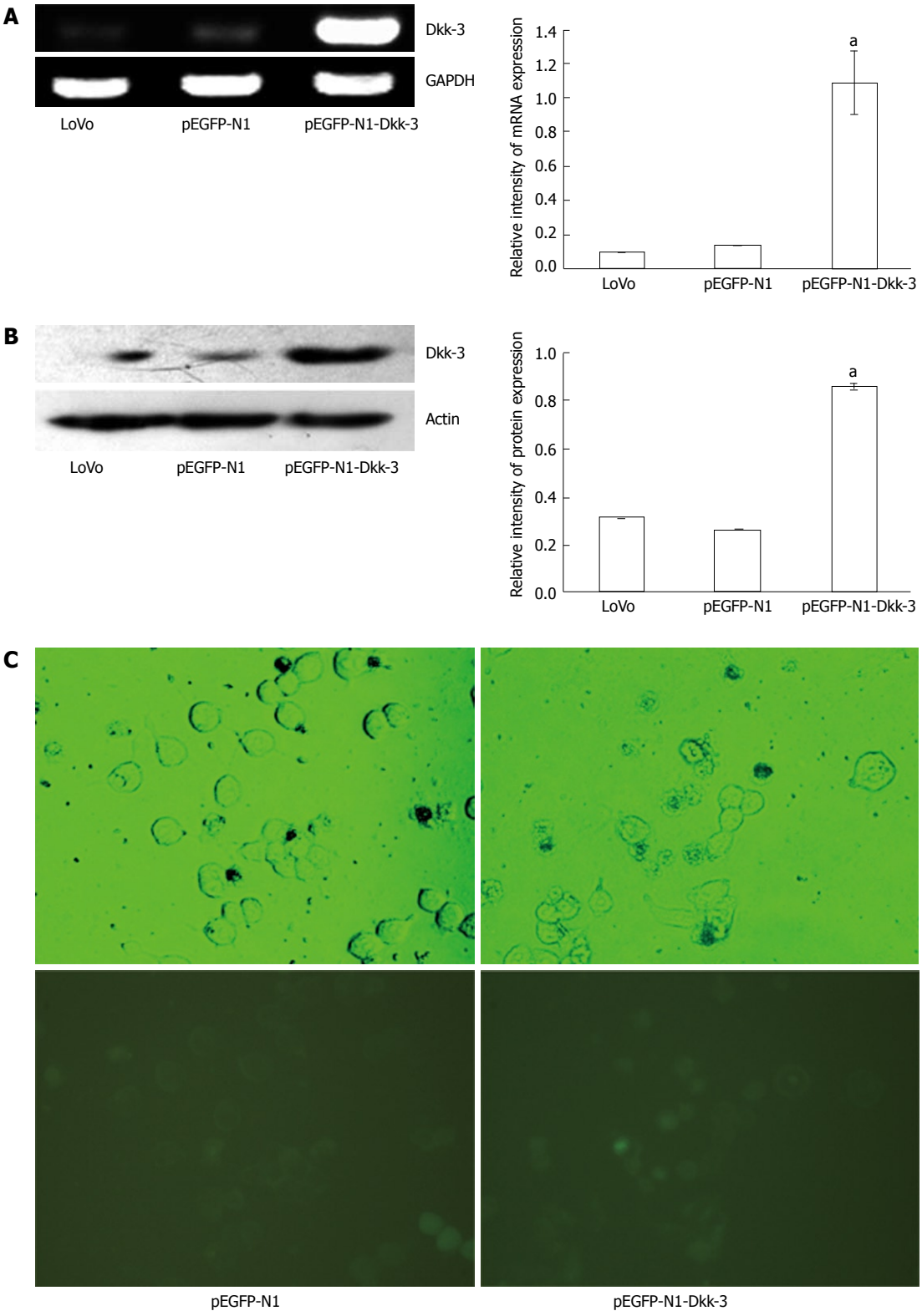

Figure 2 pEGFP-N1-Dkk-3-GFP plasmid induces overexpression of Dickkopf-3 in human colon cancer LoVo cells. A: Semi-quantitative reverse transcription polymerase chain reaction of RNA extracted from pEGFP-N1-Dkk-3-GFP plasmid transfected LoVo cells and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified as a control; B: Immunoblotting of total protein lysates extracted from pEGFP-N1-Dkk-3-GFP plasmid transfected LoVo cells, and actin was included as a loading control; C: Green fluorescent protein (GFP) was also detected under a fluorescence microscope in pEGFP-N1-Dkk-3-GFP plasmid transfected LoVo cells (× 400). $^{a}P < 0.05$ *vs* LoVo or pEGFP-N1 using Student's *t* test.

analysis. As shown in Figure 2A, analysis of the transfected cells (1.09 ± 0.11, $P < 0.05$) for Dkk-3 expression *via* semi-quantitative RT-PCR demonstrated a specific increase in mRNA levels for each gene relative to the pEGFP-N1 plasmid-transfected cells (0.14 ± 0.02) or untreated LoVo cells (0.10 ± 0.02). Immunoblot analysis

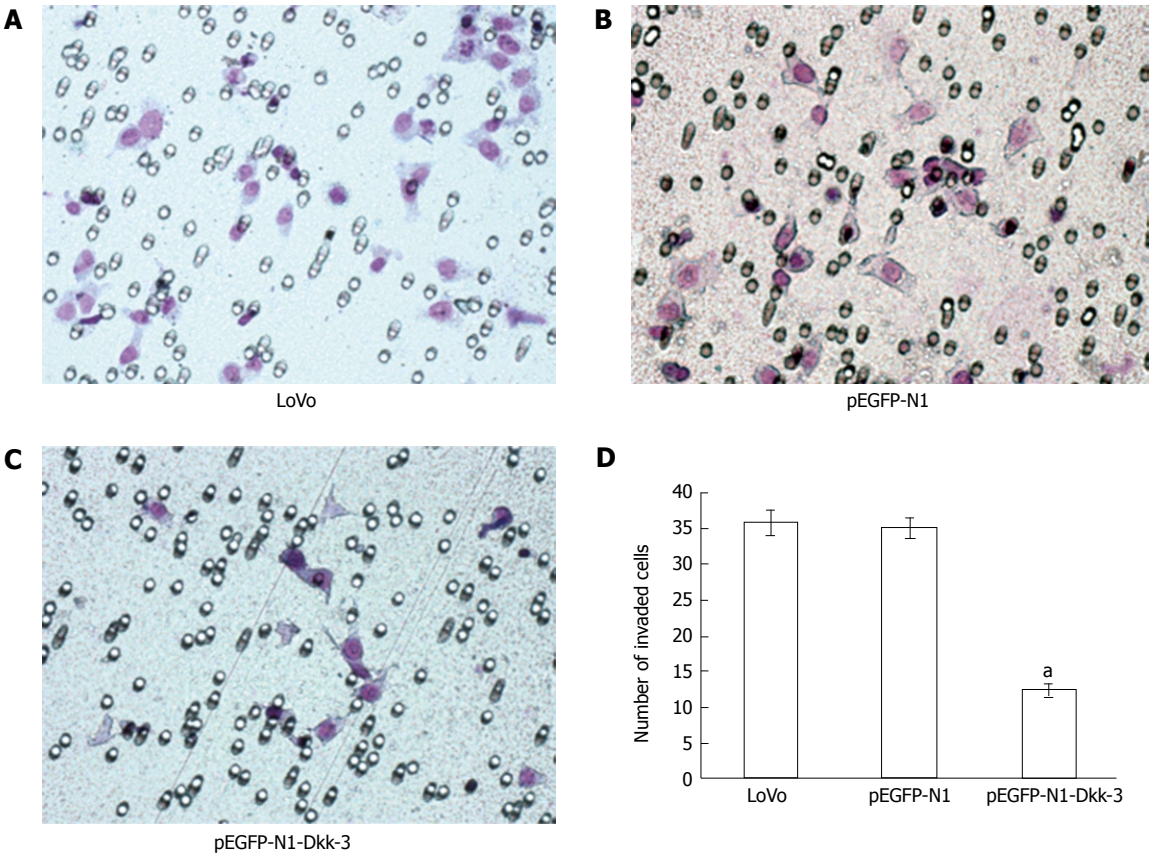

Figure 3 Overexpression of Dickkopf-3 inhibits invasion in human colon cancer LoVo cells. Representative number of invading cells through the Matrigel was counted under microscope in ten random fields at × 400 magnification. Each bar represented the mean ± SD. $^{a}P < 0.05$ *vs* LoVo or pEGFP-N1 using Student's *t* test. The results are representative of three separate experiments.

of cell extracts was carried out to determine whether increased mRNA expression, as observed, correlated with increased translation of the gene product. Figure 2B shows that the protein expression level of Dkk-3 was significantly increased in pEGFP-N1-Dkk-3 group (0.86 ± 0.12, $P < 0.05$) compared with the pEGFP-N1 group (0.26 ± 0.04) or untreated LoVo cells (0.31 ± 0.04). A similar trend was observed by immunoblot analysis with the result of RT-PCR. The transient expression of GFP was observed under a fluorescence microscope after 48 h transfection (Figure 2C). Figure 2C indicates that the efficient transduction of pEGFP-N1-Dkk-3-GFP plasmid was approximately 70% after 48 h transfection.

Effect of Dickkopf-3 overexpression on invasion in human colon cancer LoVo cells

To evaluate the impact of Dkk-3 overexpression on invasion of human colon cancer LoVo cells, a Matrigel invasion assay was performed. When compared with normal LoVo cells (36.00 ± 1.85) or cells transfected with pEGFP-N1 plasmid (36.25 ± 1.49), pEGFP-N1-Dkk-3-GFP plasmid-transfected cells (12.50 ± 0.96, $P < 0.05$) showed a substantial reduction in invasive ability. Invasion of LoVo cells was reduced to about 70% of the controls by pEGFP-N1-Dkk-3-GFP plasmid (Figure 3). Thus, LoVo cell invasion into Matrigel was substantially regulated by Dkk-3 function. Dkk-3 expression was required for colon cancer cell invasion leading to tumor metastasis.

Effect of Dickkopf-3 overexpression on proliferation in human colon cancer LoVo cells

To assess the potential effects of Dkk-3 overexpression on proliferation in human colon cancer LoVo cells, we investigated cell growth *in vitro*. Using the tetrazolium salt (WST-8) cell viability assay (see "Materials and Methods"), we generated a time-response curve by incubating cultures of transfected LoVo cells for 12, 24, 36, 48, 60 and 72 h, which showed a time-dependent inhibition of cell viability (Figure 4A). pEGFP-N1 transfection had no effect on the proliferative ability of LoVo cells, whereas pEGFP-N1-Dkk-3-GFP plasmid transfection caused a dramatic reduction in the proliferation of LoVo cells ($P < 0.05$). On the other hand, we performed colony formation assays using LoVo cells transfected with a *Dkk-3* gene construct (pEGFP-N1-Dkk-3-GFP) or with an empty vector (pEGFP-N1). The number of colonies formed was counted after 2 wk culture. When compared with the pEGFP-N1 plasmid-transfected cells (154.67 ± 5.86), we observed that *Dkk-3* overexpression (77.00 ± 2.65, $P < 0.05$) decreased markedly the number of colonies (Figure 4C).

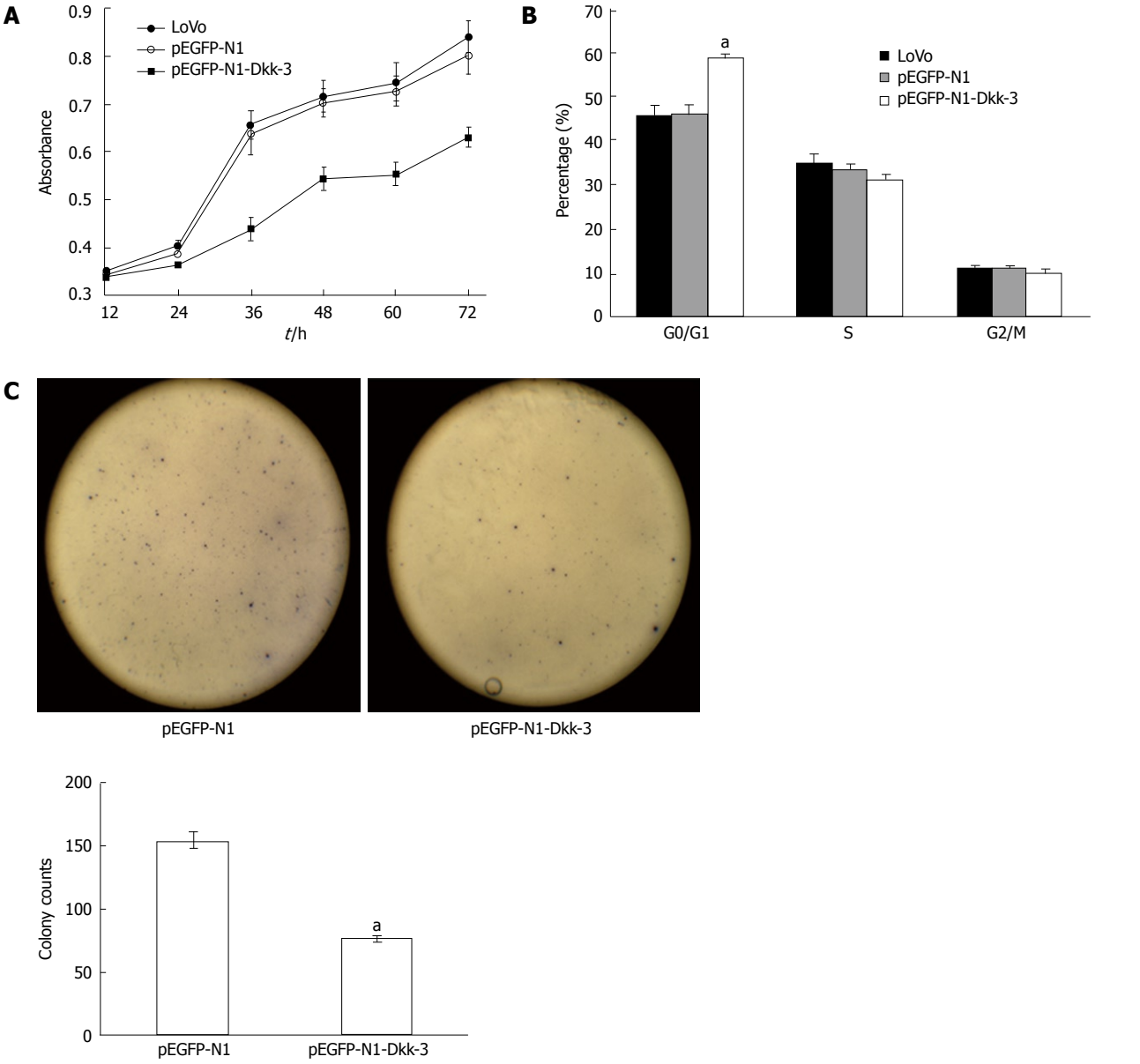

Figure 4 Overexpression of Dickkopf-3 inhibits proliferation and induces G0/G1 arrest in human colon cancer LoVo cells. A: Dickkopf-3 (Dkk-3) inhibits proliferation in human colon cancer LoVo cells; B: Forty-eight hours after transfection, LoVo was used for cell cycle analysis using a FACSCalibur flow cytometer; C: The dickkopf homolog 3 gene Dkk-3 inhibited tumor cell colony formation. LoVo cells were transfected with pEGFP-N1-Dkk-3-GFP plasmid or with pEGFP-N1 and were maintained in the presence of G418 sulfate for 2 wk. Quantitative analysis of colony numbers are shown as the mean ± SD. $^{a}P < 0.05$ *vs* pEGFP-N1 using Student's *t* test.

Effect of Dickkopf-3 overexpression on cell cycle in human colon cancer LoVo cells

To investigate the precise mechanisms of the decreased cell viability observed in LoVo transient *Dkk-3* transfectants, we analyzed the cell cycle distribution profile by flow cytometry with propidium iodide. After 48 h transfection, the cells were fixed and stained with the DNA intercalating fluorescent dye propidium iodide. As shown in Figure 4B, untreated LoVo cells had normal cell cycle profiles with approximately 45% of cells in G_0/G_1 phase containing 2N DNA content and 12% of cells in G_2/M phase containing 4N DNA content. The percentage of cells in the G_0/G_1 phase of the cell cycle was significantly higher in Dkk-3 transfected LoVo cells (0.59 ± 0.01, $P < 0.05$).

Effect of Dickkopf-3 overexpression on apoptosis in human colon cancer LoVo cells

The morphological changes of the apoptotic cells were detected by Hoechst 33258 staining (Figure 5). After 48 h transfection, cells were fixed and stained with Hoechst 33258 at room temperature. In the untreated LoVo cells (0.67 ± 0.52) and pEGFP-N1 group (1.33 ± 1.21), the nuclei were stained weak homogeneous blue, while in the group transfected with pEGFP-N1-Dkk-3-GFP plasmid (63.67 ± 7.71, $P < 0.05$), bright chromatin condensation and nuclear fragmentation were found.

Effect of Dickkopf-3 overexpression on cytoplasmic β-catenin accumulation in human colon cancer LoVo cells

Dkk-3 has been reported to induce changes in β-catenin

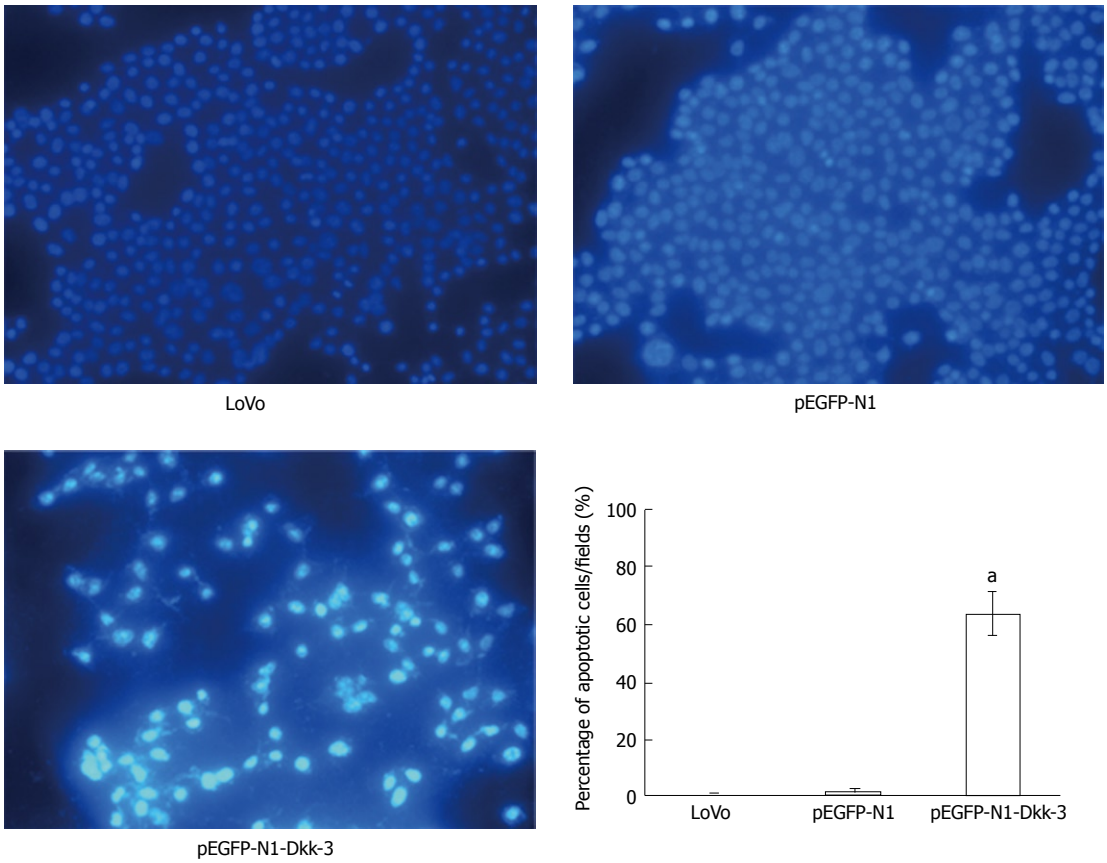

Figure 5 Detection of apoptosis by Hoechst 33285. The apoptotic feature was assessed by observing chromatin condensation and fragment staining. $^{a}P < 0.05$ *vs* control cells (untreated LoVo cells or pEGFP-N1-transfected LoVo cells).

localization consistent with an increase in cell-cell adhesion[9]. To address this question in colon cancer, we examined the β-catenin expression level in mock and *Dkk-3* transfectants. As shown in Figure 6A, transient transfection of *Dkk-3* affected Wnt signaling by reducing the accumulation of cytosolic fraction of β-catenin. β-catenin often yields doublets on Western blot analysis, perhaps as a result of being phosphorylated (most often tyrosine or serine phosphorylation)[9].

Overexpression of Dickkopf-3 induces apoptosis through activation of mitochondrial pathway in LoVo cells

To further investigate the detailed apoptotic mechanism, we examined the effect of *Dkk-3* overexpression on mitochondrial pathway. As shown in Figure 6B, *Dkk-3* overexpression caused a decline in survivin levels, a member of the inhibitors of apoptosis proteins family, which is known to block apoptosis by inhibiting caspases and mitochondria-mediated apoptosis[18]. It has been proposed that one of the main regulatory steps of programmed cell death is controlled by the ratio of antiapoptotic and proapoptotic members of the Bcl-2 family of proteins[19,20]. The role of mitochondrial damage in apoptosis was suggested to be mediated by the release of cytochrome c[21]. Upon cleavage by upstream proteases in an intracellular cascade, the activation of caspase-3 is considered as a hallmark of the apoptotic process[22]. *Dkk-3* overexpression also induced an increase in Bax protein levels and a decrease in Bcl-2 levels in LoVo cells, which led to a decrease in the antiapoptotic/proapoptotic (Bcl-2/Bax) ratio (Figure 6B). In addition, the expression levels of the cytosolic cytochrome c which was suggested to be involved in mitochondrial damage, the activated caspase-3 and the activated caspase-9 were significantly increased with *Dkk-3* overexpression.

DISCUSSION

The Wnt signalling pathway has long been known to direct growth and patterning during embryonic development[23,24]. Recent evidence also implicates that Wnt signalling pathway is involved in the postembryonic regulation of stem-cell number in epithelia, such as those of the skin and intestine, which undergo constant renewal[24]. The pathway is composed of canonical Wnt signaling *via* Wnt/β-catenin and noncanonical Wnt signaling *via* the Wnt/Ca^{2+} pathway and Wnt/c-JNK (planar cell polarity)[4,5]. Wnt signaling pathway is often activated in many cancers and the expression of Wnt antagonists is often downregulated epigenetically[4,7,24-29]. The extracellular antagonists of the Wnt signalling pathway can be divided into two broad classes. Both classes of molecule prevent ligand-receptor interactions, but by different mechanisms: members of the first class, including

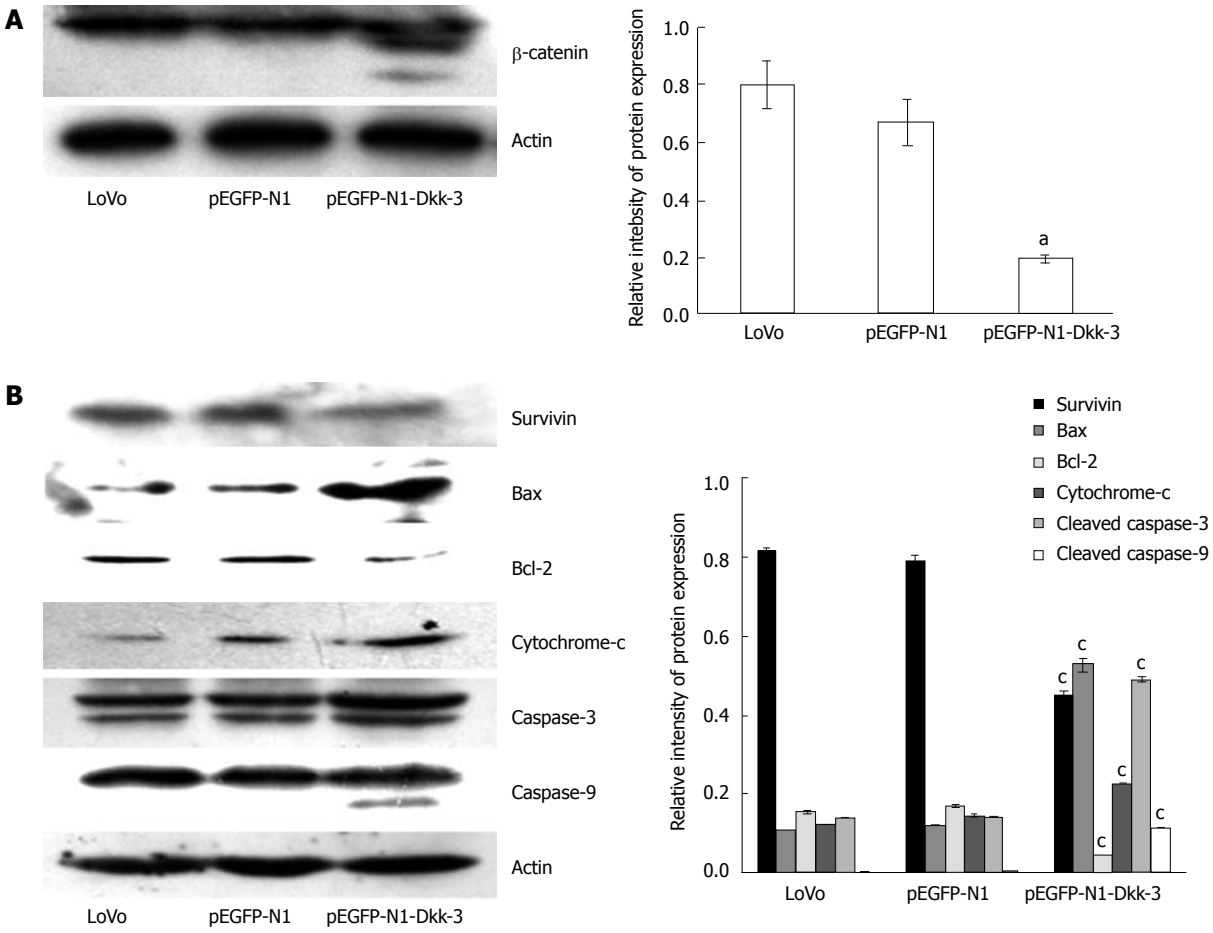

Figure 6 Overexpression of Dickkopf-3 inhibits downstream signaling and induces apoptosis in LoVo cells through mitochondrial pathway. A: Dickkopf-3 (Dkk-3) reduces cytoplasmic accumulation of β-catenin; B: Western blotting analysis of survivin, Bcl-2, Bax, cytochrome c, caspase-3 and caspase-9 protein after 48 h transfection with pEGFP-N1-Dkk-3-GFP plasmid in LoVo cells. Actin was used as a loading control. $^{a}P < 0.05$ *vs* LoVo or pEGFP-N1 using Student's *t* test.

the sFRP family, Wnt inhibitory factor-1 and Cerberus, primarily binds to Wnt proteins; the second class comprising certain members of the Dkk family, binds to one subunit of the Wnt receptor complex[6].

Human Dkk-related genes are composed of Dkk-1, Dkk-2, Dkk-3, and Dkk-4, together with a unique Dkk-3 related protein termed Soggy (Sgy)[7]. Dkk-3, the most divergent family member, is proposed to function as a secreted tumor suppressor since it is downregulated in a number of cancer cells[28]. Dkk-3 has been reported to be silenced or down-regulated in 12 (70.6%) of 17 gastric cancer cell lines and in 3 (33.3%) of 9 colon cancer cell lines, and the loss of gene expression was associated with promoter methylation, which could be restored by demethylating agents[30]. Tissue microarrays have shown that the number of microvessels in Dkk-3-positive CRC samples was significantly higher than that in Dkk-3-negative samples ($P = 0.001$), and Dkk-3 expression was also increased with rising numbers of microvessels ($P < 0.0001$)[29]. In addition, Dkk-3 has been revealed to inhibit cancer proliferation and induce apoptosis in several cancers[25,26,31-36]. Overexpression of Dkk-3 in normal fibroblasts suppresses tumor growth *via* induction of interleukin-7[32]. In malignant glioma[37], Dkk-3 transfection led to apoptosis due to the activation of phosphorylated jun proto-oncogene, caspase-9, and caspase-3 and the reduction of β-catenin. In renal cell carcinoma[25], prostate cancer[26,31,35], testicular cancer[38], bladder cancer[34] and breast cancer[33], overexpression of Dkk-3 was found to lead to apoptotic cell death in a c-JNK phosphorylation-dependent manner and/or endoplasmic reticulum stress. In osteosarcoma[9], transfection of Dkk-3 and dominant-negative LRP5 significantly lowers the cell invasion capacity and cell motility, and also induces changes in β-catenin localization consistent with an increase in cell-cell adhesion. However, Dkk-3 functional analysis and the regulation mechanism have not been reported in colorectal cancer.

In the present study, we focused on proliferation and apoptosis of colon cancer cells. We examined the anti-proliferation ability of Dkk-3 overexpression by pEGFP-N1-Dkk-3-GFP plasmid in human colon cancer LoVo cells, and measured the extent of cell proliferation by the WST-8 assay (Figure 4A). Interestingly, overexpression of Dkk-3 effectively suppressed cellular proliferation of colon cancer cells in a time-dependent fashion (Figure 4A). On the other hand, overexpression of Dkk-3 inhibited tumor cell colony formation in LoVo

cells (Figure 4C).

To investigate the precise mechanisms responsible for the Dkk-3 overexpression-mediated abortive cell divisions, we sought to examine the cell cycle distribution profile of Dkk-3 transfectants. The percentage of cells arrested in G_0/G_1 phase of the cell cycle was also increased in Dkk-3 transfectants. It was also revealed that Dkk-3 overexpression resulted in apoptosis (Hoechst 33258) in Dkk-3 transfectants (Figure 5). The morphological features in LoVo cells of apoptotic *vs* necrotic cell death can be distinguished under microscopy[39]. Apoptotic LoVo cells were identified by observing chromatin condensation and fragment staining by Hoechst 33258. It suggests that if early apoptotic cells are not ingested by phagocytes in time, secondary necrosis would proceed[40].

One of the main regulatory steps of programmed cell death is controlled by the ratio of antiapoptotic and proapoptotic members of the Bcl-2 family of proteins[19,20]. Overexpression of antiapoptotic Bcl-2 family members can tip the delicate balance in favor of survival, thereby conferring drug resistance, at least in some cellular tumor model systems[41-43]. On the other hand, overexpression of proapoptotic Bax or Bak is sufficient to increase the sensitivity of malignant cancer cells to apoptosis and to overcome drug resistance[43-45]. Bcl-2 in the unphosphorylated form complexes with Bax, and thus its phosphorylation releases Bax from the Bcl-2-Bax complex[22,46-48]. Unbound Bax translocates from cytosol to the mitochondrial membrane to signal triggering of the downstream apoptotic cascade, such as release of cytochrome c and activation of executionery caspases[22,44-46]. The activation of caspase-3, upon its cleavage by upstream proteases, is considered as a hallmark of the apoptotic process[41]. In agreement with the hypothesis, activated caspase-3 and -9 were detected in Dkk-3 transfectants. Our results showed that overexpression of Dkk-3 decreased Bcl-2/Bax ratio, caused the release of cytochrome c, and the activation of caspase-3 and caspase-9 in LoVo cells (Figure 6B). Further studies are required to determine the exact mechanism whether Dkk-3 enhances apoptosis-inducing effects on human colon cancer cells, which is cross-talking activation of death receptors pathway of apoptosis.

In conclusion, Dkk-3 is anti-proliferative and pro-apoptotic in colon cancer LoVo cells. Overexpression of Dkk-3 caused 2N DNA accumulation in LoVo cells, suggesting that the Dkk-3 transfectants arrest in G_0/G_1 phase preceding cell death. These abnormal cells probably trigger activation of programmed cell death that is mitochondrially-driven and executed through the activated caspase by the cleavage of downstream targets. The LoVo cell death program is also mediated through downregulation of survivin. Therefore, Dkk-3 functions as a tumor suppressor in colon cancer cells and its downregulation may be involved in colon cancer progression. Moreover, Dkk-3 may be involved in the Wnt/β-catenin signaling pathways in colon cancer cells.

COMMENTS

Background

The Wnt signal transduction pathway is activated in many cancers and the expression of Wnt antagonists are often downregulated epigenetically. Wnt antagonists can be divided into two functional classes, the secreted frizzled related proteins and the Dickkopf (Dkk). The *Dkk* gene family of secretory modulators of canonical Wnt/β-catenin signaling is involved in the control of proliferation, polarity and migration, cell fate specification and differentiation. Dkk-3, also known as reduced expression in immortalized cells, is the most divergent family member and proposed to function as a secreted tumor suppressor since it is downregulated in a number of cancer cells.

Research frontiers

Recently, Wnt antagonists have received increasing and specific attention due to their potential role in carcinogenesis. Dkk-3 has been revealed to inhibit cancer proliferation and induce apoptosis in malignant glioma, breast cancer, osteosarcoma, renal cell carcinoma, prostate cancer, testicular cancer and bladder cancer.

Innovations and breakthroughs

Few studies have described the correlation between Wnt antagonists and the development of colon cancer. The results of this study suggest that Dkk-3 may act as negative regulators of Wnts and may be involved in the Wnt/β-catenin signaling pathways in colon cancer cells. Dkk-3 may be a crucial Wnt signaling regulator in colon cancer and an important component of the gastrointestinal proliferative regulatory net work.

Applications

In this study, the mRNA and protein expressions of Dkk-3 were investigated in colon cancer cells and that the aberrant expression of Wnt antagonists may play an important role in carcinogenesis of colon cancer. This finding may help improve early diagnosis and new therapies by blocking this pathway in the treatment of colon cancer.

Terminology

The Wnt signaling pathway is one of evolutionarily-conserved signal transduction pathways to direct growth and patterning during embryonic development, from Hydra to humans. Wnt signals regulate many aspects of development which include the proliferation, fate specification, polarity, and migration of cells. Moreover, overactivation of Wnt signaling by mutation is an important factor in oncogenesis in the human colon and other tissues. The pathway is composed of canonical Wnt signaling *via* Wnt/β-catenin and noncanonical Wnt signaling or pathways that are β-catenin independent.

Peer review

The authors investigated the mechanisms of the biological roles of Dkk-3 in colon cancer. It revealed that Dkk-3 played an important role in mitochondria-mediated apoptosis and Dkk-3 may be involved in the Wnt/β-catenin signaling pathways in colon cancer cells. The article is a good attempt to work on the hypothesis and the results may represent a molecular mechanism of colon carcinogenesis.

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ORIGINAL ARTICLE

Toxicarioside A inhibits SGC-7901 proliferation, migration and invasion *via* NF-κB/bFGF signaling

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Abstract

AIM: To investigate the inhibitory role of toxicarioside A on the gastric cancer cell line human gastric cancer cell line (SGC-7901) and determine the underlying molecular mechanism.

METHODS: After SGC-7901 cells were treated with toxicarioside A at various concentrations (0.5, 1.5, 4.5, 9.0 μg/mL) for 24 h or 48 h, cell viability was determined by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay, and the motility and invasion of tumor cells were assessed by the Transwell chamber assay. Immunofluorescence staining, reverse transcription polymerase chain reaction and Western blotting were performed to detect the expression of basic fibroblast growth factor (bFGF) and fibroblast growth factor receptor-1 (FGFR1), and nuclear factor-kappa B (NF-κB) activation was examined by electrophoretic mobility shift assay.

RESULTS: The results showed that toxicarioside A was capable of reducing cell viability, inhibiting cell growth, and suppressing cell migration and invasion activities in a time- and dose-dependent manner in SGC-7901 cells. Further analysis revealed that not only the expression of bFGF and its high-affinity receptor FGFR1 but also the NF-κB-DNA binding activity were effectively blocked by toxicarioside A in a dose-dependent manner compared with the control group ($P < 0.05$ or $P < 0.01$). Interestingly, application of the NF-κB specific inhibitor, pyrrolidinedithiocarbamate (PDTC), to SGC-7901 cells significantly potentized the toxicarioside A-induced down-regulation of bFGF compared with the control group ($P < 0.05$).

CONCLUSION: These findings suggest that toxicarioside A has an anti-gastric cancer activity and this effect may be achieved partly through down-regulation of NF-κB and bFGF/FGFR1 signaling.

Key words: Anti-migration; Anti-proliferation; Basic fibroblast growth factor; Gastric cancer; Nuclear factor-kappa B; Toxicarioside A

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INTRODUCTION

Antiaristoxicaria (Pers.) *Lesch* (Moraceae) is a well known precious species widespread in the tropical rain forest of Southeast Asia. Its latex and seeds contain a complex mixture of cardenolide glycosides and is therefore toxic[1]. Representative toxicariosides A-L have recently been identified from the latex and seeds of *Antiaristoxicaria* in our laboratory and by others[2-5]. Classically, cardenolides are used to treat congestive heart failure and arrhythmia[6-8]. Additionally, certain cardenolides extracted from some plants or animals have been demonstrated to be capable of blocking tumor cell proliferation through regulation of cell signal transduction[9-15].

Currently, gastric cancer is one of the leading malignancies in China. However, the treatment outcome is not satisfactory because early diagnosis of gastric cancer remains difficult and most patients have already developed metastatic lesions when diagnosed[16].

Basic fibroblast growth factor (bFGF) has been shown to be a multifunctional growth factor for tumor development[17-20], and it exerts its biological effects mainly through interaction with its high-affinity receptor, fibroblast growth factor receptor-1 (FGFR1)[21-24]. Compiling evidence has demonstrated that bFGF signaling is involved in the development of gastric cancer[25,26].

Nuclear factor-kappa B (NF-κB) is a ubiquitous dimeric transcription factor that plays pivotal roles in regulating the expression of genes encoding cytokines and chemokines that are involved in tumor proliferation, angiogenesis, and synthesis of anti-apoptotic proteins[27,28]. It has been documented that NF-κB can mediate bFGF signaling[29] and some types of cardiac glycosides can block the activation of NF-κB[30,31]. As a result, we hypothesize that cardiac glycosides may suppress gastric tumor growth *via* a decrease in NF-κB activity and blocking of the bFGF signaling pathway. In the present study, we attempted to test this hypothesis in an *in vitro* cell culture model.

MATERIALS AND METHODS

Plant material

Latex of *Antiaristoxicaria* (Pers.) *Lesch* collected in Lingshui county of Hainan Province, China in November 2005 was identified with the assistance of Professor Zhunian Wang at the Institute of Crops Genetic Resources, Chinese Academy of Tropical Agricultural Sciences. The specimen was numbered as No. AN200511.

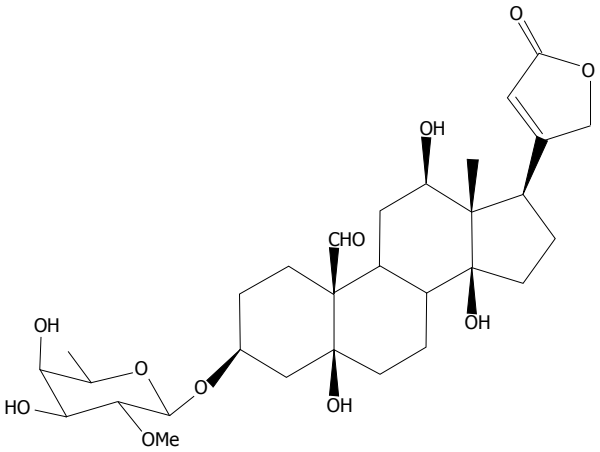

Figure 1 The structure of toxicarioside A.

Chemicals and reagents

Rabbit-anti human bFGF and FGFR1 were purchased from Santa Cruz (Santa Cruz, CA, United States). Rhodamine (TRITC)-conjugated mouse anti-rabbit immunoglobulin G (IgG), fluorescein isothiocyanate (FITC)-conjugated mouse anti-rabbit IgG, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), trypan blue and pyrrolidinedithiocarbamate (PDTC) were obtained from Sigma (Sigma Aldrich, St Louis, MO, United States). Fetal bovine serum (FBS), RPMI 1640 medium and trypsin were procured from Gibco (Gibco, Carlsbad, CA, United States).

Extraction and isolation of toxicarioside A

With 95% EtOH, 4.0 L of the latex of *Antiaristoxicaria* were extracted thrice at room temperature and filtered. The combined extract was evaporated in vacuo to yield a syrup (263.8 g), which was fractionated sequentially with petroleum ether, EtOAc, and n-BuOH. The EtOAc fraction (8.68 g) that showed potent cytotoxic activity in the bioassay was passed through pressure-reduced column chromatography using step-wise elution with $CHCl_3$-MeOH (50:1, 20:1, 10:1, 5:1, 2:1, 1:1 and 0:1, v/v), generating seven corresponding fractions, A1-A7. Fraction A7 (2.55 g) was further separated on silica gel column chromatography, from which compound 1 (788.1 mg) was eluted with $CHCl_3$-MeOH (14:1, v/v). On the basis of spectral data and chemical analyses, compound 1 was defined as toxicarioside A (Figure 1).

Cell culture

Human gastric cancer cell line (SGC-7901) was obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 IU/mL penicillin and 100 mg/mL streptomycin at 37 ℃ in a humidified atmosphere with 5% CO_2. Cells at the logarithmic phase were

used for experiments.

Proliferation assay

MTT assay and trypanblue staining were used to determine the growth and viability of SGC-7901 cells. For the MTT assay, SGC-7901 cells in logarithmic growth were trypsinized and harvested and then the cells were seeded onto a 96-well plate. After 24 h, fresh RPMI 1640 medium containing different concentrations of toxicarioside A (0.5, 1.5, 4.5, 9.0 μg/mL) was added at 100 μL per well, respectively, and 6 replicate wells were used for each of the concentrations. After incubation for different time intervals, 10 μL of MTT (5 mg/mL) was added to each well and the cells were further incubated at 37 ℃ for 4 h. The supernatant was then removed and 100 μL DMSO was added into each well. Absorbance (A value) at a wavelength of 490 nm was measured with a Bio-TekEXL808 microplate reader (Bio-Rad, Hercules, CA, United States). For trypanblue staining, SGC-7901 cells were trypsinized and seeded into 24-well plates at a density of 0.5×10^4/mL. After 4.5 μg/mL toxicarioside A was added, the cells were collected and counted using trypan blue staining under an inverse light microscope for 3 consecutive days.

Invasion and migration assay

Invasion assays were performed in a 24-well Transwell chamber (Corning, Lowell, MA, United States) as previously described[32]. Briefly, each Transwell chamber was coated with 15 μg Matrigel, 5×10^4 cells were seeded to pre-coated filters in 200 μL of serum-free medium containing different concentrations of toxicarioside A (0.5, 1.5, 4.5, 9.0 μg/mL) in triplicate. The lower parts of the chambers were filled with 500 μL of RPMI 1640 medium containing 10% FBS. After incubation in a 5% CO_2 humidified incubator at 37 ℃ for 24 h, the cells on the upper surface were gently removed with a cotton swab, and the filters were fixed with 95% alcohol for 15-20 min and stained with hematoxylin-eosin for 15 min. The number of cells on the lower surface of the filters was quantified under a microscope. The same procedures were followed for the migration assay except the Transwell chambers were not coated with Matrigel.

Immunofluorescence staining

To detect the expression of bFGF as well as its receptor FGFR1 in SGC-7901 cells, the rabbit antibody (1:100) against bFGF and FGFR1 were used. The antigenic sites were localized by TRITC-conjugated mouse anti-rabbit IgG and FITC-conjugated mouse anti-rabbit IgG, and images of antigenic sites were captured under a laser scanning confocal microscope (FV500, Olympus, Tokyo, Japan).

RNA extraction and reverse transcription polymerase chain reaction

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, United States) following the manufacturer's protocols. Reverse transcription polymerase chain reaction was carried out using pairs of primers (Invitrogen) as follows for semiquantitative assessment: bFGF (NM_002006.4) sense, 5'-AAG AGC GAC CCT CAC ATC AA-3'; anti-sense, 5'-TCG TTT CAG TGC CAC ATC CGT CAA TA CC-3', yielding a 225 bp product; FGFR1 (M34641) sense, 5'-CTT CTGT TTC AG-3'; anti-sense, 5'-TCC ACA ATG CAG GTG TAG TT-3', yielding a 354 bp product. β-actin (NM_001101) sense, 5' -GTT GCG TTA CAC CCT TTC TT-3', anti-sense, 5' -CGA AGG CTC ATC ATT CAA AA-3', yielding a 443 bp product. The products were separated by electrophoresis on a 1.5% agarose gel and visualized under UV using the gel documentation system (Bio-Rad Gel Doc1000, Bio-Rad). The mRNA levels of bFGF, FGFR1 were calculated based on the denstometric values of the specific bFGF, FGFR1 bands after adjustment with that of the β-actin band.

Western blotting analysis

This was performed as previously described with minor modifications[33]. Cells were homogenized and separated by sodium dodecyl sulfate-polyacrylamide gel (12.5%) electrophoresis followed by electrophoretic transfer of proteins from the gel to a nitrocellulose membrane blot (Bio-Rad). The blot was incubated with a rabbit anti-bFGF antibody (1:500) or a rabbit anti-FGFR1 antibody (1:500) at 4 ℃ overnight, followed by incubation with the corresponding horseradish peroxidase-conjugated anti-biotin antibody (1:2000) at room temperature for 1 h. The immunoreactive signals were visualized with enhanced chemiluminescence reagents (Pierce, Rockford, IL, United States).

Electrophoretic mobility shift assay

To determine NF-κB activation, electrophoretic mobility shift assay (EMSA) was conducted essentially as described previously[34]. In brief, nuclear proteins (10 μg) were incubated with the reaction buffer for 20 min at room temperature, followed by incubation with oligonucleotide containing the consensus sequence for the NF-κB-DNA binding site (5'-AGAGTGGGAATT TCCACTCA-3')[35] (synthesized by Invitrogen, Shanghai, China). The reaction mixture was separated in a non-denaturing polyacrylamide gel (6%) that was later stained by SYBR Green EMSA staining solution from Molecular Probes (Invitrogen) with continuous, gentle agitation for about 20 min, protected from light. The gel was then washed in 150 mL of dH_2O_2 and the stained nucleic acids were visualized and the image documented under UV using the gel documentation system (Bio-Rad Gel Doc1000).

Statistical analysis

All data are expressed as mean ± SE. For a comparison between two groups, the Student's *t* test was performed. For comparisons among multiple groups, an ANOVA was carried out, followed by a Student-Newman-Keuls test. Differences were considered significant when $P < 0.05$.

Table 1 The inhibition rates of human gastric cancer cell line cells treated with different concentrations of toxicarioside A for different time intervals

Groups	24 h		48 h	
	A value	Inhibitory rate (%)	*A* value	Inhibitory rate (%)
Control	0.879 ± 0.048	0.00 ± 0.00	0.932 ± 0.036	0.00 ± 0.00
Toxicarioside A (μg/mL)				
0.5	0.793 ± 0.062	9.79 ± 7.63	0.752 ± 0.073	19.13 ± 5.38
1.5	0.646 ± 0.041	27.83 ± 4.78[a]	0.596 ± 0.113	36.19 ± 3.81[a]
4.5	0.528 ± 0.078	40.18 ± 3.32[a]	0.443 ± 0.056	49.32 ± 5.17[b]
9.0	0.352 ± 0.092	61.84 ± 6.61[b]	0.301 ± 0.049	66.94 ± 7.03[b]

Data representative of six independent experiments were expressed as mean ± SE. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group.

Table 2 Effect of toxicarioside A on cell migration and invasion

Groups	Dose (μg/mL)	Migration		Invasion	
		Cell number	Inhibition rate (%)	Cell number	Inhibition rate (%)
Control	0.0	69.40 ± 6.38	0.00 ± 0.00	65.60 ± 7.20	0.00 ± 0.00
Toxicarioside A	0.5	62.50 ± 8.90	10.01 ± 9.78	57.80 ± 4.32	10.89 ± 8.64
	1.5	54.80 ± 7.30	22.38 ± 10.64[a]	49.70 ± 5.68	24.03 ± 9.06[a]
	4.5	41.60 ± 5.88	39.58 ± 11.62[a]	36.40 ± 7.94	44.68 ± 9.19[a]
	9.0	35.80 ± 8.32	48.13 ± 10.12[b]	30.10 ± 6.46	54.38 ± 8.17[b]

Data representative of three independent experiments were expressed as mean ± SE. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group.

RESULTS

Effect of toxicarioside A on SGC-7901 cell proliferation

To assess the effect of toxicarioside A on the growth of gastric cancer, SGC-7901 cells were treated at various concentrations (0.5, 1.5, 4.5, 9.0 μg/mL) for 24-48 h and cell viability following these treatments was determined by MTT assays. As shown in Table 1, toxicarioside A reduced SGC-7901 cell viability in a time- and dose-dependent manner. Cell growth curves also showed that toxicarioside A significantly inhibited SGC-7901 cell growth as compared with the control (Figure 2).

Effect of toxicarioside A on SGC-7901 cell migration and invasion

The results of Transwell cell migration and invasion are presented in Table 2 and Figure 3. Clearly, the addition of toxicarioside A to the medium in the upper chamber resulted in significant suppression of SGC-7901 migration and invasion in a dose-dependent manner at 1.5, 4.5 and 9.0 μg/mL, toxicarioside A inhibited SGC-7901 migration by 22.38% ± 10.64%, 39.58% ± 11.62% and 48.13% ± 10.12%, respectively ($P < 0.05$), and inhibited SGC 7901 invasion by 24.03% ± 9.06%, 44.68% ± 9.19% and 54.38% ± 8.17%, respectively ($P < 0.01$), as compared with the control group.

Effect of toxicarioside A on bFGF and FGFR1 in SGC- 7901 cells

At the protein level, the expression of bFGF and FGFR1 was predominantly detected in the cytoplasm of SGC-7901 cells and toxicarioside A significantly decreased this expression, as assessed by immunofluorescence staining (Figure 4A) and Western blotting analysis (Figure 4C). At the mRNA level, the expression of bFGF and FGFR1 was decreased by toxicarioside A in a dose-dependent manner in SGC-7901 cells (Figure 4B).

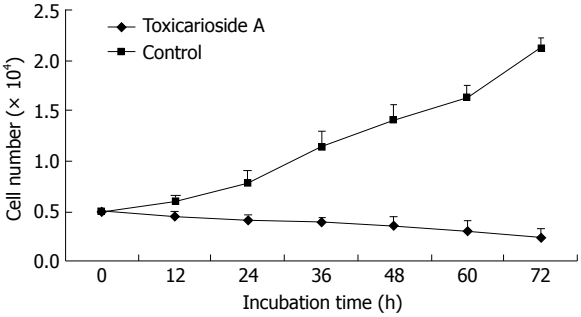

Figure 2 Effect of toxicarioside A on the growth curve of human gastric cancer cell line cells. Cells were plated in 24-well plates at a density of 1 × 10^4/mL and treated with 4.5 μg/mL toxicarioside A for 72 h. The results shown are representative of three independent experiments.

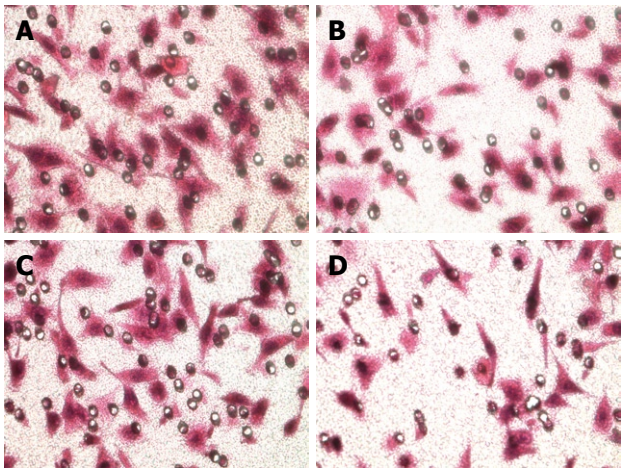

Figure 3 Representative figures of cell migration and invasion in non-treated and toxicarioside A-treated human gastric cancer cell line cells. A: Migration in the control group; B: Migration in the thetoxicarioside A-treated (4.5 μg/mL) group; C: Invasion in the control group; D: Invasion in the toxicarioside A-treated group.

Effect of toxicarioside A on NF-κB-DNA binding activity in SGC-7901 cells

To determine the effect of toxicariosideA on NF-κB activation, the NF-κB-DNA binding activity was determined in both toxicarioside A-treated and control SGC-7901 cells by EMSA. As shown in Figure 5, after treatment with toxicarioside A at various concentrations for 48 h, the NF-κB-DNA binding activity was decreased in a dose-dependent manner as compared with the control group ($P < 0.05$ or $P < 0.01$, Figure 5).

Effect of PDTC on toxicarioside A-induced downregulation of bFGF

To further determine whether NF-κB activation was necessary for bFGF expression, and was involved in toxicarioside A-induced downregulation of bFGF, a specific in-

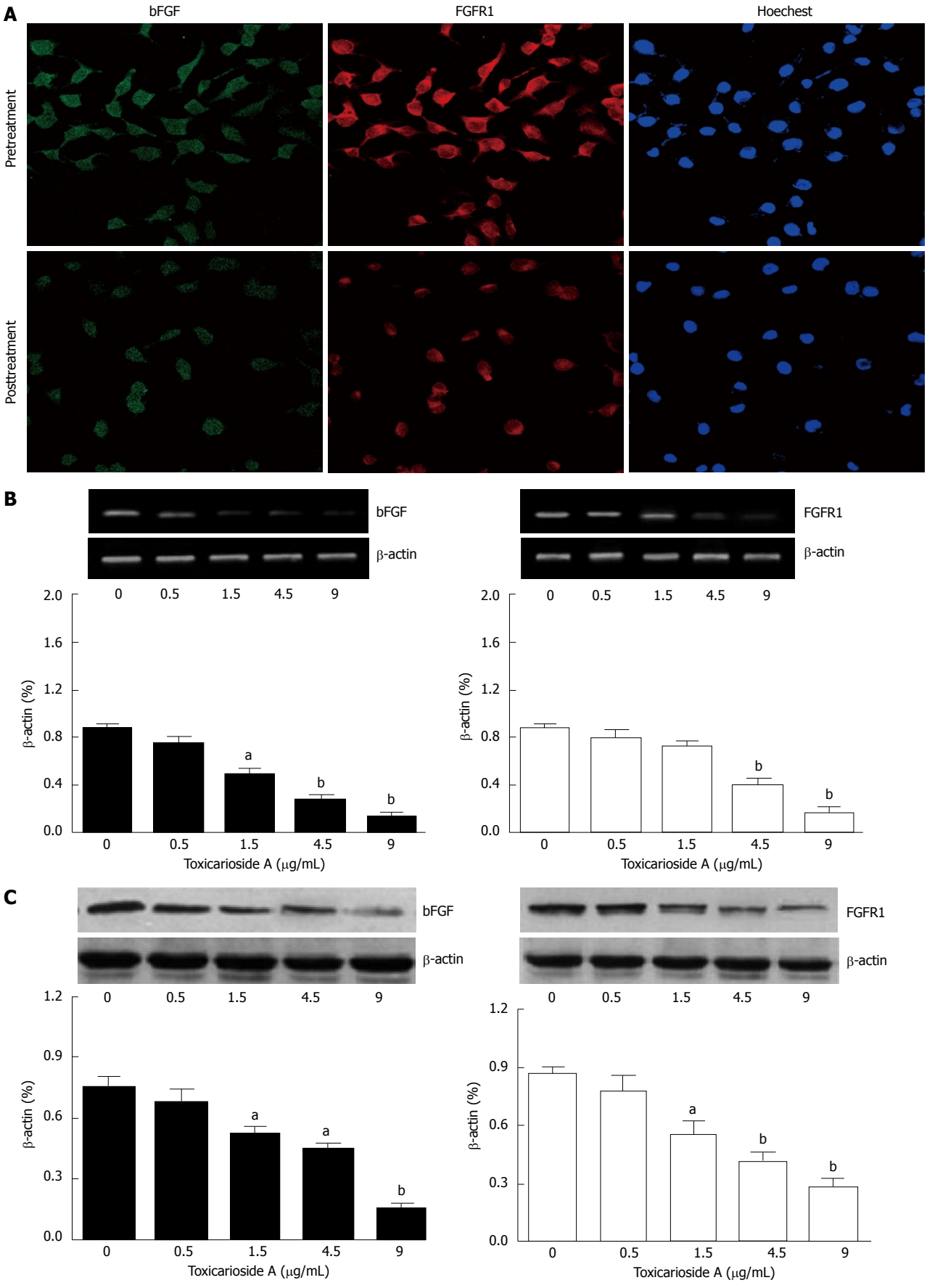

Figure 4 Basic fibroblast growth factor and fibroblast growth factor receptor-1 expression in human gastric cancer cell line cells. A: The expression of basic fibroblast growth factor (bFGF) and fibroblast growth factor receptor-1 (FGFR1) were detected using rhodamine and fluorescein isothiocyanate-conjugated mouse anti-rabbit immunoglobulin G in non-treated and toxicarioside A (4.5 μg/mL)-treated cells; B: bFGF and FGFR1 mRNA expression by reverse transcription polymerase chain reaction; C: bFGF and FGFR1 protein levels by Western blotting analysis. Results are depicted as mean ± SE of three independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

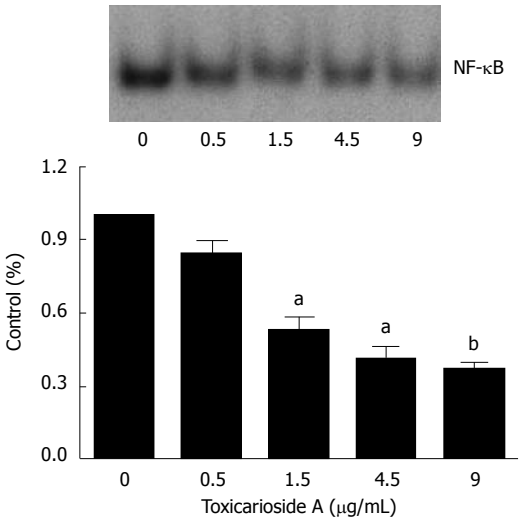

Figure 5 Effect of toxicarioside A on nuclear factor-kappa B-DNA binding activity in human gastric cancer cell line cells. After cells were incubated with various concentrations of toxicarioside A for 48 h, nuclear proteins were isolated and electrophoretic mobility shift assay was performed to determine nuclear factor-kappa B (NF-κB)-DNA binding activity. Results are depicted as mean ± SE of three independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

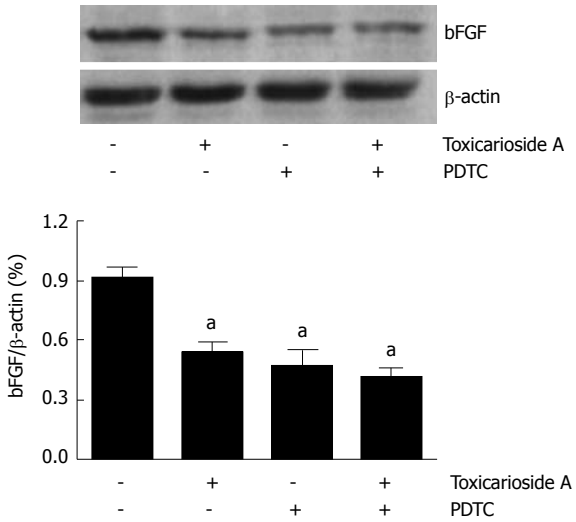

Figure 6 Effect of inhibitor, pyrrolidinedithiocarbamate, on basic fibroblast growth factor protein expression. Administration of pyrrolidinedithiocarbamate (PDTC) (50 μmol/L) reinforced the toxicarioside A (4.5 μg/mL)-induced downregulation of basic fibroblast growth factor (bFGF). Results are depicted as mean ± SE of three independent experiments. $^{a}P < 0.05$ *vs* control group.

hibitor of NF-κB activation, PDTC, was used. As shown in Figure 6, PDTC treatment significantly blocked bFGF expression, which was potentized when both PDTC and toxicarioside A were added to SGC-7901 cells.

DISCUSSION

Antiaristoxicaria (Pers.) *Lesch* (Moraceae) is widespread in the tropical rain forest of southeastern Asia, and is best known for its remedial properties against injuries due to poisoned arrows, darts and blowdarts[36]. The latex-sap and seeds of *Antiaristoxicaria* consists of a complex mixture of active cardenolide glycosides, from which several cardenolides have been isolated in our laboratory and other research groups[2-5]. Besides the classical effect of the cardenolides on inhibition of the ubiquitous cell surface Na^+, K^+-ATPase, the effect of cardiac glycosides on the growth of human malignant tumor cells has been reported in the recent past[11-15]. In the present work, we investigated the anti-cancer activity of toxicarioside A isolated from the latex of *Antiaristoxicaria*. Both the MTT assay and the growth curve analysis revealed that toxicarioside A resulted in inhibition of gastric cancer cell proliferation in a dose- and time-dependent manner. Malignant tumors are characterized by invasion and metastasis, an extremely complex process involving multi-steps. In this study, we assessed the migrating and invasive capabilities of SGC-7901 cells using the Transwell chamber assay. The results demonstrated that toxicarioside A not only suppressed cell motility, but also significantly reduced its ability to degrade the recombinant basement membrane in SGC-7901 cells.

To further investigate the molecular mechanism underlying the anti-tumor properties of cardenolides, we assessed the effect of toxicariosideA on bFGF expression in SGC-7901 cells. It is well known that bFGF, a regulatory factor secreted from cells, is involved in a variety of biological processes including cell differentiation, cell growth, migration, angiogenesis, and tumor formation[19,20]. The biological effect of bFGF is achieved mainly through interaction with its high-affinity receptor, FGFR1[21-24]. To elucidate whether the bFGF/FGFR1 signaling pathway was a target of toxicarioside A in gastric cancer cells, we evaluated changes in the expression of bFGF and FGFR1 in SGC-7901 cells after treatment with toxicarioside A at various concentrations. The results demonstrated that toxicarioside A down-regulated the expression of bFGF and FGFR1 at both mRNA and protein levels in SGC-7901 cells in a dose-dependent manner.

Next, we sought to investigate the molecules involved in the toxicarioside A-induced down-regulation of bFGF in SGC-7901 cells. The NF-κB signaling pathway is a central common regulator for the process of inflammation, viral replication, tumorigenesis, and apoptosis[37,38], and as a result has emerged as a potential target of numerous pharmaceutical agents[39,40]. Our results showed that toxicarioside A had an obvious suppressive effect on NF-κB-DNA binding activity in a dose-dependent manner, and treatment with an NF-κB specific inhibitor augmented the toxicarioside A-induced bFGF down-regulation in SGC-7901 cells, suggesting that the activated NF-κB may be partly necessary for bFGF expression in gastric cancer.

In summary, toxicarioside A weakened the abnormal activation of NF-κB to down-regulate the expression of bFGF, which in turn, interfered with bFGF/FGFR1 signal transduction subsequently leading to suppression ofproliferation, migration and invasion in SGC-7901 cells. Future research will focus on identification of new targets to provide the theoretical basis for the potential

application of toxicarioside A in the clinical treatment of gastric cancer.

COMMENTS

Background

The latex and seeds of *Antiaristoxicaria* contain a complex mixture of cardenolide glycosides, and representative toxicariosides A-L have recently been identified in our laboratory and by others. Some cardenolides have been demonstrated to be capable of blocking tumor cell proliferation through regulation of cell signal transduction.

Research frontiers

Gastric cancer is one of the leading malignancies in China. However, the treatment outcome is not satisfactory because early diagnosis of gastric cancer remains difficult and most patients have already developed metastatic lesions when diagnosed. It is important to investigate the strategies that could inhibit gastric cancer effectively.

Innovations and breakthroughs

To date, little is known about the underlying mechanism regarding the anti-cancer effects of toxicarioside A. Therefore, this study was conducted to investigate the anti-cancer activity of toxicarioside A on gastric cancer growth and migration and the underlying molecular mechanisms *in vitro*.

Applications

This study indicates the first evidence of the underlying molecular mechanisms of the anti-cancer activity of toxicarioside A in gastric cancer. These results provide the theoretical basis for the potential application of toxicarioside A in the clinical treatment of gastric cancer.

Terminology

Antiaristoxicaria (Pers.) *Lesch* (Moraceae) is a well known precious species widespread in the tropical rain forest of Southeast Asia, and the latexsap and seeds of *Antiaristoxicaria* consist of a complex mixture of active cardenolide glycosides.

Peer review

This manuscript showed toxicarioside A inhibits the proliferation, invasion and migration in a gastric cancer cell line, and these phenomena was correlated with down-regulation of nuclear factor-kappa B/basic fibroblast growth factor signaling. The design of study is solid and experiments were elegantly performed.

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BRIEF ARTICLE

Medical treatment for sphincter of oddi dysfunction: Can it replace endoscopic sphincterotomy?

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Abstract

AIM: To report the results of a medical management of sphincter of oddi dysfunction (SOD) after an intermediate follow-up period.

METHODS: A total of 59 patients with SOD (2 men and 57 women, mean age 51 years old) were included in this prospective study. After medical treatment for one year, the patients were clinically re-evaluated after an average period of 30 mo.

RESULTS: The distribution of the patients according to the Milwaukee's classification was the following: 11 patients were type 1, 34 were type 2 and 14 were type 3. Fourteen patients underwent an endoscopic sphincterotomy (ES) after one year of medical treatment. The median intermediate follow-up period was 29.8 ± 3 mo (3-72 mo). The initial effectiveness of the medical treatment was complete, partial and poor among 50.8%, 13.5% and 35%, respectively, of the patients. At the end of the follow-up period, 37 patients (62.7%) showed more than 50% improvement. The rate of improvement in patients who required ES was not significantly different compared with the patients treated conservatively (64.2% *vs* 62.2%, respectively).

CONCLUSION: Our study confirms that conservative medical treatment could be an alternative to endoscopic sphincterotomy because, after an intermediate follow-up period, the two treatments show the same success rates.

Key words: Sphincter of oddi dysfunction; Cholecystectomy; Endoscopic sphincterotomy; Biliary scintigraphy

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INTRODUCTION

Sphincter of oddi dysfunction (SOD) is a functional gastrointestinal abnormality characterized by pancreatobiliary pain that can be debilitating and may impair the quality of life. The cause of SOD remains speculative, but it could be due to hormonal or neurological disturbances of the sphincter of oddi (SO), leading to its intermittent obstruction despite the absence of organic

abnormalities[1-4]. The best way to establish diagnosis and manage SOD remains controversial, mainly in SOD type 2 and 3 of the Milwaukee classification[5,6]. The diagnosis of SOD is usually based on a high index of clinical suspicion. Direct endoscopic manometry of the SO is an invasive procedure that remains the gold standard for the diagnosis of SOD[7-9]. However, dynamic biliary scintigraphy has been used as a non-invasive tool for evaluation of the SO by providing indirect evidence of increased sphincter resistance. The contribution of the biliary scintigraphy appears to predict, with good effectiveness, the clinical success of biliary endoscopic sphincterotomy in SOD types 1 and 2 of the Milwaukee classification[10,11]. The management of SOD is also controversial, and it is based on the relaxation of the SO, which should improve the symptoms of SOD. The treatment can be accomplished pharmacologically either by an endoscopic procedure or surgically[12,13]. The surgical procedure is often replaced by an endoscopic procedure[14-16]. Several studies have suggested that there is a benefit from endoscopic sphincterotomy (ES) in SOD patients having high SO basal pressures at the time of manometry. However, procedural pancreatitis cannot be completely avoided, and surgical treatment will be necessary in some cases. Morbidity and mortality after ES have been reported to be as high as 9.8% and 2.3%, respectively. Moreover, long-term data regarding the rate of restenosis and complications resulting from repeated therapy are limited. If SO manometry findings are abnormal, the relief of pain after sphincterotomy occurs in 90%-95% of patients with type 1 SOD, 85% of patients with type 2 SOD, and 55%-60% of patients with type 3 SOD. However, almost uniformly and despite ES, some patients continue to have pain that is consistent with nonspecific chronic pain disorders, suggesting a multifactorial cause for SOD[17-19]. Endoscopic stenting is no longer recommended as a routine method of treatment of SOD because endoscopic stenting is associated with poor symptomatic relief and a high risk of stent-induced pancreatitis[20]. Many pharmacologic agents that are known to relax the SO have already been used in the management of SOD. However, although medical therapy may be an attractive initial approach in patients with sphincter of Oddi dysfunction, data on intermediate clinical outcomes associated with pharmacological treatment is scant[21-31]. In a recent study[32], we showed that a one-year treatment period with trimebutine could significantly reduce pain in patients suffering from SOD, reducing the need for ES. Our aim in the current study was to determine the efficacy of medical therapy in relieving symptoms of SOD compared to ES after a prolonged follow-up period.

MATERIALS AND METHODS

As described in our previous study[32], fifty-nine patients complaining of SOD were included in this prospective monocentric study between 1999 and 2005. The patients included 57 women and 2 men, with a mean age of 50.5 ± 12.3 years old (range 20-75 years old), which were followed for a mean duration of 29 mo after the end of the endoscopic or medical treatment. The main inclusion criterion was biliary or pancreatic pain after cholecystectomy after ruling out the diagnosis of residual lithiasis or the presence of a tumor. The following data were collected: age, sex, cholecystectomy, the time elapsing since the onset of the symptoms, pain and rate of SOD occurrence. All of the patients underwent biliopancreatic endoscopic ultrasonography in order to rule out the diagnosis of a residual lithiasis or the presence of a tumor (none of the patients underwent secretin-magnetic resonance cholangiopancreatography). The diameters of the main biliary pathway and the canal de Wirsung were systematically measured during these procedures. An initial biological check-up was carried out when the first painful episode occurred after the medical consultation. The bological check-up included the measurement of the following proteins: transaminases (alanine aminotransférase and aspartate aminotransférase), gamma-glutamyltransferase, alkaline phosphatases, total and conjugate bilirubin, amylase and lipase. PD-SOD was defined as the association between recurent pancreatic pain with an increased level of serum lipase (> 3N). An initial biliary scintigraphy was carried out systematically on all of the patients. An initial hepatic and pancreatic biological assessment was done at the first event of abdominal pain. In addition, a biliary scintigraphy was performed for all patients in order to determine the isotopic hile to duodenum transit time (HDTT). According to the Milwaukee classification of SOD, the patients were subdivided into three groups. All of the patients were clearly informed about the various therapeutic modalities to manage their disease, the chances of success and the rates of complications of the medical therapy or endoscopic treatment. For each patients, we proposed an initial management of SOD by medical treatment, planned for a one-year duration; it consisted of a combined treatment, an association of trimebutine (200 mg three times per day) and nitrates taken sublingually when needed, mainly at the onset of abdominal pain. In the case of intolerance or counter-indication to the nitrates, a treatment with trimebutine alone was proposed. If previous treatment with trimebutine had been unsuccessful, only the nitrate derivatives were prescribed. If the painful attacks occurred too frequently (> 1 per week), a transdermal nitrate treatment (5 mg/d) was prescribed. The patients attended clinical follow-up consultations every four mo for a period of one year. After one year of medical therapy, an evaluation of painful symptoms was performed for each patient. The efficacy of the treatment was considered complete if there was a complete disappearance of the painful symptoms; the efficacy was considered partial if there was a greater than 50% reduction in the frequency and intensity of the pain and considered poor when the frequency and intensity of the pain decreased by less than 50%. In the cases where the medical treat-

Table 1 Patient's characteristics *n* (%)

Age, mean ± SD, yr	50.5 ± 12
Sex, M/F	2/57
Symptoms	
Biliary	47 (79.6)
Pancreatic	9 (15.2)
Biliopancreatic	3 (5)
Occurrence of painful episodes	
Once a week	9 (15.8)
Once a month	20 (35.1)
Every 3 mo	9 (15.8)
Every 6 mo	3 (5.3)
Once a year	7 (12.3)
Dilatation of biliary or pancreatic ducts (bile duct > 12 mm; pancreatic duct > 4 mm)	
Main biliary duct dilatation	29 (49.1)
Main pancreatic duct dilatation	1 (1.7)
Milwaukee classification repartition	
Type 1	11 (18.6)
Type 2	34 (57.6)
Type 3	14 (23.7)
Initial elevation of laboratory data	34 (57.6)
Lengthening of the isotopic transit time	32 (54.2)

ment was only partially successful or poor, endoscopic sphincterotomy was proposed to the patients, but not to the remaining patients. If medical treatment was successful, endoscopic treatment was not proposed. When the indications for endoscopic treatment had been chosen, the patients received clear information concerning the procedure. The endoscopic treatment was performed under general anaesthesia and consisted of an endoscopic retrograde cholangiopancreatography, with realization of a biliary sphincterotomy on patients with isolated biliary symptoms or dual sphincterotomy (biliary and pancreatic) on patients with both biliary and pancreatic symptoms. All of the patients were called by telephone to propose a follow-up examination with a hepatic biological assessment and an abdominal ultrasound in order to measure the diameter of the main biliary and pancreatic ducts; among the patients who refused this assessment, a simple evaluation of the clinical symptoms was proposed. The management was considered successful if there was an improvement greater than 50% compared to the initial symptoms; it was considered a failure if there was an absence of significant improvement by the patient. This study was approved by our local ethics committee.

Statistical analysis

Statistical analysis was carried out on the data with the Statview software program (Abacus concept, Inc., Berkeley, CA, 1992). Quantitative data were expressed as the mean ± SD. The significance of the differences was tested using the Student's *t* test or paired *t* tests to make comparisons between groups. Nonparametric tests (Mann Whitney tests) were performed when the Student's *t* test was not appropriate. The qualitative variables were compared using the chi-square test with Yate's correction when appropriate and with Fisher's exact test for 2 × 2 contingency tables. The significance threshold was set at $P < 0.05$.

Table 2 One-year outcome after medical treatment *n* (%)

Global effect		
Complete or partial	38 (64.3)	
Poor	21 (35)	$P < 0.05$
Complete or partial effect according the Milwaukee group		
Type 1	5 (45)	
Type 2	23 (67)	$P = 0.31$
Type 3	10 (71)	
Complete or partial effect according to the increase of HDTT		
With prolongation of HDTT	21 (55.3)	
Without prolongation of HDTT	17 (44.7)	$P = 0.77$

HDTT: Hile to duodenum transit time.

RESULTS

Characteristics of the SOD and treatment

The patients developed the symptoms of SOD after a mean period of 9.3 ± 1.2 years (range, 1-38 years) after the cholecystectomy. The patient's main characteristics are presented in Table 1. According to the three Milwaukee groups, there were no significant differences in age (type 1, 56.2 ± 2.1 years old; type 2, 48.1 ± 2.1 years old; and type 3, 52.1 ± 3.5 years old, $P = 0.22$) or the time elapsing from diagnosis to the cholecystectomy (type 1, 11 ± 3.6 years; type 2, 8.6 ± 1.6 years; and type 3, 10 ± 2.4 years; $P = 0.93$). Five patients were treated with nitrates only (8.4%), 12 were treated with trimebutine only (20.3%), and 42 patients had a combined treatment of nitrates and trimebutine (71.1%). The mean duration of the treatment period was 11.5 ± 0.8 mo. The efficacy of the medical therapy was considered to be complete or partial in 38 patients (64.3%) and poor in 21 patients (35%). Details concerning the success of the medical treatment are presented in Table 2. Among the 21 patients with poor results from the medical management, 14 (23.7%) agreed to undergo ES, including 12 with an isolated biliary sphincterotomy and 2 with an associated pancreatic sphincterotomy (dual sphincterotomy). In patients with a dual sphincterotomy, a pancreatic duct stenting was performed. A lengthening of the isotopic time of transit was present before the ES in 11 patients of the 14 who underwent this intervention (78.5%) and in 21 patients of the 45 without ES (46.6%) ($P = 0.02$). Other characteristics concerning ES are presented in Table 3. The complications of the ES were severe acute pancreatitis in two cases, which had resolved favourably with medical treatment. Among the patients requiring an ES, three patients had mixed biliary and pancreatic symptoms, and 9 patients had an isolated pancreatic pattern; among these patients, only 3 had a lengthening of the HDTT on the biliary scintigraphy corresponding to the 3 patients with isolated pancreatic symptoms as defined in the Materials and Methods section.

Intermediate follow-up period

Five patients refused to participate in the follow-up period. The intermediate follow-up period was 29.8 ± 3 mo (3-72 mo). Thirty-seven patients either did not pres-

Table 3 Results of the endoscopic treatment (n = 14), (%)

Time between the beginning of medical therapy and endoscopic treatment (mo)	12.2 ± 1.5
Patients concerned	
Patients with poor response to medical treatment	12 (57.1)
Patients with a partial response	2 (25)
Indication according to Milwaukee group	
Type 1	4 (36)
Type 2	9 (27)
Type 3	1 (7)
Results according to Milwaukee group	
Type 1	3/4 (75)
Type 2	6/9 (66)
Type 3	0/1 (0)

Table 4 Results after intermediate follow-up (29.8 ± 3 mo) n (%)

Rate of improvement		
Total rate	37 (62.7)	
Without endoscopic sphincterotomy	28 (62)	P = 0.88
With endoscopic sphincterotomy	9 (64.2)	
According to initial lengthening of HDTT		
With lengthening	19 (59.4)	
Without lengthening	18 (66.7)	P = 0.59
According to the Milwaukee group		
Type 1	6/11 (54.5)	
Type 2	23/34 (67.6)	P = 0.75
Type 3	8/14 (57.1)	

HDTT: Hile to duodenum transit time.

ent any additional painful events or improved by more than 50% (62.7%). Twenty-two patients still presented painful episodes (38.6%). The characteristics of the patients following endoscopic treatment are presented in Table 3. An abdominal ultrasound was performed in 45 patients and highlighted a dilation of the main biliary duct in 10 patients (22%). A biological laboratory assessment was also performed in these 45 patients. Among these patients, 6 still presented abnormal liver and pancreatic enzyme serum levels. At the end of the follow-up period, there was no significant difference in the size of the main biliary duct (at the beginning of the study, 49% of patients had dilation, and at the end of the study, 22% still had dilation of the main biliary duct) (P = 0.49); these characteristics are presented in Table 4.

DISCUSSION

The present study demonstrates that medical management with trimebutine may improve pain in patients suffering from SOD. Moreover, our study shows that after an intermediate follow-up period, the success of medical treatment (62%) does not differ from that of ES (64%). The medical treatment of SOD is usually disappointing, although nitrates or calcium-channel blockers can decrease basal pressure of the SO[32-36]. A positive effect of erythromycin on the motility of the SO has been suggested, but its clinical efficacy has not been demonstrated[29]. Somatostatin also modifies the SO activity but in the direction of an increase in the frequency of the phasic contractions of basal pressure. Therefore, somatostatin would be of little benefit for the indication of SOD and would not be suitable as a preventive measure after endoscopic retrograde cholangiopancreatography. The injection of botulinum toxin within the sphincter was tested in humans and pigs and promoted a significant reduction in the basal pressure in 50% of the cases[37-41]. On the clinical level, the injection of botulinum toxin was beneficial in 55% of the patients who suffered from biliary pain post-cholecystectomy without disturbance of the hepatic enzymes or dilation of the bile duct. However, 90% of the patients who experienced improvement presented a recurrence of symptoms after 6 mo, making the effect of botulinum toxin inconstant and transitory[37-41]. Endoscopic management is mainly based on the ES, requiring SOD diagnosis to be formally established, i.e., based on the description of a basal high pressure of the SO. However, despite its high rate of success (86%-91%), ES is associated with high morbidity (19%-30%). Moreover, the natural course of SOD has not been well documented so far, so that the use of this endoscopic strategy remains highly controversial[42-49].

Our research group previously described the effects of trimebutine on Oddi motility in a prospective study on patients with post-cholecystectomy pain[29]. We also reported in another study[32] that a one-year medical management could avoid the need for ES and had a success rate of 64%. Kovács *et al*[21] previously suggested that, depending on the Milwaukee classification, medical treatment should be first attempted and its efficacy subsequently reassessed; if this treatment fails or is poorly tolerated, ES can then be proposed, especially for type 1 or 2 SOD patients, based on the presence of a lengthening of the HDTT. The lengthening of the HDTT as a factor for predicting a favourable response to ES decreases with the Milwaukee types, as mentioned by Cicala *et al*[17]. In our study, 100% of the type 1 patients, 78% of the type 2 patients and none of the type 3 patients showed a lengthening of the HDTT and underwent ES successfully, whereas the immediate efficacy of ES was only 86%. This ES success ratein is quite similar to the success rate reported in the literature, which ranged between 86% and 91%[18,50-52]. Few studies have addressed the intermediate natural history of SOD. The available data suggest that the clinical course is variable depending, in part, on the initial biliary classification. In a one-year follow-up study, seven SOD type 2 patients with abnormal SO pressure treated by a sham procedure continued to have symptoms, which ended only after subsequent ES. All patients continued to do well four years later. Five other SOD type 2 patients with abnormal SO pressure refused ES; after four years, three were unimproved while two had "fair" improvement. The clinical course was unpredictable after a sham or ES treatment in patients with SOD type 3 biliary pain. In another report, 11 such patients were followed for two years after ES. Four improved symptomatically, while

seven had no change in their symptoms. Eleven other patients had a sham procedure, five of which improved, while six had no change in their symptoms during a two-year follow-up period[53]. In our study, the monitoring of the response rate to ES after an average period of 29 mo showed a loss of efficacy of the ES in 3 patients. Therefore, the rate of patient improvement did not differ significantly in the presence or absence of a previous ES according to the performance or not of an ES.

In conclusion, our study confirms that a conservative medical treatment could be an alternative to ES because, after an intermediate follow-up period, the two treatments show the same success rates.

COMMENTS

Background

Sphincter of oddi dysfunction (SOD) is a symptom characterized by recurrent abdominal pain. It has been showed that and endoscopic treatment called endoscopic sphincterotomy (ES) can improve symptoms but with a high level of complications. Pharmacologic agents have been tested in this pathology but are still disappointing.

Research frontiers

Many ways of treatment (medical, endoscopic) are still studied because none of them offer a satisfying and safe rate of prolonged improvement.

Innovations and breakthroughs

The authors here demonstrate that, after a long follow-up (29 mo), a medical treatment with trimebutine can have the same success rate than ES.

Applications

The demonstration of a long-term efficacy of a medical conservative treatment is important because it can avoid the need for ES which is associated with morbidity and mortality after ES of 9.8% and 2.3% respectively. This study demonstrates that a medical conservative treatment with trimebutine may be an effective alternative to the endoscopic sphincterotomy since, after an intermediate follow-up, the two treatments show the same success rate.

Terminology

The sphincter of oddi is a muscular valve that controls the flow of digestive juices (bile and pancreaticjuice) through the ampulla of Vaterinto the second part of the duodenum. ES is an endoscopic technique developed to examine and treat abnormalities of the bile ducts, pancreas and gallbladder. The procedurew as developed as an extension to the diagnostic examination, endoscopic retrograde cholangio pancreatography; with the addition of "sphincterotomy", abnormalities found during the study could be treated at the same time without the need for invasive surgery.

Peer review

This study is about the results of a medical management of SOD after an intermediate follow-up. It is well-written.

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BRIEF ARTICLE

Impact of comorbidities on the severity of chronic hepatitis B at presentation

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Abstract

AIM: To evaluate the clinical relevance of each cofactor on clinical presentation of chronic hepatitis B.

METHODS: Out of 1366 hepatitis B surface antigen (HBsAg) positive subjects consecutively observed in 79 Italian hospitals, 53 (4.3%) showed as the only cofactor hepatitis D virus (HDV) infection [hepatitis B virus (HBV)/HDV group], 130 (9.5%) hepatitis C virus (HCV) (group HBV/HCV), 6 (0.4%) human immunodeficiency virus (HIV) (group HBV/HIV), 138 (10.2%) alcohol abuse (group HBV/alcohol); 109 (8.0%) subjects had at least two cofactors and 924 were in the cofactor-free (CF) group.

RESULTS: Compared with patients in group CF those in group HBV/alcohol were older and more frequently had cirrhosis ($P < 0.001$), those in group HBV/HDV were younger ($P < 0.001$), more frequently resided in the south of the country and had cirrhosis ($P < 0.001$), those in group HBV/HCV were older ($P < 0.001$) and more frequently had cirrhosis ($P < 0.001$). These cofactors were all independent predictors of liver cirrhosis in HBsAg positive patients. Multivariate analysis showed that an older age [odds ratio (OR) 1.06, 95% CI: 1.05-1.08], alcohol abuse with more than 8 drinks daily (OR 2.89, 95% CI: 1.81-4.62) and anti-HDV positivity (OR 3.48, 95% CI: 2.16-5.58) are all independently associated with liver cirrhosis. This association was found also for anti-HCV positivity in univariate analysis, but it was no longer associated (OR 1.23, 95% CI: 0.84-1.80) at multivariate analysis.

CONCLUSION: Older age, HDV infection and alcohol abuse are the major determinants of severe liver disease in chronic HBV infection, while HCV replication plays a lesser role in the severity of hepatic damage.

Key words: Chronic hepatitis B; Hepatitis B virus/hepatitis D virus dual infection; Hepatitis B virus/hepatitis C virus dual infection; Alcohol abuse

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INTRODUCTION

Seroepidemiological studies on the aetiology of chronic hepatitis in Italy performed in the 1980s, 1990s[1-3] and in 2001[4] showed a progressive reduction in the prevalence of hepatitis B surface antigen (HBsAg) positive cases from 60.7% observed in cases collected from 1976 to 1981[1] to 13% found in 2001[4]. Despite this, infection by the hepatitis B virus (HBV) is still responsible for a sizable number of cases of chronic hepatitis and/or hepatocellular carcinoma (HCC), and the burden of HBV infection on the healthcare system in Italy is still heavy.

In a multicentre study carried out on 1829 patients with cirrhosis in 1992, the prevalence of HBV-related cases was 13.8%; in particular, HBV was the only aetiological factor in 4.2% of cases, most cases being associated with cofactors (9.6%): hepatitis D virus (HDV) in 3.4%, hepatitis C virus (HCV) in 3.2% and alcohol abuse in the remaining 3%[5]. In human immunodeficiency virus (HIV)/HBV dual infection, found in 3.7%-4.6% of HIV positive subjects in Italy but estimated around 10% in people with HIV infection worldwide[6], the prevalence of patients with cirrhosis is reported as higher than in HIV-negative/HBV-positive cases[7]. Cofactors may therefore play a substantial role in the progression of HBsAg positive chronic hepatitis to the more severe clinical forms[8-10].

The gradual reduction in the percentage of HBsAg positive cases was associated with a dramatic decrease in the prevalence of cases with HBV/HDV dual infection[11-13]. About a quarter of HBsAg positive patients with chronic hepatitis were anti-HDV positive in 1978-1981[14] and in 1987[15]; this prevalence was lower in 1992 (14.4%)[16], in 1997 (8.3%)[17] and in 2001 (9.9%)[4]. No data to this effect are available on HBV/HCV dual infection and on the association of HBV infection/alcohol intake[18].

Our survey on the largest series of patients with chronic hepatitis ever studied in Italy showed an overall low HDV prevalence in HBsAg chronic carriers (9%), a high prevalence of patients with HBV/HCV dual infection (16.9%) and alcohol abuse (10.4%)[4]. The data from this study allow us to evaluate the current main characteristics of patients with HBV/HDV or HBV/HCV chronic infection or HBV infection/alcohol abuse compared with a large control group of HBsAg chronic carriers with no evident cofactor.

MATERIALS AND METHODS

The study design was more extensively described in a previous paper[4]. Seventy-nine centres participated in the study, 25 in the North, 24 in the Centre and 30 in the South of Italy and the two main islands (Sicily and Sardinia).

All subjects consecutively referred from February 1 to July 31, 2001 as in-patients or out-patients to one of the 79 Italian centres were recruited; 9997 patients with chronic hepatitis were enrolled. Both tertiary and peripheral centres were randomly selected by a systematic cluster sampling procedure. For each of these three geographical areas, all of the hospitals were identified and listed numerically according to an assigned number. In each list, a single hospital was considered as a cluster. The first cluster was randomly chosen, whereas the others were selected with a probability proportional to the required number of hospitals at systematic intervals. Both prevalent and incident cases were recruited. We defined as "incident cases" all new diagnoses of chronic liver disease made during the enrolment period, and as "prevalent cases" all subjects with a previous diagnosis of chronic liver disease observed during the study period.

For each subject, the demographic, clinical and aetiological data were recorded using a pre-coded questionnaire. The amount of alcohol intake was determined using a standard questionnaire containing information on the daily intake of various alcoholic beverages and lifetime duration of alcohol consumption. An alcohol intake of more than 40 g daily for males and 30 g daily for females for at least 10 years was considered as an aetiological cofactor[19,20].

HBV serum markers, HBsAg, anti-HBc, hepatitis B e antigen (HBeAg), anti-HBeAg and anti-HDV were determined by commercial immunoenzymatic assays. Antibodies to HCV were detected by 3rd generation commercial immunoenzymatic assays. Antibodies to HIV 1 and 2 were determined by commercial enzyme-linked immunosorbent assay (Diasorin Biomedica, Saluggia, Vercelli, Italy; Abbott Labs, North Chicago, Illinois, United States) and the positive results were confirmed by a Western Blot analysis (Genelabs Diagnostics, Science Park Drive, Singapore). Patients were enrolled in 2001 when various methods of different sensitivity were used to detect HBV viral load in different centres in Italy. For this reason, we preferred not to evaluate the predicting value of HBV viral load; data on HBV-DNA were given as positive or negative.

Data on HDV-RNA and HDV-Ag were not available. We classified patients as asymptomatic carriers when alanine aminotransferase (ALT) values were persistently normal in the absence of clinical, biochemical and ultrasound signs of chronic liver disease. Chronic hepatitis was diagnosed only on the basis of liver histology. Liver cirrhosis was diagnosed from a liver biopsy or the presence of unequivocal clinical, biochemical and ultrasound signs[20]. HCC diagnosis was based on histology, imaging techniques or biochemical parameters (α_1-feto protein greater than 400 ng/mL)[21,22]. Patients with serum markers suggesting autoimmune liver disease and those with liver disease associated with genetic disorders were excluded from the study.

Statistical analysis

Continuous variables were summarised as mean ± SD or median and interquartile range, and categorical variables as absolute and relative frequencies. Differences in the means were evaluated by an unpaired Student *t* test or Kruskal-Wallis one-way analysis of variance, and the χ^2 test was applied to categorical variables. Crude odds ratios (OR) and their 95% CI for the association of liver cirrhosis with potential risk factors were calculated by univariate analysis. Adjusted OR were calculated by stepwise logistic regression analysis to identify factors independently associated with liver cirrhosis. Only factors associated with liver cirrhosis by univariate analysis were included in the logistic regression analysis. In the logistic model liver cirrhosis was the outcome variable, while age, sex, anti-HDV, anti-HCV, alcohol intake, and body mass index were the independent variables.

RESULTS

Of the 1366 HBsAg positive patients, 924 (67.6%) lacked all the cofactors investigated (HDV, HCV, HIV and alcohol abuse); this group of patients was named the cofactor-free (CF) group. Fifty-nine (4.3%) patients showed HDV infection (anti-HDV positive) as the only cofactor (group HBV/HDV), 130 (9.5%) HCV infection (group HBV/HCV), 6 (0.4%) HIV infection (group HBV/HIV), 138 (10.2%) alcohol abuse (group HBV/alcohol) and 109 (8.0%) had more than one cofactor (group with two or more cofactors). Overall, 333 (24.4%) patients had no liver biopsy at presentation; of the remaining 1033 cases, 278 subjects (26.9%) were classified as asymptomatic carriers, 453 (43.9%) as having chronic hepatitis, 249 (24.9%) as having liver cirrhosis and 53 (5.1%) patients had a diagnosis of HCC. Interferon treatment was given in 365 (26.7%) patients and 244 (17.9%) patients received Lamivudine. Excluding cases under immunosuppressive therapy, approximately all HBeAg positive and half of HBeAg negative cases showed active HBV replication. Fifty-seven (4.2%) patients were born outside Italy, and only 4 of them were born in China.

Characteristics of the CF group

The 924 HBsAg positive subjects with none of the cofactors investigated were more frequently males (66.5%) and observed as out-patients (81.1%) and as prevalent cases (83.2%); they aged 48.1 ± 14.3 years, were infrequently HBeAg positive (12.6%) and frequently showed a mild clinical presentation: 26.9% of cases were asymptomatic carriers, 56.9% of patients showed chronic hepatitis, 12.4% liver cirrhosis and 3.8% had HCC.

Comparison according to anti-HDV status

The 59 anti-HDV positive patients were younger than those in group CF (46.7 ± 11.8 years *vs* 48.1 ± 14.3 years; $P < 0.001$) and showed higher ALT levels (128 ± 116 IU/L *vs* 98 ± 290 IU/L, $P = 0.008$); they were more frequently observed as in-patients (25.4% *vs* 18.9%, $P = 0.3$), were more frequently born in southern Italy or on one of the two main islands (67.8% *vs* 52.9%, $P = 0.029$) and more frequently had liver cirrhosis (37.3% *vs* 12.4%, $P \leqslant 0.001$); none of the 59 anti-Delta positive patients had HCC (Table 1).

Comparison according to anti-HCV status

The 130 subjects co-infected with HCV were older than those in group CF (55.2 ± 14.7 years *vs* 48.1 ± 14.3 years, $P < 0.0001$), and more frequently showed liver cirrhosis (23.1% *vs* 12.4%, $P < 0.001$); this group contained the highest prevalence of patients with HCC observed in the study (6.2%) (Table 1). Active HCV replication as evaluated by positive HCV-RNA by RT-PCR was found in 115 subjects (88.5%).

Comparison according to anti-HIV status

Only 6 patients showed HBV/HIV dual infection; all of these patients were males and younger than those in group CF. Four of them were HBeAg positive and none had liver cirrhosis (Table 1).

Comparison according to alcohol abuse

Compared with patients in group CF, the 138 HBsAg positive patients with alcohol abuse as the only cofactor were older (52.6 ± 11.8 years *vs* 48.1 ± 14.3 years, $P < 0.0001$), were more frequently males (92.6% *vs* 66.5%, $P < 0.0001$), had had fewer years of schooling (85.9% *vs* 67.0%, $P < 0.001$) and more frequently showed liver cirrhosis (31.1% *vs* 12.4%, $P < 0.0001$) and HCC (5.1%).

Comparison according to the presence of two or more cofactors

Of the 109 patients in this group, 21 had HBV/HDV/HCV concurrent infection, 12 HBV/HDV dual infection plus alcohol abuse, 64 had HBV/HCV dual infection plus alcohol abuse, and 12 had HBV/HDV/HCV concurrent infection plus alcohol abuse. Compared with group CF, patients with two or more cofactors were more frequently males (86.8% *vs* 66.5%, $P < 0.001$), in-patients (28.0% *vs* 18.9%, $P = 0.003$) and had liver cirrhosis (35.8% *vs* 12.4%, $P < 0.001$). They more frequently belonged to larger families (24.5% *vs* 14.6%, $P < 0.01$) and had had fewer years of schooling (84.3% *vs* 67.0%, $P < 0.001$). Only 4 patients showed HCC (Table 1).

Variables associated with the presence of cirrhosis

At the univariate analysis older age, anti-HCV, HCV-RNA positivity, alcohol abuse, anti-HDV and anti-HIV positivity were all associated with liver cirrhosis. Multivariate analysis showed that age (OR 1.10, 95% CI: 1.08-1.11), alcohol abuse (between 4 and 8 drinks daily: OR 2.30, 95% CI: 1.29-4.10; more than 8 drinks daily: OR 2.42, 95% CI: 1.31-4.46), HDV positivity (OR 2.68, 95% CI: 1.56-4.61) and anti-HIV (OR 5.78, 95% CI: 1.50-22.27) were independent predictors of the development of cirrhosis, whereas anti-HCV and HCV-RNA positivity were no longer associated (Table 2).

Table 1 Comparison of baseline features of hepatitis B surface antigen positive patients, according to different cofactors (mean ± SD) *n* (%)

Variable	HBsAg positive (*n* = 924)	HBsAg/anti-HDV positive (*n* = 59)	HBsAg/anti-HCV positive (*n* = 130)	HBsAg/anti-HIV positive (*n* = 6)	HBsAg/alcohol abuse (*n* = 138)	HBsAg/two or more cofactors (*n* = 109)	*P* value
Age (yr)	48.4 ± 14.0	46.5 ± 11.7	55.2 ± 14.7	37.0 ± 3.6	52.7 ± 11.7	45.8 ± 12.1	< 0.001
Males	606 (66.5)	41 (71.9)	87 (68.0)	6 (100)	126 (92.6)	92 (86.8)	< 0.001
In-patients	171 (18.9)	15 (25.4)	29 (22.7)	0	20 (14.7)	30 (28.0)	0.060
Out-patients	732 (81.1)	44 (74.6)	99 (77.3)	6 (100)	116 (85.3)	77 (72.0)	
Prevalent cases	769 (83.2)	53 (89.8)	111 (85.4)	6 (100)	115 (83.3)	95 (87.2)	0.500
Incident cases	155 (16.8)	6 (10.2)	19 (14.6)	0	23 (16.7)	14 (12.8)	
Born in Italy							
North	192 (21.0)	11 (18.6)	36 (27.7)	3 (50.0)	32 (23.7)	35 (32.4)	0.010
Centre	195 (21.3)	5 (8.5)	16 (12.3)	0	26 (19.3)	21 (19.4)	
South/islands	484 (52.9)	40 (67.8)	76 (58.5)	2 (33.3)	73 (54.1)	49 (45.4)	
Born Abroad	44 (4.8)	3 (5.1)	2 (1.5)	1 (16.7)	4 (2.9)	3 (2.8)	
Asymptomatic carrier	233 (33.5)	3 (6.0)	17 (17.7)	1 (100)	19 (17.6)	5 (6.0)	< 0.001
Chronic hepatitis[1]	313 (45.0)	25 (50.0)	41 (42.7)	0	39 (36.1)	35 (42.2)	
Liver cirrhosis	115 (16.5)	22 (44.0)	30 (31.3)	0	43 (39.8)	39 (47.0)	
HCC	34 (4.9)	0	8 (8.3)	0	7 (6.5)	4 (4.8)	
ALT (× ULN) (median, IQR)	1.0 (1.0-1.8)	2.2 (1.1-4.8)	1.4 (1.0-2.1)	1.5 (1.1-1.8)	1.1 (1.0-2.1)	1.8 (1.0-2.9)	< 0.001
BMI (kg/m^2)	25.0 ± 3.4	25.5 ± 5.0	24.8 ± 3.1	22.8 ± 2.0	26.1 ± 3.5	25.6 ± 4.1	0.006
Years of schooling							< 0.001
< 6	239 (26.3)	9 (16.4)	46 (36.5)	1 (16.7)	62 (46.3)	31 (28.7)	
6-13	369 (40.7)	31 (56.4)	53 (42.1)	1 (16.7)	53 (39.5)	60 (55.6)	
> 13	299 (33.0)	15 (27.2)	27 (21.4)	4 (66.6)	19 (14.2)	17 (15.7)	
HBeAg positive	116 (12.6)	9 (15.3)	10 (7.7)	4 (66.7)	10 (7.2)	15 (13.8)	< 0.001
HBeAg negative	808 (87.4)	50 (84.7)	120 (92.3)	2 (33.3)	128 (92.8)	94 (86.2)	

[1]Only subjects with liver biopsy. HBsAg: Hepatitis B surface antigen; HDV: Hepatitis D virus; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; HCC: Hepatocellular carcinoma; ALT: Alanine aminotransferase; × ULN: Times the upper limit of the normal; IQR: Interquartile range; BMI: Body mass index; HBeAg: Hepatitis B e antigen.

Comparison according to the severity of liver cirrhosis

The Child-Pugh Score was calculated for 229 (92.0%) of the 249 patients with cirrhosis: 104 cases in group CF, 43 cases in group HBV/Alcohol, 19 cases in group HBV/HDV, 30 cases in group HBV/HCV and 33 cases in the HBV/two or more cofactors group. The prevalence of patients in the Child-Pugh Classes B or C was lower in group CF (31.7%), than in group HBV/Alcohol (44.2%), in group HBV/HCV (43.3%) and in the HBV/two or more cofactors group (75.8%, $P < 0.001$) (Table 3).

DISCUSSION

This study is based on three strong points: the first, a large sample size, the second, the validity of the random selection including both tertiary and peripheral hospitals all over the country, which may have avoided the selection of "difficult-to-treat" patients, and lastly, the homogeneity of each single cofactor group and of the comparative CF group, making this study the first to investigate the clinical presentation of CF HBsAg positive chronic hepatitis and the influence of each single cofactor (HDV, HCV and alcohol intake).

Excluding the 442 patients with one or more cofactors enabled us to examine a group of almost a thousand patients with HBsAg positive chronic hepatitis and no cofactor. Most of these patients showed a mild clinical presentation and only a minority of them had liver cirrhosis (12.4%), prevalently in the Child A stage. The low prevalence of HCC (3.9%) refers to HCC at the time of diagnosis. No information is available on the risk of occurrence of HCC, death or orthotopic liver transplantation over time, since the present study is cross-sectional and no evaluation of the clinical outcomes was made.

Despite the decreasing endemicity levels, HDV infection maintains its geographical distribution in our country, i.e., more frequent in southern Italy and on the two main islands, as previously described[15-17]. Compared with those in group CF, patients in group HBV/HDV showed a more severe clinical presentation: they were younger, more frequently hospitalised and with evidence of cirrhosis. In accordance with the low prevalence of patients with HCC in previous studies[5], no patient in our HBV/HDV group showed HCC, most probably reflecting a more severe course of the illness with a rapid transition to death or to the need for liver transplantation before HCC becomes evident[23]. On the other hand, patients in group HBV/HCV were older than those in group CF, more frequently had liver cirrhosis and showed an almost double prevalence of HCC. These differences, although not statistically significant, may suggest that subjects with long-lasting HBV/HCV dual infection are at a higher risk of developing liver cirrhosis with or without liver cancer[24-26].

Most patients in group HBV/Alcohol were males with a low educational level, a combination more frequently associated with a high risk of alcohol abuse. The prevalence of patients with cirrhosis was 2.5 times higher in group HBV/alcohol than in group CF, a dif-

Table 2 Risk factors associated with cirrhosis in hepatitis B surface antigen positive patients *n* (%)

Variable	Chronic hepatitis	Cirrhosis	Crude OR (95% CI)	Adjusted OR (95% CI)	*P* value
Age (mean ± SD, yr)	44.9 ± 12.0	56.2 ± 11.6	1.06 (1.05-1.07)	1.10 (1.08-1.11)	< 0.001
Gender					
Male	371 (81.9)	192 (77.1)	1		
Female	82 (18.1)	57 (22.9)	1.34 (0.92-1.97)		
HBeAg					
Negative	387 (85.4)	225 (90.4)	1		
Positive	66 (14.6)	24 (9.6)	0.63 (0.38-1.03)		
Anti-HCV					
Negative	382 (84.3)	187 (75.1)	1	1	0.300
Positive	71 (15.7)	62 (24.9)	1.78 (1.22-2.62)	1.23 (0.84-1.80)	
HCV-RNA					
Negative	382 (86.2)	187 (77.3)	1	1	0.200
Positive	61 (13.8)	55 (22.7)	1.84 (1.23-2.76)	1.44 (0.65-1.72)	
Anti-HDV					
Negative	410 (90.5)	207 (83.1)	1	1	< 0.001
Positive	43 (9.5)	42 (16.9)	1.94 (1.23-3.06)	2.68 (1.56-4.61)	
Anti-HIV					
Negative	449 (99.1)	241 (96.8)	1	1	0.010
Positive	4 (0.9)	8 (3.2)	3.73 (1.11-12.50)	5.78 (1.50-22.27)	
Alcohol (drinks/d)					
0	247 (54.5)	117 (47.0)	1	1	
< 4	143 (31.6)	56 (22.4)	0.83 (0.57-1.21)	0.69 (0.48-1.07)	0.080
4-8	35 (7.7)	37 (14.9)	2.23 (1.34-3.72)	2.30 (1.29-4.01)	0.005
> 8	28 (6.2)	39 (15.7)	2.94 (1.73-5.01)	2.42 (1.31-4.46)	0.005
BMI (kg/m^2)					
< 25	247 (54.5)	121 (48.6)	1		
25-30	173 (38.2)	107 (43.0)	1.26 (0.91-1.75)		
> 30	33 (7.3)	21 (8.4)	1.30 (0.72-2.34)		

Crude and adjusted odds ratios (OR) deriving from multiple logistic regression analysis. Patients with HCC were excluded from the analysis. HCC: Hepatocellular carcinoma; HDV: Hepatitis D virus; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; BMI: Body mass index.

Table 3 Percentages of cases in different child-pugh classes in 229 of the 249 patients with liver cirrhosis, by aetiology *n* (%)

Child-pugh class	CF group (*n* = 104)	HBV/alcohol group (*n* = 43)	HBV/HDV group (*n* = 19)	HBV/HCV group (*n* = 30)	HBV/ two or more cofactors group (*n* = 33)
A	71 (68.3)	24 (55.8)	13 (68.4)	17 (56.7)	8 (24.2)
B + C	33 (31.7)[b]	19 (44.2)	6 (31.6)	13 (43.3)	25 (75.8)[b]

[b]$P < 0.001$ *vs* hepatitis B virus (HBV)/two or more cofactors. Patients with hepatocellular carcinoma are not included. CF: Cofactor-free; HCV: Hepatitis C virus; HDV: Hepatitis D virus.

ference most probably due to alcohol intake rather than to the association of the two aetiological factors, since, as described in a previous paper[4], 41.8% of 761 patients showing alcohol abuse as the only aetiological factor had liver cirrhosis. The group of patients with two or more cofactors is a miscellany of 4 subgroups that are too small to be analysed or compared with group CF or with the other cofactor groups. This miscellaneous group with more than one cofactor may be more frequently exposed to the aetiological agents of liver disease and to a higher risk of developing liver cirrhosis and/or HCC.

As observed in previous Italian studies[4,17,18], the majority of HBsAg positive patients in this study were found to be HBeAg negative. Patients with HBV/HCV dual infection and those with HBV plus alcohol abuse showed a lower prevalence of HBeAg positive cases than those in the other aetiological groups, probably because they were older and HBeAg loss is, at least in part, a time-dependent phenomenon.

In conclusion, HBV chronic infection was frequently associated with a mild or moderate clinical condition. Liver cirrhosis and HCC were detected in less than one sixth of cases, and viral and metabolic cofactors unfavourably influenced the clinical course in patients with chronic HBV infection since their presence was associated with an increased risk of cirrhosis, an association proven by multivariate logistic regression analysis for HDV infection and alcohol abuse.

COMMENTS

Background

Many cofactors play a substantial role in the progression of hepatitis B surface antigen positive chronic hepatitis to more severe clinical forms.

Research frontiers

The study aimed to evaluate the clinical relevance of each cofactor on the severity of the clinical presentation of chronic hepatitis B.

Innovations and breakthroughs

Older age, hepatitis D virus co-infection and alcohol abuse are the major determinants of severe liver disease in chronic hepatitis B virus (HBV) infection. Conversely, hepatitis C virus replication plays a lesser role in the severity of hepatic damage.

Applications

Removal of some risk factors may hamper the progression of chronic HBV-related liver disease in many patients.

Peer review

This is a large-size, multicentre study showing the importance of comorbidities in exacerbating hepatocellular necroinflammation and playing a substantial role in the progression to a more advanced stage of liver disease.

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BRIEF ARTICLE

No evidence of circulating autoantibodies against osteoprotegerin in patients with celiac disease

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Abstract

AIM: To investigate risk factors for low bone mineral density (BMD) in celiac disease (CD) patients, focusing on circulating autoantibodies against osteoprotegerin (OPG).

METHODS: Seventy asymptomatic CD adult patients on gluten-free diet (GFD) and harbouring persistent negative CD-related serology were recruited. Conventional risk factors for osteoporosis (e.g., age, sex, menopausal status, history of fractures, smoke, and body mass index) were checked and BMD was assessed by dual energy X ray absorptiometry. Serum calcium and parathyroid hormone (PTH) levels were evaluated. Thirty-eight patients underwent repeat duodenal biopsy. Serum samples from a selected sub-group of 30 patients, who were also typed for human leukocyte antigen (HLA) DQ2 and DQ8 haplotype, were incubated with homodimeric recombinant human OPG and tested by western blotting with an anti-OPG antibody after immunoprecipitation.

RESULTS: Despite persistent negative CD-related serology and strict adherence to GFD, 49 out of the 70 (74%) patients displayed low BMD. Among these patients, 13 (24%) showed osteoporosis and 36 (76%) osteopenia. With the exception of age, conventional risk factors for osteoporosis did not differ between patients with normal and low BMD. Circulating serum calcium and PTH levels were normal in all patients. Duodenal mucosa healing was found in 31 (82%) out of 38 patients who underwent repeat duodenal biopsy with 20 (64%) still displaying low BMD. The remaining 7 patients had an incomplete normalization of duodenal mucosa with 6 (84%) showing low BMD. No evidence of circulating antibodies against OPG was found in the serum of 30 celiac patients who were tested for, independent of BMD, duodenal histology, and HLA status.

CONCLUSION: If any, the role of circulating autoantibodies against OPG in the pathogenesis of bone derangement in patients with CD is not a major one.

Key words: Celiac disease; Osteoprotegerin; Bone mineral density; Gluten-free diet; Osteoporosis; Osteopenia

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INTRODUCTION

Celiac disease (CD) is a permanent gluten intolerance in genetically predisposed individuals who display an inflammatory process in the small intestinal mucosa with villous atrophy, crypt hyperplasia, and increased number of lymphocytes[1].

Evidences indicate that a low bone mineral density (BMD) is found in 20%-50% newly diagnosed patients with CD[2]. By means of dual energy X ray absorptiometry (DEXA) it can be now rapidly and easily obtained semi-quantitative values of BMD[3].

Osteoporosis is a quantitative and qualitative alteration in the components of bone tissue, in which the process of demineralization becomes intense and prolonged and minerals are used up more quickly than they can be replaced resulting in bones fragility and increased risk of fractures[4,5]. Individual's gender, constitution and age as well as variations in endocrine systems associated with factors such as the menopause, and the presence of other pathologies, can all interact with lifestyle factors, including smoking, lack of exercise and low dietary calcium intake, to determine the onset of osteoporosis[6].

Impaired absorption of calcium during CD is thought to result principally from loss of villous in the proximal intestine, where calcium is most actively absorbed, and also from the unabsorbed fatty acids, which bind calcium in the intestinal lumen and may reduce dietary vitamin D absorption[7]. Adherence to a strict gluten-free diet (GFD) will reverse the histological changes in the intestine and also the biochemical evidence of calcium malabsorption[8], resulting in normal BMD in these treated patients[9]. Nevertheless, there may be long-term impairment of bone mineralization in some otherwise healthy CD patients adhering to GFD[10]. Furthermore, osteopenia has been found in treated CD patients who showed improvement or even complete healing of intestinal mucosa[11]. These findings suggest that other mechanisms of bone injury than calcium malabsorption are probably involved in patients with CD[12].

Mediators of inflammatory immune-mediated responses (e.g., cytokines), parathyroid hormone (PTH), estrogens, androgens, corticosteroids and vitamin D are all acknowledged to affect BMD by modulating the receptor activator of nuclear factor B/receptor activator of nuclear factor B-ligand/osteoprotegerin [RANK/RANK-L/osteoprotegerin (OPG)] system[13]. Furthermore, neutralizing auto-antibodies against OPG have been recently shown in the sera of a few patients with CD, leading to the hypothesis that blocking the inhibitory effect of OPG on RANKL may have a role in the development of bone derangement in these patients[14]. Nevertheless, this finding has not yet been confirmed by others, so the role of auto-antibodies against OPG, if any, in the pathogenesis of reduced BMD in patients with CD remains to be established.

This study aimed at investigating risk factors associated with low BMD in patients with CD, focusing on circulating auto-antibodies against OPG.

MATERIALS AND METHODS

Patients recruitment

Seventy consecutive outpatients with CD (13M, 57F; median age 40.5 years, range 20-68 years) who reported no current symptoms, claimed to be adherent to a GFD for at least 2 years and harboured persistent (at least 18 mo) negative CD-related serology (anti-endomysium and anti-transglutaminase IgA antibodies) were recruited. Diagnosis of CD was performed on the basis of clinical presentation, positive CD-related serology and suggestive histological findings on duodenal biopsy[15]. Dietary compliance was assessed by periodic interview during follow-up visits and classified as good according to Leffler *et al*[16]. Data on height, weight, time since diagnosis, symptoms beginning, age at menarche, cycle regularity, menopausal status, drug use, calcium intake, life style, smoking, and history of fracture were collected. Blood samples were collected in the morning after a 12 h fast in order to measure serum calcium and parathormone levels. Thirty-eight patients (8M, 30F; median age 41 years, range 20-60 years) underwent repeat duodenal biopsy after a period of at least 12 mo since GFD beginning.

A subgroup of 30 patients (8M, 22F; median age 44 years, range 21-60 years), who were typed for HLA-DQ2 and DQ8 haplotype, were selected in order to measure antibodies against OPG.

Histology

At least four duodenal biopsies were collected during upper gastrointestinal endoscopy. Intraepithelial lymphocytes have been identified using CD3 immunostaining and a value ≤ 25 lymphocytes/100 epithelial cells was considered normal. Histological changes were classified according to Marsh criteria (stage 0: Normal mucosa; stage 1: Increased number of intra-epithelial lymphocytes; stage 2: Crypts proliferation; stage 3a-3b-3c: Respectively mild, moderate and severe villous atrophy)[17].

Measurement of BMD

All patients underwent lumbar spine and femoral neck BMD evaluation by means of DEXA. A T-score 1 to 2.5 and > 2.5 distinguished osteopenia and osteoporosis, respectively.

Measurement of antibodies against osteoprotegerin

Non-fasting serum samples were obtained from the selected subgroup of 30 CD patients. Measurement was performed according to Riches *et al*[14]. Briefly, serum samples were incubated at a 1:100, 1:50, and 1:25 dilution with 12.5 ng of homodimeric recombinant human OPG (R and D Systems, Minneapolis, United States) and also with protein G-coated agarose beads (Calbiochem, Darmstadt, Germany) that had been pre-incubated with 5% albumin to reduce non-specific binding. After incubation for 1 h at 37 ℃, the beads were washed five times with phosphate-buffered saline, suspended in 30 μL of reducing sample buffer, and incubated at 90 ℃ for 5 min. After brief centrifugation,

Table 1 Characteristics of the 70 treated celiac disease patients with negative serology according to bone mineral density as assessed by dual energy X ray absorptiometry

Variables	Overall (*n* = 70)	Normal BMD (*n* = 21)	Low BMD (*n* = 49)	*P*
Sex (M/F)	13/57	2/19	11/38	0.31
Age (yr)	40.5 ± 10.5	31.0 ± 9.7	43.0 ± 9.7	0.00
Time since diagnosis	2.8 ± 0.6	2.5 ± 0.5	2.4 ± 0.4	0.37
BMI	22.2 ± 1.4	22.5 ± 1.3	22.0 ± 1.4	0.40
Smoke	13	3	10	0.74
Fracture	0	0	0	-
Menopausal status	6	0	6	0.17

BMD: Bone mineral density; BMI: Body mass index; M: Male; F: Female.

the supernatant was loaded onto a 12% polyacrylamide gel, subjected to electrophoresis at 200 V for 60 min, transferred to membrane, and therefore probed with a mouse monoclonal antibody against human OPG (Abcam, Cambridge, United Kingdom). A peroxidase-conjugated donkey anti-mouse antibody (Jackson, Suffolk, United Kingdom) at a 1:5000 dilution was used for detection. Equal loading was assessed by probing the blot with peroxidase-conjugated goat anti-human antibody (Jackson) at a 1:5000 dilution. Immunolabeled bands were detected with the use of a chemiluminescent substrate and a chemiluminescence imager. A homodimeric recombinant human OPG (R and D Systems) was used as positive control. A 55-kDa band indicated the presence of antibodies against OPG.

The study was approved by the local research Ethical Committee, and informed consent was obtained from all participants.

Statistical analysis

Comparison of proportions was performed using χ^2 test. A multivariate analysis was performed using Multivariate Analysis of Variance (MANOVA) to identify variables associated with low BMD. Difference was considered significant if the *P* value was < 0.05. Data were analyzed using the Statistical Package for Social Services, Version 16.0 (SPSS Inc., IL, United States).

RESULTS

Forty-nine out of the 70 (74%) CD patients displayed low BMD, with 13 (24%) accounting for osteoporosis and 36 (76%) for osteopenia (Table 1). Multiple logistic regression analysis showed that age was the only one variable which positively correlated with low BMD (Table 1). Serum calcium and PTH levels were normal in all patients. A complete healing of duodenal mucosa was found in 31 out of 38 (82%) patients who underwent repeat intestinal biopsies. In this specific subgroup, 20 (64%) patients showed a low BMD compared to 6 out of 7 (86%) patients who were found to carry an incomplete duodenal mucosa healing (*n* = 1 Marsh 1, *n* = 2 Marsh 2, *n* = 4 Marsh 3) (Table 2, *P* = 0.4).

No evidence of the 55-kDa band was found in serum samples of the subgroup of 30 patients who were tested for, indicating no presence of auto-antibodies against OPG (Figure 1). The characteristics of this subgroup of CD patients, including HLA DQ2 and DQ8 status, are shown in Table 3.

Table 2 Bone mineral density according to dual energy X ray absorptiometry in celiac disease patients with and without duodenal mucosa healing after gluten free diet

	Duodenal mucosa healing	Duodenal mucosa lesions	Total
Low BMD	20	6	26
Normal BMD	11	1	12
Total	31	7	38

BMD: Bone mineral density.

Table 3 Characteristics of the 30 celiac disease patients who underwent measurement of serum antibodies against osteoprotegerin

Variables	Overall (*n* = 30)	Normal BMD (*n* = 6)	Low BMD (*n* = 24)
Sex (M/F)	8/22	1/5	7/17
Age (yr)	43.5 (21-60)	31.0 (21-54)	44.5 (32-60)
Time since diagnosis (yr)	2.8 (1.0-3.5)	2.7 (1.8-3.2)	2.9 (1.0-3.5)
BMI (kg/m^2)	22.3 (19.2-25.1)	21.8 (19.2-24.7)	22.4 (20.0-25.1)
Smoke	4	0	4
Fracture	0	0	0
Menopausal status	1	0	1
Duodenal mucosa histology (*n* = 22)			
Healing	17	5	12
Lesions	5	1	4
HLA status			
DQ2	23	4	19
DQ8	7	2	5

BMD: Bone mineral density; BMI: Body mass index; HLA: Human leukocyte antigen.

DISCUSSION

Currently, serology is employed to select individuals needing to underwent intestinal biopsy for diagnosing CD as well as to monitor adherence and response to GFD[18,19]. However, confirming a previous observation[20], this study shows that, despite a persistent negative serology, 18% of CD patients with good adherence to GFD have an incomplete normalization of intestinal mucosa (e.g., 82% negative predictive value in detecting intestinal mucosal recovery).

A GFD normally gains mucosal damage in CD patients restoring calcium absorption, and this can support an improvement in bone mineralization in one year[21]. Nevertheless, a GFD rarely normalizes BMD in adult patients, so nutritional supplementation may be necessary[22,23]. Findings of this study show a higher prevalence (74%) of bone demineralization in adulthood diagnosed CD patients notwithstanding long-term strict adherence to GFD and persistent negative CD-related serology.

No differences in acknowledged risk factors for osteoporosis have been found between patients with low

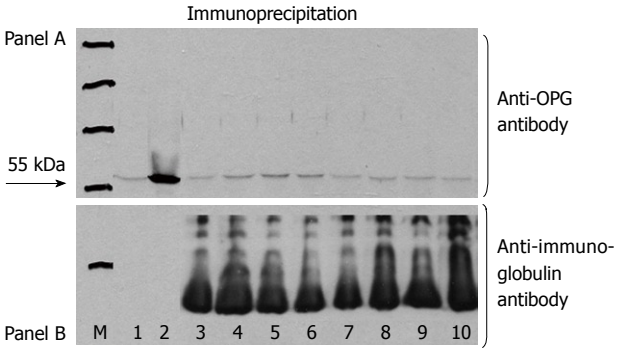

Figure 1 No evidence of the 55-kDa band was found in serum samples of the subgroup of 30 patients who were tested for, indicating no presence of auto-antibodies against osteoprotegerin. A: Western blotting (representative serum samples from a series of 30 celiac disease patients) showing the absence of antibodies against osteoprotegerin (OPG) after immunoprecipitation. Positive control indicates a 55 kDa band (arrow, lane 2) as the presence of antibodies against OPG. Negative control is shown in the lane 1; B: Western blotting confirms the equal loading of the gel by means of the addition of staining for immunoglobulins. Molecular-weight markers are shown in lane M.

and normal BMD except for age, suggesting that CD is a major one. Even though serum calcium levels may not adequately reflect calcium absorption, no patient showed low levels of serum calcium, and this was in accordance with the finding that calcium absorption returns to normal setting after one year GFD[24]. Furthermore, a significant proportion of patients (64%) on GFD showed low BMD, even if they displayed complete recovery of duodenal lesions as assessed by Marsh classification[25]. Nevertheless, recent observations suggested that normal Marsh grade does not exclude villous atrophy when assessed morphometrically[26].

Despite the high frequency of low BMD, there is still not a consensus about the timing for BMD evaluation in CD patients[27]. A novel finding of this study is that DEXA performed 92% negative predictive value in detecting intestinal mucosa recovery. So, based on this finding, the use of DEXA could be proposed for its additive value in this specific issue (e.g., a non-pathological DEXA has 92% probability to predict intestinal mucosa recovery in CD patients on GFD).

Insight of the molecular mechanism regulating osteoclast formation and activation progressed a lot in the past 10 years, with the identification of the RANKL/RANK signaling system as well as the discovering of OPG, a protein that appeared to protect from excessive bone reabsorption[28,29]. Fiore *et al*[30] demonstrated that OPG/RANKL ratio was significantly lower in CD patients with normalization of duodenal histology than in healthy controls and it positively correlated with low BMD. It has been hypothesized that in some patients OPG is bound to a plasma protein(s) and this could inactivate it[31].

In this study, circulating antibodies against OPG were not found in 30 CD patients. This contrasts with findings of Riches *et al*[14] who showed auto-antibodies against OPG in a man with CD on GFD presenting with severe osteoporosis and high bone turnover. As authors demonstrated, these auto-antibodies had the potential to block the inhibitory effect of OPG on RANKL and this lead to the hypothesis that they may play a role in the development of bone derangement. In the same report, authors detected these circulating auto-antibodies in three among 15 additional patients with CD and low BMD, while there was no evidence of them in serum specimens from 10 healthy controls and 14 patients with autoimmune hypothyroidism. If these CD patients were or were not on GFD was not indicated by the authors and data on duodenal mucosa histology were not provided.

It is unlikely that discrepancy between Riches *et al*[14] and findings of this study relies on the selection of patients neither on the used methodology. Indeed, it seems that the subgroup of 30 CD patients who were tested for antibodies against OPG is representative enough with respect to the variables that may affect the possible appearance of auto-antibodies (e.g., BMD, duodenal histology, HLA). Furthermore, Riches *et al*[14] anti-OPG antibodies measurement methodology has been strictly followed. A positive control has been checked in order to validate the procedure and several serum sample dilutions have been tested in order to increase sensitivity. Nevertheless, that a long-term GFD, as is the case of this study, may reduce the production of circulating auto-antibodies against OPG towards undetectable levels may be a possibility. At this regard, the occurrence of a limited amount of mucosal antibodies could be taken into account. Furthermore, genetic background affecting the immune system (e.g., auto-antibody development) may be another issue. Indeed, HLA DQ2 heterodimer has been shown to be more involved than HLA DQ8 heterodimer in complicated CD[32]. While for HLA-DQ2 a single deamidation in a gluten peptide is enough to produce a CD4+ T cell response, for HLA-DQ8 it is necessary a deamidation at two positions in the gluten peptide, resulting in a more limited generation of strong antigenic gluten peptides than HLA DQ2 haplotype[33]. Furthermore, the HLA-DQ8 peptidic domain is more easily degraded limiting the availability for antigen presentation[34]. With this in mind, in this study all 30 CD patients who were screened for antibodies against OPG were also tested for HLA DQ2/DQ8 alleles. As expected[35], a proportion of 77% and 23% for DQ2 and DQ8 haplotype, respectively, was found, indicating an unselected sample at this regard. Anyway, although the role of HLA molecules and the association to particular genotypes has been well established in CD pathogenesis, HLA is estimated to contribute only for the 35% of the genetic risk, suggesting that more genetic risk factors had to be involved in CD susceptibility[36].

In conclusion, the negative results of this study indicate that auto-antibodies against OPG, if any, do not play a major role in the pathogenesis of bone demineralization in patients with CD, suggesting that other mechanisms should be investigated.

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COMMENTS

Background

Evidences indicate that a low bone mineral density (BMD) is found in 20%-50% newly diagnosed patients with celiac disease (CD). Adherence to a strict gluten-free diet (GFD) will reverse the histological changes in the intestine and also the biochemical evidence of calcium malabsorption, resulting in normal BMD in these treated patients. Nevertheless, there may be long-term impairment of bone mineralization in some otherwise healthy CD patients adhering to GFD. Furthermore, osteopenia has been found in treated CD patients who showed improvement or even complete healing of intestinal mucosa.

Research frontiers

Other mechanisms of bone injury than calcium malabsorption are probably involved in patients with CD. Mediators of inflammatory immune-mediated responses (e.g., cytokines), parathyroid hormone (PTH), estrogens, androgens, corticosteroids and vitamin D are all acknowledged to affect BMD by modulating the receptor activator of nuclear factor B/receptor activator of nuclear factor B-ligand/osteoprotegerin [RANK/RANK-L/osteoprotegerin (OPG)] system. Recently, circulating neutralizing autoantibodies against OPG have been shown in CD patients with low BMD leading to the hypothesis that blocking the inhibitory effect of OPG on RANKL may have a role in the development of bone derangement in these patients.

Innovations and breakthroughs

This study aimed at investigating risk factors associated with low BMD in patients with CD, focusing on circulating auto-antibodies against OPG. Findings confirm a high prevalence of low BMD in CD patients despite strict adherence to GFD, persistent negative serology, and healing of duodenal lesions. Since auto-antibodies against OPG have not been found in serum samples from a subgroup of patients who were tested for, it can be argued that, if any, the role of antibodies against OPG in the pathogenesis of reduced BMD in patients with CD remains to be established.

Applications

The study further supports the importance of early diagnosis in order to avoid bone complications in CD patients. Furthermore, it remarks that autoantibodies against OPG do not play a major role in the pathogenesis of bone demineralization in patients with CD, suggesting that other mechanisms of bone derangement should be investigated.

Peer review

This is an interesting study showing antibodies to OPG are not present at least systemically to explain persistent low bone density in coeliac subjects. Authors guess there is a small possibility that mucosal antibodies could still be present which should still be alluded to.

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BRIEF ARTICLE

Mucosa-associated bacteria in two middle-aged women diagnosed with collagenous colitis

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Abstract

AIM: To characterize the colon microbiota in two women histologically diagnosed with collagenous colitis using a culture-independent method.

METHODS: Biopsies were taken from the ascending colon and the total DNA was extracted. Universal bacterial primers were used to amplify the bacterial 16S rRNA genes. The amplicons were then cloned into competent *Escherichia coli* cells. The clones were sequenced and identified by comparison to known sequences.

RESULTS: The clones could be divided into 44 different phylotypes. The microbiota was dominated by Firmicutes and Bacteroidetes. Seven phylotypes were found in both patients and constituted 47.5% of the total number of clones. Of these, the most dominating were clones similar to *Bacteroides cellulosilyticus, Bacteroides caccae, Bacteroides thetaiotaomicron, Bacteroides uniformis* and *Bacteroides dorei* within Bacteroidetes. Sequences similar to *Faecalibacterium prausnitzii* and *Clostridium citroniae* were also found in both patients.

CONCLUSION: A predominance of potentially pathogenic *Bacteroides spp.*, and the presence of clones showing similarity to *Clostridium clostridioforme* were found but the overall colon microbiota showed similarities to a healthy one. Etiologies for collagenous colitis other than an adverse bacterial flora must also be considered.

Key words: Microscopic colitis; Collagenous colitis; Lymphocytic colitis; Colonic microbiota; 16S rRNA sequencing

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INTRODUCTION

Collagenous colitis (CC), an idiopathic inflammatory bowel disease, is a subtype of microscopic colitis (MC) together with lymphocytic colitis (LC)[1]. It is considered as a common cause of chronic diarrhea. In Sweden the incidence is approx four to five cases per 100 000[2]. The incidence for

both CC and LC in Europe and North America is almost as high as for Crohn's disease and ulcerative colitis[2].

CC is clinically characterized by chronic non-bloody diarrhea, often combined with abdominal pain and weight loss[2]. The colonic mucosa appears macroscopically normal or near-normal and the diagnosis is made by microscopic examination of mucosal biopsies that reveals diagnostic histopathological changes. CC was first described in 1976 by Lindström[3] in a woman with chronic watery diarrhea in whom histological examination revealed a thick subepithelial collagenous deposition in the rectum. In 1989, Lazenby *et al*[4] proposed the term lymphocytic colitis in a group of patients with chronic diarrhea and normal colonoscopy with only minor histological changes, where the microscopic evaluation of colonic biopsy specimens revealed modestly increased inflammation in the lamina propria without subepithelial collagen deposition or other mucosal changes.

The peak incidence of MC is in individuals between 55 years and 70 years of age. The female:male ratio is about 7:1 for CC. For LC the female predominance is less pronounced, with a female:male ratio of 2-3:1[5]. However, the disease can occur at all ages, and a few children with CC have been reported[6,7]. Bile acid malabsorption is found in about 27%-44% of patients with CC and 9%-60% in patients with LC[5,8-9]. Treatment with bile acid binding medications is effective in patients with bile-acid malabsorption but can also be effective in patients without bile-acid malabsorption[10].

Both etiology and pathogenesis of MC are uncertain. The most widely held hypothesis is that a noxious agent in the lumen, probably originating from the bacterial microflora, may have a major pathogenic role in the chronic intestinal inflammation. This is supported by regression of symptoms and histopathological changes after diversion of the fecal stream, and recurrence after restoration of intestinal continuity[11,12]. Other observations supporting this hypothesis are the sudden onset of diarrhea and that treatment with antibiotics may have positive effects[2,13]. The increased infiltration of lymphocytes in the mucosa also indicates a proinflammatory component in the lumen. There are case reports of linking pathogenic bacteria such as *Clostridium difficile*, *Yersinia enterocolitica*, *Campylobacter jejuni* and *Aeromonas hydrophila* to MC[2,7,14-16].

The human microbiota in healthy persons as well as in patients with inflammatory bowel disease has been analyzed in several studies using culture-independent methods[17-19]. However, to our knowledge no such studies have been performed on patients diagnosed with CC. The aim of the present study was to characterize the mucosa-associated microbiota in the ascending colon in two women histologically diagnosed with CC, by cloning and sequencing of the bacterial 16S rRNA genes.

MATERIALS AND METHODS

Subjects and samples

Two female patients, 51 years and 60 years old (A and B) with a known diagnosis of MC, took part in the study. Patient A, otherwise healthy, started to experience watery, non-bloody diarrhea after an antibiotic treatment for gastroenteritis 10 years earlier. Colonoscopy was performed and she was diagnosed with LC. She was treated with Loperamid® (Merck NM AB, Stockholm, Sweden). Two years later she had a relapse of watery, non-bloody diarrhea and a second colonoscopy was performed, still indicating LC. This time she improved spontaneously. At the time of the present study, after a period of stress and a viral gastroenteritis, she started to lose weight and had frequent, watery, non-bloody diarrhea. The present colonoscopy showed a slightly swollen mucosa and increased vascular pattern. The histological examination revealed a thickened subepithelial collagen layer as well as inflammation in the lamina propria and a damaged surface epithelial layer. Patient B had a history of chronic thyreoiditis but was otherwise healthy. She was diagnosed with CC as well as with bile acid malabsorption 4 years before the study. At that time she improved spontaneously but had a recurrence after a period of major stress. Previously, she was treated with non-steroidal anti-inflammatory drugs due to muscular stiffness and actually experienced an improvement of her bowel function by this treatment. At the time of the present colonoscopy her symptoms had improved due to dietary fat reduction. Colonoscopy showed an increased vascular pattern in the right colon but was otherwise normal. Histological examination could verify a collagenous colitis.

Neither patient had any medication at the time of the colonoscopy. Celiac disease had been excluded in both women. They were both non-smokers.

The patients were asked to avoid fiber-rich foods such as fruits, vegetables, grains and seeds some days before the colonoscopy. The day before the examination they ate a plain breakfast, and no solid food was allowed after noon. Intestinal cleansing was carried out with Phosphoral® (Clean Chemical Sweden AB), a salt preparation with osmotic effects. Colonoscopy was performed and serial biopsies throughout the colon as well as two extra biopsies from the right colon were collected. The histological examination followed routine procedures. The latter were placed in tubes with TE-buffer [10 mmol Tris-HCl, 1 mmol ethylenediaminetetraacetic acid (EDTA), pH 8.0], frozen immediately in liquid nitrogen and stored at -80 ℃. The study was approved by the Ethics Committee at Lund University. The women gave written, informed consent before entering the study.

DNA extraction and amplification

Frozen tissue samples were thawed on ice and a single biopsy was transferred to a 1.5 mL tube with 190 μL Buffer G2 (DNA Tissue Kit; Qiagen, Gmbh, Hilden, Germany) and 10 μL of Proteinase K (Qiagen). Eight to ten sterile glass (2 mm) beads were added and the cells were lysed at 56 ℃ for 3-4 h in a shaking water bath. Tubes were cooled on ice and shaken for 30 min on an Eppendorf Mixer 5432 (Eppendorf, Hamburg, Germany) at 4 ℃

to disintegrate all bacteria. After centrifugation at 300 × *g* for one minute, the solution was transferred to a Qiagen sample tube, and total DNA was extracted by using Biorobot EZ1 (Qiagen) according to the manufacturer's instructions. DNA was eluted in 200 μL.

Polymerase chain reaction amplification and cloning

The bacterial 16S rRNA genes were amplified by the universal primers ENV1 and ENV2 annealing to positions 8-27 and 1492-1511, respectively, according to *Escherichia coli* (*E. coli*) numbering[20]. The reaction mixture contained 5 μL of 10× polymerase chain reaction (PCR) buffer (100 mmol Tris-HCl, 15 mmol $MgCl_2$, 500 mmol KCl, pH 8.3), each deoxynucleotide phosphate at a concentration of 200 μmol, 2.5 U of Tag DNA Polymerase (Roche Diagnostics, GmbH, Mannheim, Germany) and 10 pmol of each primer. To each tube, 5 μL of extracted sample DNA was added and sterile water was added to 50 μL. As negative controls, water was added to the reaction mixture instead of DNA. Amplification was performed on an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany). Initially, the reaction was heated to 94 ℃ for 3 min, followed by 25 cycles of denaturing at 94 ℃ for 1 min, annealing at 50 ℃ for 45 s and elongation at 72 ℃ for 2 min. Finally, the reaction was held at 72 ℃ for 7 min before cooling down to 4 ℃. Six PCR tubes were prepared from each sample and then pooled. Forty-two μL of the pooled reaction mixture from one sample was separated on a 1.5% (w/v) agarose gel (Agarose Type Ⅲ; Sigma Aldrich, St Louis, Mo., United States) in TBE-buffer (89 mmol Tris, 89 mmol boric acid and 2.5 mmol EDTA, pH 8.3). The agarose gel was stained with ethidium bromide (0.5 mg/L) and the band was cut out from the gel. DNA was purified by using Wizard® SV Gel and PCR Clean-Up System (Promega Corp., Madison, WI, United States). For cloning Promega pGEM®-T Vector System and *E. coli* JM 109 (Promega Corp.) competent cells were used as described previously[20]. Colonies were selected randomly and recultivated on LB-agar containing ampicillin, and then harvested and stored in freezing buffer at -80 ℃.

Sequencing

Selected clones were single-strand sequenced by MWG Biotech (Ebersberg, Germany). ENV1 primer was used as sequencing primer. Sequences were edited using Bioedit Sequence Alignment editor 7.0.5.3[21]. Sequences were identified by comparing them to sequences using the option "seqmatch" available at the Ribosomal Database Project[22]. Sequences were checked for chimeric artifacts by using the Bellerophon server[23] and by creating phylogenetic trees of both 5'- and 3'- ends of the sequences. DNAdist calculations were performed using the Phylip DNAdist program using the "similarity table" option (available at: http://mobyle.pasteur.fr/ cgi-bin/portal.py?form=dnadist)[24]. Sequences representing the different phylotypes have been submitted to Genbank and the accession numbers are HQ992999- HQ993042.

Diversity calculations

Shannon and Simpson's indices were used for diversity calculations. The Shannon index is based on the proportional abundance of species and accounts for both evenness and species richness. Simpson's index is the dominance measure where the abundance of commonest species is considered more than species richness[25]. The Simpson's index was expressed as 1/D.

RESULTS

Two clone libraries were constructed, one for patient A with 87 clones and one for patient B with 90 clones. Five clones were suspected chimeras and were removed from the dataset before analysis. The lengths of the sequenced fragments were approximately 750 bp. Sequences showing > 98% similarity to each other were assigned to a single phylotype and a total of 44 phylotypes were identified (Table 1).

Sequences could be grouped into 22 phylotypes in patient A and 29 phylotypes in patient B. Shannon's and Simpsons diversity indices were calculated and both the patients showed similar values. The Shannon index was 2.61 for patient A and 2.78 for patient B, and the Simpson index was 8.13 for A and 9.29 for patient B. Firmicutes and Bacteroidetes were the dominating phyla with 50.6% and 47.2% in patient A and 57.8% and 42.2% in patient B, respectively (Figure 1).

In patient A Porphyromonadaceae constituted 1.2% of the clones and in patient B, Porphyromonadaceae and Rikenellaceae constituted 11.1% of the clones. Only two clones (2.3%) similar to Enterobacteriaceae were found in patient A.

The most common phylotypes were sequences similar to *Blautia wexlerae* (23 clones), *Faecalibacterium prausnitzii* (13 clones) and *Clostridium citroniae* (9 clones) within Firmicutes, and *Bacteroides dorei* (29 clones), *Bacteroides caccae* (16 clones) and *Bacteroides cellulosilyticus* (9 clones) within Bacteroidetes (Table 1). These phylotypes showed > 97% similarity to the closest type strain except for *C. citroniae*. Out of the 44 phylotypes identified, the two patients had 7 in common and 5 of these were assigned to Bacteroidetes and two to the Firmicutes. The phylotypes in common constituted 84 clones (47.5%) of the total number of clones. Sequences similar to *F. prausnitzii* and *C. citroniae* were found in both patients (Table 1). Within Bacteroidetes the shared phylotypes were most similar to, *Bacteroides thetaiotaomicron*, *Bacteroides uniformis, B. cellulosilyticus, B. caccae* and *B. dorei*.

DISCUSSION

In the present study the microbiota of the ascending colon in the two female patients with CC showed similarities to a normal colon microbiota with Firmicutes and

Table 1 Sequences grouped into phylotypes at 98% similarity

Phylotype No.	Closest type strain	Acc. No[1]	Similarity (%)[2]	No. of clones[3]	Distribution of clones[4]	Assignment of clones
1	*Faecalibacterium prausnitzii*	AJ413954	98.4-98.5	9	8 (A); 1 (B)	Ruminococcaceae
2	*Faecalibacterium prausnitzii*	AJ413954	98.4-99.1	4	4 (B)	Ruminococcaceae
3	*Subdoligranulum variabile*	AJ518869	97.0	1	1 (B)	Ruminococcaceae
4	*Anaerofilum agile*	X98011	90.6	1	1 (B)	Ruminococcaceae
5	*Ruminococcus lactaris*	L76602	94.0	1	1 (B)	Ruminicoccaceae
6	*Oscillibacter valericigenes*	AB238598	92.5	1	1 (B)	Ruminicoccaceae
7	*Ruminococcus lactaris*	L76602	95.9	2	2 (A)	Ruminococcaceae
8	*Ruminococcus lactaris*	L76602	95.1	2	2 (B)	Ruminococcaceae
	Clostridium jejuense	AY494606	94.7			Lachnospiraceae
9	*Marvinbryantia formatexigens*	AJ505973	95.7	1	1 (B)	Lachnospiraceae
10	*Roseburia intestinalis*	AJ312385	94.1	1	1 (B)	Lachnospiraceae
11	*Anaerostipes caccae*	AJ270487	99.2	2	2 (A)	Lachnospiraceae
12	*Anaerostipes caccae*	AJ270487	95.8	1	1 (B)	Lachnospiraceae
13	*Roseburia intestinalis*	AJ312385	100.0	2	2 (A)	Lachnospiraceae
14	*Roseburia faecis*	AY305310	96.9	2	2 (B)	Lachnospiraceae
	Roseburia intestinalis	AJ312385	97.1			Lachnospiraceae
15	*Pseudobutyrivibrio ruminis*	X95893	94.2-94.3	2	2 (A)	Lachnospiraceae
16	*Dorea longicatena*	AJ132842	94.9-95.2	3	3 (A)	Lachnospiraceae
17	*Dorea longicatena*	AJ132842	96.4-97.0	5	5 (A)	Lachnospiraceae
18	*Dorea longicatena*	AJ132842	100.0	3	3 (B)	Lachnospiraceae
19	*Dialister pneumosintes*	X82500	99.6	1	1 (A)	Veillonellaceae
20	*Eubacterium plautii*	AY724678	91.5	1	1 (B)	Eubacteriaceae
21	*Streptococcus thermophilus*	AY188354	99.9	1	1 (B)	Streptococcaceae
22	*Blautia wexlerae*	EF036467	99.1-99.9	23	23 (B)	Insertae cedis XIV
23	*Clostridium citroniae*	DQ279737	95.1-96.2	9	7 (A); 2 (B)	Unclass Clostridiales
	Clostridium asparagiforme	AJ582080	95.1-95.5			Unclass Clostridiales
24	*Clostridium clostridioforme*	M59089	95.0-95.2	5	5 (A)	Unclass Clostridiales
	Clostridium citroniae	DQ279737	95.0			Unclass Clostridiales
25	*Clostridium clostridioforme*	M59089	99.4-99.7	3	3 (A)	Unclass Clostridiales
26	*Clostridium aldenense*	DQ279736	99.1	1	1 (A)	Unclass Clostridiales
27	*Clostridium asparagiforme*	AJ582080	95.7	1	1 (A)	Unclass Clostridiales
28	*Clostridium asparagiforme*	AJ582080	96.5	1	1 (B)	Unclass Clostridiales
29	*Clostridium clostridioforme*	M59089	95.1-95.9	5	5 (B)	Unclass Clostridiales
30	*Clostridium ramosum*	X73440	100.0	2	2 (A)	Unclass firmicutes
31	*Escherichia fergusonii*	AF530475	99.7-99.9	2	2 (A)	Gammaproteobacteria
32	*Barnesiella intestinihominis*	AB267809	99.1-99.3	2	2 (B)	Porphyrmonadaceae
33	*Barnesiella viscericola*	AB267809	92.1	1	1 (B)	Porphyrmonadaceae
34	*Barnesiella viscericola*	AB267809	90.0	1	1 (A)	Porphyrmonadaceae
35	*Parabacteroides distasonis*	AB238922	99.4-100.0	4	4 (B)	Porphyrmonadaceae
36	*Bacteroides cellulosilyticus*	AJ583243	97.6-98.9	9	4 (A); 5 (B)	Bacteroidaceae
37	*Bacteroides caccae*	X83951	99.4-99.9	16	2 (A); 14 (B)	Bacteroidaceae
38	*Bacteroides xylanisolvens*	AM230650	97.7	1	1 (A)	Bacteroidaceae
39	*Bacteroides thetaiotaomicron*	AE015928	99.9	6	4 (A); 2 (B)	Bacteroidaceae
40	*Bacteroides thetaiotaomicron*	AE015930	99.3	1	1 (B)	Bacteroidaceae
41	*Bacteroides uniformis*	AB050110	99.7-100.0	6	3 (A); 3 (B)	Bacteroidaceae
42	*Bacteroides dorei*	AB242142	97.3-98.7	29	26 (A); 3 (B)	Bacteroidaceae
43	*Alistipes putredinis*	L16497	92.4-92.7	2	2 (B)	Rikenellaceae
44	*Alistipes onderdonkii*	AY974071	99.7	1	1 (B)	Rikenellaceae

The type strain showing the highest similarity to the sequence is shown. Assignment of the clones to bacterial family level was done using the "sequence match" option in the Ribosomal data base[22]. [1]Accession number for the type strain; [2]Similarity to the closest type strain; [3]The total number of clones assigned to the phylotype; [4]Number of clones found in patient A and B, respectively.

Bacteroidetes as dominating phyla, making up 97.7% and 100.0% of the clones in patient A and B, respectively. Only two clones close to Enterobacteriaceae were found in patient A. In several studies, the microbiota of healthy persons have been analyzed by sequencing of the 16S rRNA genes using either fecal samples or tissue samples from the intestinal mucosa[17,18,26-28]. All these studies showed a predominance of Firmicutes and Bacteroidetes while Verrucomicrobia, Actinobacteria and gamma proteobacteria were detected at lower frequency.

The proportion of clones belonging to *Bacteroides* was 47.0% in patient A and 31.1% in patient B. These were higher figures than Wang *et al*[18], using a similar methodology, found in biopsies taken from the ascending colon from a healthy, 54-year old woman where *Bacteroides* constituted 24.4% of the clones. Hayashi *et al*[17] analyzed fecal samples of 3 healthy men aged 27, 34 and 54 years, and the proportion of *Bacteroides* was 4.2%, 3.4% and 14.9%, respectively. In another study of fecal samples from a healthy 40-year old man, *Bacteroides* constituted 14.4% of the total number of clones[26]. Delgado *et al*[27] analyzed clones from the descending colon from

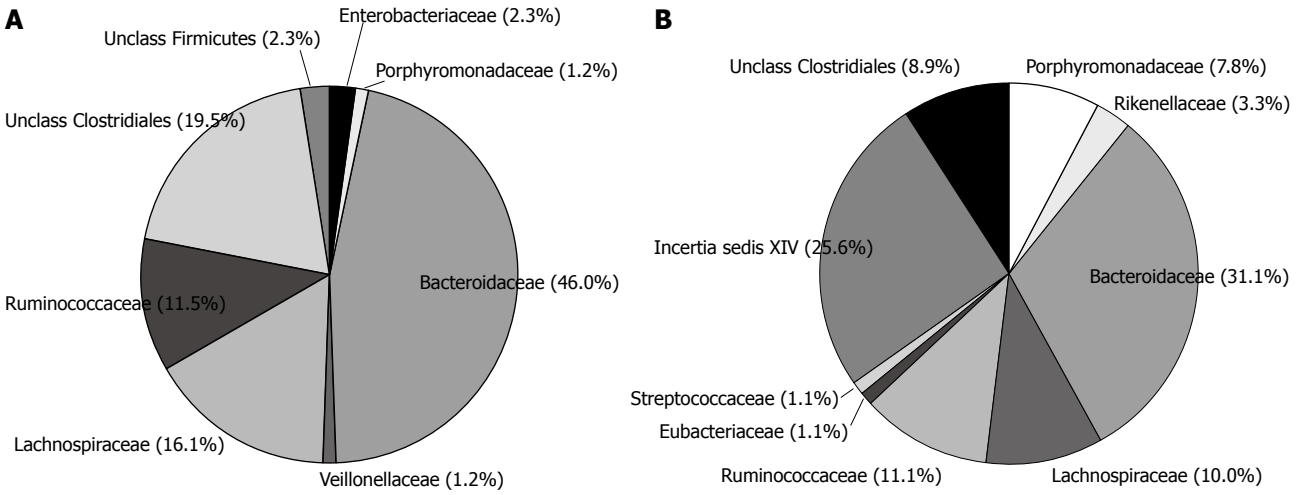

Figure 1 Distribution of clones at family level. Assignment of the clones were done using the Ribosomal Data Base Project Release 10 and the option "seqmatch"[22]. A: Patient A; B: Patient B.

a healthy 45-year old man and found one clone out of 20 (5%) belonging to *Bacteroides*. Of the 44 phylotypes found here, the two patients had only 7 in common. However, these shared phylotypes constituted 47.5% of the total number of clones. Within *Bacteroides* five phylotypes were common to both patients. Of these the most dominating were clones similar to *B. caccae* and *B. dorei* making up 25.4% of the total number of clones (Table 1). Both species belong to the *Bacteroides fragilis* group that are opportunistic pathogens isolated from a variety of anaerobic infections and cause about 50% of all anaerobic bacteremias[29,30].

A subgroup of *B. fragilis*, enterotoxigenic *B. fragilis* (ETBF), that can secrete a proinflammatory enterotoxin, has been found to be implicated in traveller´s diarrhea[31]. In a study by Zhang *et al*[32], significantly more ETBF were found in patients with watery diarrhea (26.8%) than in the control group (12.4%). ETBF was also found at a higher frequency in patients over 30 years of age compared to the control group. Additonally, it was shown that 27.0% of patients over the age of 60 carried ETBF compared to 3.7% for the control group. It has been suggested that *Bacteroides fragilis* toxin can bind to receptors on the epithelial cells, leading to a signal cascade and clevage of cadherine promoting an increased intestinal permeability[33]. An increased intestinal permeability was shown in one patient with CC, using an Ussing chamber[12]. Permeability was measured on biopsies taken from the sigmoid colon and it was shown that the intestinal integrity was improved after a fecal diversion by an ileostomy, but after restoration of the bowel continuity the permeabilty increased again[12]. Some improvement has been reported when CC patients were treated with metronidazole, penicillin or erythromycin[34]. *Bacteroides* are sensitive to metronidazole and that might point to *Bacteroides* as a possible disease-provoking agent[30]. On the other hand, the positive effect shown with penicillin and erythromycin speaks against *Bacteroides*[34].

It has been shown that *Akkermansia mucinciphila* and strains of *Clostridium, Prevotella* and *Bacteroides* are able to degrade mucin[35,36]. The type strain *B. thetaiotaomicron* NCTC 10582 was shown to express glycosidases and glycosulphatase and could degrade pig gastric mucin[37]. In the present study 4 clones from patient A and 3 clones from patient B showed high similarity (99.3%-99.9%) to the type strain *B. thetaiotaomicron* NCTC 10582 (Table 1). Clones belonging to *Akkermansia muciniphila* were not found. However, it has been shown that this species represents only about one percent of the microbiota in healthy children and adults[38]. One might speculate that specific components present within the microbiota of the CC patients, i.e., *Bacteroides spp.*, that has an impact both on the colonic mucin layer and the intestinal permeability, leading to an immune response.

The clones resembling *Clostridium clostridioforme*, *Clostridium citroniae*, *Clostridium aspargiforme* and *Clostridium aldenense* were distributed into 7 phylotypes showing 95%-99.7% similarity to the different type strains. Four clones from patient A showed high similarity to *C. clostridioforme* and *C. aldenense*. Also in patient B, 5 clones resembling C. *clostridioforme* were found, but they showed lower similarity to the type strain. They are all related and belong to cluster XIVa as defined by Collins *et al*[39], Warren *et al*[40] and Mohan *et al*[41]. Strains of *C. clostridioforme* and closely related species have been involved in a variety of infections[42]. In a study of autistic children, all of whom had gastrointestinal symptoms, high counts of fecal isolates showing 95% similarity to *C. clostridioforme* were found in the diseased children but not in the controls. It cannot be excluded that the presence of sequences resembling *C. clostridioforme* might play a role in the disease in the patients analyzed here.

Clones identified as *F. prausnitzii* of the Ruminococcaceae family were found in both patients and constituted about 7% of the total number of clones. These bacteria together with *Eubacterium rectale* and *Roseburia spp.* are known as butyrate producers and usually make up about 5%-10% of the human microbiota and can be re-

garded as commensals[43]. No clones resembling *Lactobacillus* nor Actinobacteria or Verrucomicrobia were found. This can probably be explained by the fact that too few clones were sequenced and that they usually constitute a minor part of the microbiota. Previously published case reports have suggested *Clostridium difficile*, *Yersinia enterocolitica*, *Campylobacter jejuni* and *Aeromonas hydrophila* to CC as possible pathogens[2,7,14-16]. This could not be confirmed in the present study. As different pathogens are described, and the fact that the colonic microbiota was similar to a healthy one, the etiology to CC may not primarily depend on abnormal microbiota, and antibiotics may not be the treatment of choice in this entity, as it is sometimes considered[34].

This study has some limitations. Only two patients were examined and the method applied here only detects the dominant bacteria. Future research needs to examine the presence of common pathogens in the bowel, but also etiologies of CC other than bacteria must be considered.

To the best of our knowledge, this is the first study of the intestinal microbiota in patients with a histologically diagnosed CC, by a culture-independent method. The overall composition of the colonic microbiota was similar to a healthy one with dominance of Firmicutes and Bacteroidetes. Due to the fact that only two patients were analyzed it is difficult to draw any conclusions, but in both patients a high proportion of potentially pathogenic species of *Bacteroides* and clones related to *C. clostridioforme* were found.

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COMMENTS

Background

Collagenous colitis (CC) is an idiopathic inflammatory bowel disease characterized by chronic non-bloody diarrhea. CC is regarded as a subtype of microscopic colitis. The etiology is unknown but a noxious agent, probably originating from the microbiota, in the intestinal lumen has been proposed to have a pathogenic role. However, no attempt to analyze the microbiota in diseased patients has been done.

Research frontiers

The intestinal mucosa is colonized by a huge number of bacteria that are important for health and disease. In several studies the gut microbiota has been analyzed by culture-independent methods in patients with intestinal inflammatory diseases such as ulcerative colitis and Crohn's disease.

Innovations and breakthroughs

Having the opportunity to obtain histologically well-defined collagenous colitis samples, the authors have characterized the dominant microbiota in two diseased patients.

Applications

Culture-independent methods can be used for analyzing the dominant mucosa-associated microbiota in collagenous colitis.

Terminology

The meaning of the word microbiota here is synonymous to the bacterial flora in the intestine.

Peer review

It is well organized. Several papers have been presented in support of microbiota from controls, but no speculation have been made about findings in this paper and clinical applications, limitations of the study and future of research in this field.

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BRIEF ARTICLE

Randomized controlled trial of pancreatic stenting to prevent pancreatitis after endoscopic retrograde cholangiopancreatography

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Abstract

AIM: To determine the effectiveness of pancreatic duct (PD) stent placement for the prevention of pancreatitis after endoscopic retrograde cholangiopancreatography (ERCP) in high risk patients.

METHODS: Authors conducted a single-blind, randomized controlled trial to evaluate the effectiveness of a pancreatic spontaneous dislodgement stent against post-ERCP pancreatitis, including rates of spontaneous dislodgement and complications. Authors defined high risk patients as having any of the following: sphincter of Oddi dysfunction, difficult cannulation, prior history of post-ERCP pancreatitis, pre-cut sphincterotomy, pancreatic ductal biopsy, pancreatic sphincterotomy, intraductal ultrasonography, or a procedure time of more than 30 min. Patients were randomized to a stent group (n = 60) or to a non-stent group (n = 60). An abdominal radiograph was obtained daily to assess spontaneous stent dislodgement. Post-ERCP pancreatitis was diagnosed according to consensus criteria.

RESULTS: The mean age (± standard deviation) was 67.4 ± 13.8 years and the male: female ratio was 68:52. In the stent group, the mean age was 66 ± 13 years and the male: female ratio was 33:27, and in the non-stent group, the mean age was 68 ± 14 years and the male: female ratio was 35:25. There were no significant differences between groups with respect to age, gender, final diagnosis, or type of endoscopic intervention. The frequency of post-ERCP pancreatitis in PD stent and non-stent groups was 1.7% (1/60) and 13.3% (8/60), respectively. The severity of pancreatitis was mild in all cases. The frequency of post-ERCP pancreatitis in the stent group was significantly lower than in the non-stent group (P = 0.032, Fisher's exact test). The rate of hyperamylasemia were 30% (18/60) and 38.3% (23 of 60) in the stent and non-stent groups, respectively (P = 0.05, χ^2 test). The placement of a PD stent was successful in all 60 patients. The rate of spontaneous dislodgement by the third day was 96.7% (58/60), and the median (range) time to dislodgement was 2.1 (2-3) d. The rates of stent migration, hemorrhage, perforation, infection (cholangitis or cholecystitis) or other complicationss were 0% (0/60), 0% (0/60), 0% (0/60), 0% (0/60), 0% (0/60), respectively, in the stent group. Univariate analysis revealed no significant differences in high risk factors between the two groups. The pancreatic spontaneous dislodgement stent safely prevented post-ERCP pancreatitis in high risk patients.

CONCLUSION: Pancreatic stent placement is a safe and effective technique to prevent post-ERCP pancreatitis. Therefore authors recommend pancreatic stent placement after ERCP in high risk patients.

Key words: Endoscopic retrograde cholangiopancreatography; Pancreatitis; Postoperative complications; Prophylaxis; Stents

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Kawaguchi Y, Ogawa M, Omata F, Ito H, Shimosegawa T, Mine T. Randomized controlled trial of pancreatic stenting to prevent pancreatitis after endoscopic retrograde cholangiopancreatography. *World J Gastroenterol* 2012; 18(14): 1635-1641 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i14/1635.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i14.1635

INTRODUCTION

Acute pancreatitis is a serious complication of endoscopic retrograde cholangiopancreatography (ERCP). The frequency of post-ERCP pancreatitis varies between 1% and 9%[1-6] in average risk patients, and its prevention remains a critical issue. Impaired drainage of the pancreatic duct (PD), leading to acinar injury, is a commonly accepted mechanism of injury[7,8]. Possible causes of impaired drainage include mechanical, chemical and thermal injury, as well as subsequent edema and spasm of the duodenal papilla.

Several prospective studies have confirmed that PD stent placement prevents post-ERCP pancreatitis, especially in high risk patients[9-13]. However, no consensus has yet been reached on the indications for prophylactic PD stent placement or on the type of stent which should be used. Therefore, we conducted a single-blind, randomized controlled trial (RCT) to evaluate the effectiveness of spontaneous dislodgement stents in preventing post-ERCP pancreatitis in high risk patients.

MATERIALS AND METHODS

Study design

This RCT was conducted in Tokai University Hospital, Japan between April 2006 and June 2010. During this period, we performed 1438 ERCPs on patients with pancreatobiliary diseases. All procedures for this study were performed by one physician (Kawaguchi Y), an experienced surgeon with more than 2000 prior ERCP cases. Independent patient-related and procedure-related risk factors for post-ERCP pancreatitis have been previously published [3-5,14-16]. Patients at high risk of post-ERCP pancreatitis who met any of the following criteria were enrolled in this RCT: (1) A previous history of post-ERCP pancreatitis; (2) Sphincter of Oddi dysfunction; (3) Difficult cannulation; (4) Pre-cutting; (5) PD biopsy; (6) Intraductal PD ultrasonography; or (7) ERCP procedure time > 30 min prior to PD stent placement. Difficult cannulation was defined as > 10 min of attempted cannulation. We performed PD guidewire placement or pancreatic sphincterotomy in some cases of difficult cannulation. Exclusion criteria were as follows: (1) Inability to provide written informed consent; (2) Performance status of 4; (3) Age 19 years or younger; (4) Pregnancy or breastfeeding; (5) Inability to access duodenal papilla endoscopically; (6) Previous endoscopic sphincterotomy or endoscopic papillary balloon dilation; (7) Inability to insert a guidewire into the PD; (8) Patients requiring PD drainage; (9) Patients requiring endoscopic papillectomy; (10) Pancreatic head cancer; or (11) Pancreas divisum.

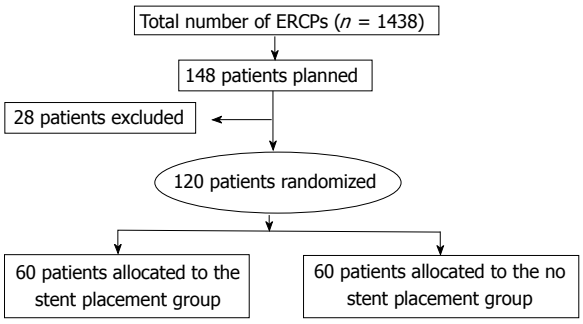

Figure 1 Trial profile.

Of 148 patients screened, 120 patients who satisfied the inclusion criteria participated in this study. Patients were randomly assigned into a stent placement group (60 patients) and a non-stent placement group (60 patients) after the completion of diagnostic or therapeutic ERCP (Figure 1). Randomization was conducted by a simple randomization method without any stratification factor. After diagnostic or therapeutic ERCP was achieved and eligibility was confirmed, a research assistant assigned patients to either the stent or non-stent group, allocating patients using a uniform random number algorithm. All patients provided written informed consent before study entry. This study was approved by Institutional Review Board of Tokai University Hospital (Clinical Trials; University Hospital Medical Information Network Identification Number in Japan, 3995).

Endoscopic procedures and patient cares

Each patient's past medical history was obtained and physical examination was performed prior to ERCP. Lidocaine gel (4%) was used for pharyngeal anesthesia. Intravenous administration of midazolam with pethidine hydrochloride was used for conscious sedation. All patients received an intravenous drip infusion of 20 mg nafamostat mesilate during the examination, starting before ERCP, with the same dose of nafamostat mesilate as well as antibiotics administered afterwards.

ERCP was performed in standard fashion with JF-240, JF-260V or TJF240 video endoscopes (Olympus, Tokyo, Japan); PR-V216Q, PR-V234Q or PR-V220Q catheters (Olympus) and Jagwire guidewires (Boston Scientific Japan, Tokyo, Japan) were used. We used 5 Fr straight polyethylene stents, 3 cm in length, unflanged on the PD side, and with two flanges on the duodenal side (GPDS-5-3; Cook Endoscopy Inc., Winston-Salem, NC,

Table 1 Patient characteristics

	Non-stent	Stent	*P* value
No. of patients	60	60	-
Mean age (range) (yr)	68 (27-92)	66 (24-88)	0.35
Sex: female/male	25/35	27/33	0.46
Reasons of high risk			
Previous post-ERCP pancreatitis	5 (8%)	5 (8%)	0.65
Sphincter of Oddi dysfunction	0 (0%)	0 (0%)	-
A difficult cannulation	10 (17%)	10 (17%)	0.68
Pre-cut	0 (0%)	0 (0%)	-
Pancreatic sphincterotomy	5 (8%)	4 (7%)	1
Pancreatic duct biopsy	5 (8%)	6 (10%)	1
IDUS for pancreatic duct	27 (45%)	25 (42%)	0.15
Procedure time greater than 30 min	29 (48%)	33 (55%)	0.50

ERCP: Endoscopic retrograde cholangiopancreatography; IDUS: Intraductal ultrasonography.

United States). Stent dislodgment was confirmed by daily serial abdominal radiography. If the stent remained on the third day, it was removed endoscopically. In the stent group, we performed PD cannulation followed by contrast injection, guidewire insertion, and stent placement under fluoroscopy. All 120 patients were hospitalized for at least 3 d after the procedure for observation of potential pancreatitis or other complication, regardless of stent placement. On the assumption that the frequency of post-ERCP pancreatitis was 2.3% and 20% in the stent and non- stent group, respectively, 60 patients were needed in each group for 80% power and 5% type I error.

Definitions

The definition of post-ERCP pancreatitis was based on Cotton's criteria[17], with a modified definition of severity. Instead of the number of hospital days, we evaluated the degree of severity of pancreatitis by number of days before resuming feeding. Post-ERCP pancreatitis was defined as pancreatic pain and hyperamylasemia within 24 h post-procedure. Pancreatic pain was defined as persistent pain in the epigastric or periumbilical region. Hyperamylasemia was defined as an amylase level greater than three times the upper limit of normal in our institution.

Outcomes

The primary outcome was frequency and severity of post-ERCP pancreatitis. As secondary outcomes, we evaluated the frequency of serum hyperamylasemia, the success rate of stent placement, time to stent dislodgement, and other complications.

Statistical analysis

This trial was designed as a superiority study to detect differences in clinical effectiveness to prevent post-ERCP pancreatitis with the addition of PD stent placement. The effectiveness of pancreatic stenting was analyzed on an intention-to-treat basis.

The χ^2 test or Fisher's exact test were used to evaluate proportional differences. The Student *t* test was used for comparing continuous variables. Univariate evaluation was made for each potential risk factor. All statistical analyses were performed with StatView Ver. 5.0 (SAS Institute, Cary, NC, United States) and StatMate 4 (ATMS, Tokyo, Japan).

Table 2 Final diagnosis *n* (%)

	Non-stent	Stent	*P* value
Biliary disease			
CBD stone	16 (27)	15 (25)	0.68
Cholangitis	2 (3)	2 (3)	0.49
Cholangiocarcinoma	4 (7)	3 (5)	1
Cholangiocellular carcinoma	2 (3)	1 (2)	1
Benign biliary stricture	1 (2)	2 (3)	1
Primary sclerosing cholangitis	2 (3)	1 (2)	1
GB stone	1 (2)	0 (0)	1
GB polyp	1 (2)	1 (2)	1
GB adenomyomatosis	1 (2)	1 (2)	0.46
Cholecystitis	1 (2)	1 (2)	0.46
GB carcinoma	3 (5)	3 (5)	0.47
Pancreatic disease			
IPMN	10 (17)	11 (18)	0.67
MCN	0 (0)	1 (2)	1
SCN	1 (2)	0 (0)	1
Chronic pancreatitis	3 (5)	3 (5)	0.47
Pancreatic cyst	1 (2)	2 (3)	1
Pancreatic carcinoma	11 (18)	13 (22)	0.65

CBD: Common bile duct; GB: Gallbladder; IPMN: Intraductal papillary mucinous neoplasm; MCN: Mucinous cystic neoplasm; SCN: Serous cystic neoplasm.

RESULTS

Patient characteristics

Tables 1 and 2 show basic patient characteristics including risk factors for post-ERCP pancreatitis and final diagnosis in both groups. The mean age (± standard deviation) of all patients was 67.4 ± 3.8 years and the male: female ratio was 68:52. In the stent group, the mean age was 66 ± 13 years and the male: female ratio was 33:27, whereas the mean age was 68 ± 14 years and the male: female ratio was 35:25 in the non-stent group. There were no significant differences between groups with respect to age, gender, final diagnosis, or type of endoscopic intervention (Table 1).

Pancreatic stenting

The placement of the PD stent was successful in all 60 patients and no complications were observed (Table 3).

Post-ERCP pancreatitis

The overall rate of post-ERCP pancreatitis was 7.5% (9/120). The rate of post-ERCP pancreatitis in the stent and non-stent groups was 1.7% (1/60) and 13.3% (8/60), respectively ($P = 0.032$, Fisher's exact test). The severity of pancreatitis was mild in all nine patients (Table 4). The rate of hyperamylasemia was 30% (18/60) and 38.3% (23/60) in the stent and non- stent groups, respectively ($P = 0.05$, χ^2 test).

Table 3 Placement of the pancreatic duct stent

No. of patients	60
Success rate in stent placement	100%
Rate of spontaneous stent dislodgement	96.7%
Duration time to dislodgement, d, (range)	2.1 (23)
Complications	
Stent migration	0%
Post-ERCP pancreatitis	1.7%
Hyperamylasemia	30%
Hemorrhage	0%
Perforation	0%
Infection (cholangitis, cholecystitis)	0%
Others	0%
Mean serum amylase level after procedures, U/L, (range)	1246 (746-1964)

ERCP: Endoscopic retrograde cholangiopancreatography.

Table 4 Overall post-endoscopic retrograde cholangiopancreatography pancreatitis in non-stent and stent groups

	Non-stent	Stent	*P* value
No. of patients	60	60	
Hyperamylasemia	23 (38.3%)	18 (30%)	0.862
Average serum amylase level (IU/L) (range)	842.4 (381-2040)	746.2 (420-1620)	0.798
Post-ERCP pancreatitis	8 (13.3%)	1 (1.7%)	0.0322
Mild	8	1	0.0322
Moderate	0	0	-
Severe	0	0	-
Average serum amylase level in pancreatitis cases (IU/L) (range)	1720 (820-2040)	1240 (746-1964)	0.04

ERCP: Endoscopic retrograde cholangiopancreatography. The Chi-square test or Fisher's exact test was used to determine the significance of associations, and $P < 0.05$ was regarded as significant.

Stent dislodgement

The rate of spontaneous dislodgement by the third day was 96.7% (58/60), and the median (range) time to dislodgement was 2.1 (range, 2-3) d (Table 3).

Other complications

The rates of stent migration, hemorrhage, perforation, infection (cholangitis or cholecystitis) or other complications were 0% (0/120) in both groups (Table 3).

Risk factors of post-ERCP pancreatitis

Table 5 shows the final diagnoses of those patients with post-ERCP pancreatitis patients in both groups. Regarding univariate analysis of risk factors for post-ERCP pancreatitis, there were no significant differences between groups with respect to various high risk factors studied (Table 6).

DISCUSSION

We conducted a single-blind, randomized controlled clinical study to evaluate the effectiveness of spontaneous dislodgement PD stents for preventing post-ERCP pancreatitis in high risk patients. The overall rate of post-ERCP pancreatitis was 7.5%. This was consistent

Table 5 Final diagnoses in post-endoscopic retrograde cholangiopancreatography pancreatitis patients

	Non-stent	Pancreatitis	Stent	Pancreatitis
Biliary disease				
CBD stone	16	3 (19%)	15	0
Cholangitis	2	0	2	0
Cholangiocarcinoma	4	1 (25%)	3	0
Cholangiocellular carcinoma	2	0	1	0
Benign biliary stricture	1	0	2	0
Primary sclerosing cholangitis	2	0	1	0
GB stone	1	0	0	0
GB polyp	1	0	1	0
GB adenomyomatosis	1	0	1	0
Cholecystitis	1	0	1	0
GB carcinoma	3	1 (33%)	3	0
Pancreatic disease				
IPMN	10	1 (10%)	11	0
MCN	0	0	1	0
SCN	1	0	0	0
Chronic pancreatitis	3	0	3	0
Pancreatic cyst	1	0	2	0
Pancreatic carcinoma	11	2 (18%)	13	1 (8%)

CBD: Common bile duct; GB: Gallbladder; IPMN: Intraductal papillary mucinous neoplasm; MCN: Mucinous cystic neoplasm; SCN: Serous cystic neoplasm.

with reported rates of post-ERCP pancreatitis, ranging from 1% to as high as 40% in patients with risk factors[1-5,7-13,18-22]. The rate of post-ERCP pancreatitis in the stent and non-stent groups was significantly different at 1.7% and 13.3%, respectively. Thus, prophylactic PD stenting may reduce the rate of post-ERCP pancreatitis from 13.3% to 1.7% in high risk patients. Our study suggested that PD stent placement prevented post-ERCP pancreatitis in high risk patients. However the severity of pancreatitis in this study was mild in all cases and we were therefore were unable to assess the efficacy of pancreatic stents for severe post-ERCP pancreatitis. Considering the sample size of this study and no cases of severe post-ERCP pancreatitis, this study had a limitation to determine any significant difference in severity between the two groups.

Several prospective studies have suggested that prophylactic PD stent placement decreases the risk of pancreatitis in high risk patients[9-14]. In contrast, Smithline *et al*[2] reported that PD stent insertion after ERCP did not have a significant beneficial effect in individuals undergoing biliary sphincterotomy for various indications. These differences may be attributed to variable levels of risk for pancreatitis in the individual study populations. However, two independent meta-analysis on the use of pancreatic stent placement for post-ERCP pancreatitis prophylaxis in patients at high risk of post-ERCP pancreatitis have demonstrated that stent placement significantly reduced the incidence of post-ERCP pancreatitis[10,23]. Based on these meta-analyses, European Society of Gastrointestinal Endoscopy guidelines recommend prophylactic pancreatic stent placement to prevent post-ERCP pancreatitis in high risk patients[24]. An updated meta-analysis of RCTs involving pancreatic stent place-

Table 6 Analysis of risk factors for post-endoscopic retrograde cholangiopancreatography pancreatitis *n* (%)

	Non-stent	Pancreatitis	Stent	Pancreatitis	Univariate *P* value
No. of patients	60	8	60	1	
Age (< 60 yr)	17 (28)	3 (18)	18 (30)	0 (0)	0.72
Female	25 (42)	3 (12)	27 (45)	0 (0)	0.78
Previous post-ERCP pancreatitis	5 (8)	1 (20)	5 (8)	0 (0)	0.68
Sphincter of Oddi dysfunction	0 (0)	0 (0)	0 (0)	0 (0)	-
A difficult cannulation	10 (17)	2 (20)	10 (17)	0 (0)	0.68
Pre-cut	0 (0)	0 (0)	0 (0)	0 (0)	-
Pancreatic sphincterotomy	5 (8)	1 (20)	4 (7)	0 (0)	0.68
Pancreatic duct biopsy	5 (8)	1 (20)	6 (10)	1 (17)	0.87
IDUS for pancreatic duct	27 (45)	3 (11)	25 (42)	1 (4)	0.74
Procedure time greater than 30 min	29 (48)	4 (14)	33 (55)	0 (0)	0.72

ERCP: Endoscopic retrograde cholangiopancreatography; IDUS: Intraductal ultrasonography.

ment also showed that pancreatic stent placement after ERCP reduced the risk of post-ERCP pancreatitis and was beneficial for patients at high risk compared to those who did not have stenting[25]. However, several areas requiring further clarification were noted, including the efficacy of pancreatic stents for severe post-ERCP pancreatitis, identification of risk factors and management of adverse events, optimal stent design and material, timing of both placement and removal, and comparison of stenting with wire-guided cannulation or pharmacoprophylaxis[25]. Several of these points warrant further investigation.

Regarding indications for stenting, prophylactic pancreatic stent placement has been shown to be cost-effective in patients at high risk of post-ERCP pancreatitis, but not in those at average risk[26]. Caution should be used when attempting prophylactic pancreatic stent placement due to the incidence of post-ERCP pancreatitis after failed attempts, which may be as high as 65%[27]. As such, prophylactic pancreatic stent placement in high risk patients is cost-effective only if the success rate of stent placement exceeds 75%. Careful selection of patients with risk factors for post-ERCP pancreatitis is therefore critical.

The type of stent used may also play an important role in prophylactic care. As for diameter of stents, most recent studies have used 3 Fr and 5 Fr diameter pancreatic stents. In two recent RCTs, 5 Fr stents proved equivalent to 3 Fr stents for most outcomes studied, though successful insertion of 5 Fr stents was achieved significantly more often[28,29]. Placement of 3-4 Fr stents require a small-caliber guidewire (0.018-0.025 inch), a procedure that is difficult and which requires a high level of experience[17,18,30,31]. In contrast, the 0.035-inch guidewire used for 5 Fr stents is relatively easy to use for stent placement. Regarding stent length, we used very short (3 cm) stents in this study. Long stents may be more difficult to place and may have higher rates of spontaneous dislodgement, causing damage to the intestines. We therefore recommend the use of shorter stents.

Regarding placement of PD stents, previous studies reported a 5%-10% failure rate and a low rate of complications (2%)[2,9,13]. It is difficult to cannulate the PD after all the procedures, and PD cannulation itself may cause pancreatitis. Unsuccessful cases were reported to be at a higher risk of pancreatitis[7,27]. In our study, we could place PD stents successfully in all patients. PD stent-related complications, such as migration or occlusion, did not occur in any patients. Based on the previous literature and our outcomes, we recommend 5 Fr diameter and very short (3 cm) stents.

As for optimal stent design, Sofuni *et al*[12] reported that the unflanged duodenal pigtail-type stent dislodged spontaneously at a higher rate, and that handling of the short duodenal pigtail stent may be complicated, such as the sudden forward movement of the stent on release, requiring close attention and experience. In addition, an internal flange may make spontaneous PD stent dislodgement difficult. In contrast, stents without an internal flange may dislodge spontaneously into the duodenum by pancreatic juice flow or friction with passing food. Since we used 5 Fr straight type stents without an internal flange, stents dislodged spontaneously in 1 d or 2 d in most cases. In our study, the spontaneous dislodgement rate was 96% at 3 d, with a median of 2.1 d. In the absence of spontaneous migration out of the PD at 5-10 d post-ERCP, prompt endoscopic stent removal is recommended due to the increased risk of post-ERCP pancreatitis (relative risk 5.2 in patients without *vs* with spontaneous stent elimination at 2 wk) and potential for stent-induced damage to the PD[28,29]. It is our recommendation to avoid the use of stents with an internal flange and to confirm stent dislodgement, or to remove stents in a timely interval, preferably within 1-2 wk after ERCP.

Finally, prophylactic pancreatic stent placement by operator and location warrants discussion. According to survey data, only a small percentage of endoscopists utilize prophylactic pancreatic stenting. The incidence of post-ERCP pancreatitis and a high ERCP volume were independently associated with the use of prophylactic pancreatic stenting[32-35]. From these surveys, endoscopists who did not place prophylactic pancreatic stents cited lack of experience in this technique as a primary reason[32]. We anticipate that a stent insertion success rate and low rate of complications can also be achieved in other institutions by ERCP specialists. Placement of

pancreatic stents is quickly becoming standard practice. However, many endoscopists remain unfamiliar with the specific techniques required to achieve safe and effective PD guidewire access and stent placement. It is critically important that all endoscopists performing ERCP become proficient in techniques for safe and effective stent placement.

Our study has several limitations. First, this study is single-blind and observation bias is inevitable. Second, we could not evaluate the risk of post-ERCP pancreatitis in patients who failed PD stent placement. Third, we could not evaluate the efficacy of PD stenting alone because nafamostat mesilate was administered in both groups.

In conclusion, pancreatic spontaneous dislodgement stent placement decreases the risk of post-ERCP pancreatitis in patients who are likely to develop post-ERCP pancreatitis.

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COMMENTS

Background

Acute pancreatitis is a serious complication of endoscopic retrograde cholangiopancreatography (ERCP) and its prevention remains a critical issue. Though several studies have confirmed that pancreatic duct (PD) stent placement prevents post-ERCP pancreatitis, no consensus has yet been reached on the indications for prophylactic PD stent placement or on the type of stent.

Research frontiers

In this study, the authors demonstrated that pancreatic stent placement was a safe and effective technique to prevent post-ERCP pancreatitis. Authors recommend pancreatic stent placement after ERCP procedures in high risk patients.

Innovations and breakthroughs

All procedures in this study were performed by one endoscopist (Kawaguchi Y), an experienced operator with more than 2000 prior ERCP cases. Independent patient-related and procedure-related risk factors for post-ERCP pancreatitis have been previously published. This study suggests that PD stenting can significantly reduce post-ERCP pancreatitis in high risk patients.

Applications

By the general use of the pancreatic stent placement after ERCP procedures in high risk patients, post-ERCP pancreatitis may decrease in the future.

Terminology

The authors used 5 Fr straight polyethylene stents, 3 cm in length, unflanged on the pancreatic duct side, and with two flanges on the duodenal side. As this stent dislodges spontaneously into the duodenum as a result of pancreatic juice flow or friction with passing food, it is termed a spontaneous dislodgement stent.

Peer review

The pancreatic spontaneous dislodgement stent placement safely prevented post-ERCP pancreatitis in high risk patients. This result is very impressive.

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BRIEF ARTICLE

Relationship between hepatitis C virus infection and type 2 diabetes mellitus: Meta-analysis

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Abstract

AIM: To investigate the association between hepatitis C infection and type 2 diabetes mellitus.

METHODS: Observational studies assessing the relationship between hepatitis C infection and type 2 diabetes mellitus were identified *via* electronic and hand searches. Studies published between 1988 to March 2011 were screened, according to the inclusion criteria set for the present analysis. Authors performed separate analyses for the comparisons between hepatitis C virus (HCV) infected and not infected, and HCV infected and hepatitis B virus infected. The included studies were further subgrouped according to the study design. Heterogenity was assessed using I^2 statistics. The summary odds ratios with their corresponding 95% CIs were calculated based on a random-effects model. The included studies were subgrouped according to the study design. To assess any factor that could potentially affect the outcome, results were further stratified by age group (proportion of $\geqslant$ 40 years), gender (proportion of male gender), body mass index (BMI) (proportion of BMI $\geqslant$ 27), and family history of diabetes (i.e., self reported). For stability of results, a sensitivity analysis was conducted including only prospective studies.

RESULTS: Combining the electronic database and hand searches, a total of 35 observational studies (in 31 articles) were identified for the final analysis. Based on random-effects model, 17 studies (n = 286 084) compared hepatitis C-infected patients with those who were uninfected [summary odds ratio (OR): 1.68, 95% CI: 1.15-2.45]. Of these 17 studies, 7 were both a cross-sectional design (41.2%) and cohort design (41.2%), while 3 were case-control studies (17.6%). Nineteen studies (n = 51 156) compared hepatitis C-infected participants with hepatitis B-infected (summary OR: 1.92, 95% CI: 1.41-2.62). Of these 19 studies, 4 (21.1%), 6 (31.6%) and 9 (47.4%) were cross-sectional, cohort and case-control studies, respectively. A sensitivity analysis with 3 prospective studies indicated that hepatitis C-infected patients had a higher risk of developing type 2 diabetes compared with uninfected controls (summary odds ratio: 1.41, 95% CI: 1.17-1.7; I^2 = 0%). Among hepatitis C-infected patients, male patients (OR: 1.26, 95% CI: 1.03-1.54) with age over 40 years (summary OR: 7.39, 95% CI: 3.82-9.38) had an increased frequency of type 2 diabetes. Some caution must be taken in the interpretation of these results because there may be unmeasured confounding factors which may introduce bias.

CONCLUSION: The findings support the association between hepatitis C infection and type 2 diabetes mellitus. The direction of association remains to be determined, however. Prospective studies with adequate sample sizes are recommended.

Key words: Hepatitis C; Type 2 diabetes mellitus; Observational studies; Meta-analysis

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INTRODUCTION

Hepatitis C virus (HCV) infections has been identified as one of the leading causes of chronic liver disease with serious sequelae such as end-stage cirrhosis and liver cancer[1]. Moreover, chronic HCV infection has been associated with several extrahepatic complications[2-4]. The suggestion that HCV may be associated with type 2 diabetes mellitus (type 2 DM) was first made by Allison in 1994. Since then, scores of observational studies assessing the association between HCV and type 2 DM have been published. However, these studies have provided inconclusive results, with some studies supporting the excess type 2 DM risk with HCV infection compared to non-HCV infected controls[3,5], and some studies showed differently[6-8]. There are narrative reviews which have assessed the association between HCV infections and type 2 DM[9-13]. In 2008, a meta-analysis of observational studies reported an excess type 2 DM risk with HCV infection[14]. After these reviews were published, new observational studies in which prevalence of type 2 DM in patients with HCV infection was assessed have been carried out in endemic countries. As the epidemiology of HCV is complex and heterogeneous, information from studies across geographic regions is important. Moreover, the current review also assesses the traditional risk factors.

The objectives were (1) to investigate the available evidence on the association between HCV infections and type 2 DM; and (2) to assess the effect of study design and traditional risk factors on the association.

MATERIALS AND METHODS

Data sources and search strategy

Published studies that assess the association between HCV and type 2 DM were searched in MEDLINE, EMBASE and PubMed databases covering the period from 1980 to March 2011. Literature search was carried out using the combination of terms "diabetes", "diabetes mellitus", "type Ⅱ diabetes mellitus", "type 2 diabetes mellitus", "type Ⅱ diabetes", "T2D", "T2DM", "type 2 DM", "non-insulin dependent diabetes", or "NIDDM" and "hepatitis", "hepatitis C", "hepatitis C virus", "HCV", "HVC", or "chronic hepatitis" and "risk", "risk factor", "case-control", "cohort", "clinical trial", "cross-sectional", "epidemiology", "observational", "meta-analysis", "systematic review", or "review". In addition, we searched Cochrane Database of Systematic Reviews, Cochrane Central Database of Controlled Trials, Database of Abstracts of Reviews of Effects, Google Scholar, European Association for the Study of the Liver, Eurosurveillance (http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=695), and GlaxoSmithKline (http://www.gsk.com/reportsandpublications.htm). We also searched the reference lists of the retrieved articles and reviews of this field[9,10,13,14]. Our search was limited to human studies and English publications. We also contacted the corresponding authors for any missing data or clarification.

Study selection

Inclusion criteria for studies were: (1) An epidemiologic study design to conduct a primary or secondary data analysis; (2) At least 1 comparison group without HCV; (3) Provision of sufficient data to calculate odds ratio (OR) or relative risk (RR) comparing type 2 DM in HCV infected patients to non-HCV infected patients; (4) Controlled for at least age and gender in the study design or analysis; and (5) Conducted with not less than 20 HCV-infected patients. HCV was confirmed with the detection of anti-HCV (tested with ELIZA) or HCV RNA (detected by reverse transcriptase polymerase chain reaction). Type 2 DM was confirmed with one of the following criteria; (1) Self-reported type 2 DM (i.e., physician diagnosed); (2) Self-reported diabetes with no history of insulin medication; (3) If fasting plasma glucose exceeding 7.0 mmol/L (126 mg/dL) on two separate occasions; or (4) Impaired fasting glycaemia was between 6.1 mmol/L and 7.0 mmol/L with no insulin medication. Where available, hepatitis B virus (HBV is confirmed with positive hepatitis B surface antigen and/or detectable serum HBV DNA. Definition of covariates such as family history of diabetes was taken directly from included studies. Studies with patients having other causes of chronic liver disease such as cirrhosis, autoimmune hepatitis, steatohepatitis, primary biliary cirrhosis, primary cholangitis, and hepatocellular carcinoma were excluded. One author (Mak JW) first screened titles and abstracts of publications using eligibility criteria.

Two authors (Naing C, Ahmed SI) independently recorded the detailed information from each primary study using piloted forms that include relevant items: author, year of publication, country, confirmation of type 2 DM, confirmation of HCV, confirmation of HBV (if presented), study design, number of controls and of cases, genotype of HCV (if provided), distribution of age and gender, family history of diabetes. Any discrepancy between these two investigators was resolved by discussion, and by consultation with another author (Maung M).

Statistical analysis

The degree of heterogeneity between studies was assessed using chi-square and I^2 test. An I^2 value greater than > 50% is considered substantial heterogeneity[15]. We used the assumptions that OR from a case control

Table 1 Preferred reporting items for systematic reviews and meta-analysis reporting

Section/topic	No.	Checklist item	Reported on page
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both	Title: Meta-analysis
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number	Abstract
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known	Introduction
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes and study design	Introduction
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number	NA
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale	Methods: Search strategy and eligibility of relevant studies
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched	Methods: Search strategy and eligibility of relevant studies
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated	Search strategy
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis)	Methods: Eligibility of relevant studies; PRISMA flowchart provided
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators	Methods: Data extraction and outcome measures
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made	
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis	
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means)	Methods: Statistical analysis
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis	

PICOS: Participants, interventions, comparisons, outcomes and study design; PRISMA: Preferred reporting items for systematic reviews and meta-analysis; NA: Not available.

study approximates the RR in a cohort study. The summary OR with their corresponding 95% CI was calculated based on a random-effects model. We performed separate analysis for the comparisons between (1) HCV infected and not infected and (2) HCV infected and HBV infected. The included studies were subgrouped by the study design. In order to assess any factor that could potentially affect the outcome, results were stratified by age group (proportion of $\geqslant$ 40 years), gender (proportion of male gender), body mass index (BMI) (proportion of BMI $\geqslant$ 27), and family history of diabetes (i.e. self reported), where there was enough data. We also examined the funnel plots for potential publication bias among the included studies. A sensitivity analysis was conducted including only prospective studies Data entry and analysis was performed using RevMan 5.1[16]. The methods and findings of the present review have been reported based on the preferred reporting items for systematic reviews and meta-analysis checklist (PRISMA) (Table 1)[17].

RESULTS

Study selection

Figure 1 provides a flowchart of the present review. We retrieved 57 full articles. Of these, 26 publications were excluded because: (1) They were reviews[10-12]; (2) They did not adequately distinguish type 2 DM from diabetes[18,19]; (3) They were conducted in a special population such as transplant patients[20,21], patients with Human immunodeficiency virus[22], with cryoglobulinemia[23], or with thalassemia[24]; (4) They did not include or provide data on patients without HCV infection[25-31]; (5) They were conducted in patients with known chronic liver disease[32-35]; (6) It had less than 20 HCV infected patients[36-38]; and (7) It had duplicate data[39]. The remaining 31 publications of 35 independent studies[5,7,8,40-67] were eligible for inclusion in the present meta-analysis. Four publications[8,55,56,66] assessed both HCV positive *vs* negative and HCV positive *vs* HBV positive.

Study characteristics

A summary of study characteristics in the present analy-

Table 2 Characteristics of the included studies

Study	Country	Type of study	Age, yr (mean ± SD)	Confirmation of HCV	Confirmation of T2D
Akbar *et al*[40]	Saudi Arabia	CC	94% had > 40[2]	Anti-HCV	FBS
Antonelli *et al*[41]	Italy	CC	65 ± 10	Anti-HCV, HCV RNA	FPG
Arao *et al*[42]	Japan	CC		Anti-HCV, HCV RNA	Random, FBG
Boschi-Pinto *et al*[43]	Japan	Cohort	65% had > 54[2]	Anti-HCV	Nil
Butt *et al*[44]	United States	Cohort	50.8[2]		ICD-9
Caronia *et al*[45]	Italy	CC	57.5 ± 8	Anti-HCV	FPG
Chehadeh *et al*[8]	Kuwait	Cohort	51 (23-73)[3]	HCV RNA	FPG
Chen *et al*[46]	Taiwan	CS		Anti-HCV	
Gulcan *et al*[47]	Turkey	CC	56.89 ± 11.9	Anti-HCV, HCV RNA	Guideline[5]
Howard *et al*[48]	United States	CS	51 (37-75)[3]	Anti-HCV, HCV RNA	Patient reported, FPG
Huang *et al*[49]	Taiwan	CC	52.7 ± 0.73	Anti-HCV, HCV RNA	FPG
Imazeki *et al*[50]	Japan	CC	45 ± 16.5	Anti-HCV, HCV RNA	FBS
Jadoon *et al*[51]	Nigeria	Cohort	48.19 ± 10.32	Anti-HCV	Clinic diagnosed
Kaabia *et al*[52]	Tunisia	CS	55.6[2]	Anti-HCV, HCV RNA	Patient reported
Knobler *et al*[53]	Israel	CC	54 ± 14	HCV RNA	FPG
Lecube *et al*[54]	Spain	Cohort	52.9 ± 14.1	Anti-HCV, HCV RNA	FPG
Li-Ng *et al*[55]	United States	Cohort	30-79[4]	HbsAg[1]	ICD-9
Mason *et al*[5]	United States	CS	72% had > 37	Anti-HCV	FPG, Random
Marzouk *et al*[56]	Egypt	Cohort	> 25[2]	Anti-HCV, HCV RNA	FBS
Mehta *et al*[57]	United States	CS	> 20[2]	Anti-HCV	FPG
Nwokediuko *et al*[58]	Nigeria	CS	55.8 ± 11.84	Anti-HCV	FPG
Okan *et al*[59]	Turkey	CS	51.9[2]	Anti-HCV, HCV RNA	Nil
Olokoba *et al*[60]	Nigeria	CS	51.5 ± 12	Anti-HCV	FBS
Papatheodoridis *et al*[7]	Greece	Cohort	48.1 ± 15.3	Anti-HCV, HCV RNA	FPG
Qureshi *et al*[61]	Pakistan	CS	42 ± 13	Anti-HCV	Random
Rouabhia *et al*[62]	Pakistan	CS	55 ± 9	Anti-HCV, HCV RNA	FPG
Ryu *et al*[63]	Korea	Cohort	44 ± 14	Anti-HCV	FPG
Sangiorgio *et al*[64]	Italy	CS		Anti-HCV	
Simó *et al*[65]	Spain	CC	46.4 ± 21.2	Anti-HCV	WHO
Wang *et al*[66]	Taiwan	Cohort	50.9 ± 14.2	Anti-HCV	FPG
Wang *et al*[67]	China	CC	50.9 ± 14.2	HCV RNA	FBS

[1]For HBV infection; [2]Mean; [3]Mean and range; [4]Range only; [5]American Diabetes Association Guideline. CC: Case-control study; CS: Cross-sectional study; FPG: Fasting plasma glucose; FBS: Fasting blood sugar; IFG: Impaired fasting glycaemic; HCV: Hepatitis C virus; T2D: Type 2 diabetes mellitus; HbsAg: Hepatitis B surface antigen; ICD-9: International Classification of Diseases, Ninth Revision; WHO: World Health Organization.

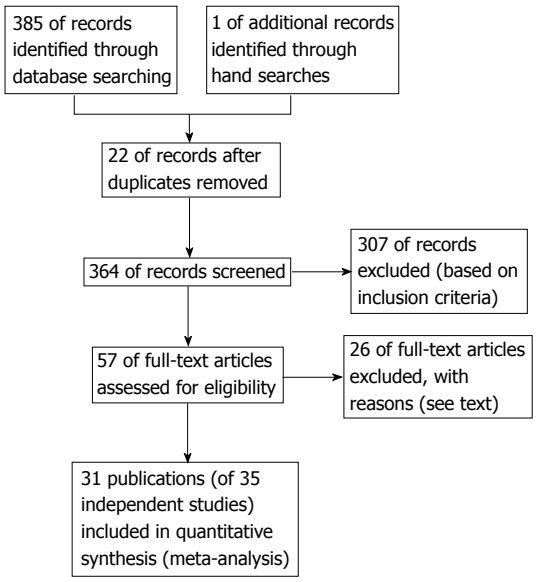

Figure 1 Flowchart of studies identified for the present review.

sis is presented in Table 2. Five studies were carried out in United States[5,44,48,55,57], and three each in Italy[41,45,64], Japan[42,43,50] and Taiwan[46,49,66], among others. Notably, 4 studies[51,60,62,67] identified for the present analysis were published between 2010 and March 2011. Of the included studies, 17 studies (n = 286 084) compared HCV-infected participants with those uninfected; 7 were both a cross-sectional design (41.2%) and cohort design (41.2%), while 3 (17.6%) were case-control studies (Figure 2). Nineteen studies (n = 51 156) compared HCV-infected participants with HBV-infected; 4 (21.1%), 6 (31.58%) and 9 (47.4%) were cross-sectional, cohort and case-control, respectively (Figure 3). The sample size of the included studies widely varied from 135[52] to 126 926 participants[43].

Main results

Of the included studies, 17 studies (n = 286 084) compared HCV-infected participants with those uninfected and the pooled OR was 1.68 (95% CI: 1.15-2.45). There was, however, substantial heterogeneity among studies (I^2 = 95%, heterogeneity P < 0.001). Nineteen studies (n = 51 156) compared HCV- infected participants with HBV-infected and the pooled OR was 1.92 (95% CI: 1.41-2.62). There was evidence of considerable heterogeneity among studies (I^2 = 91%, heterogeneity P < 0.001). Among HCV-infected patients, based on available data, male patients

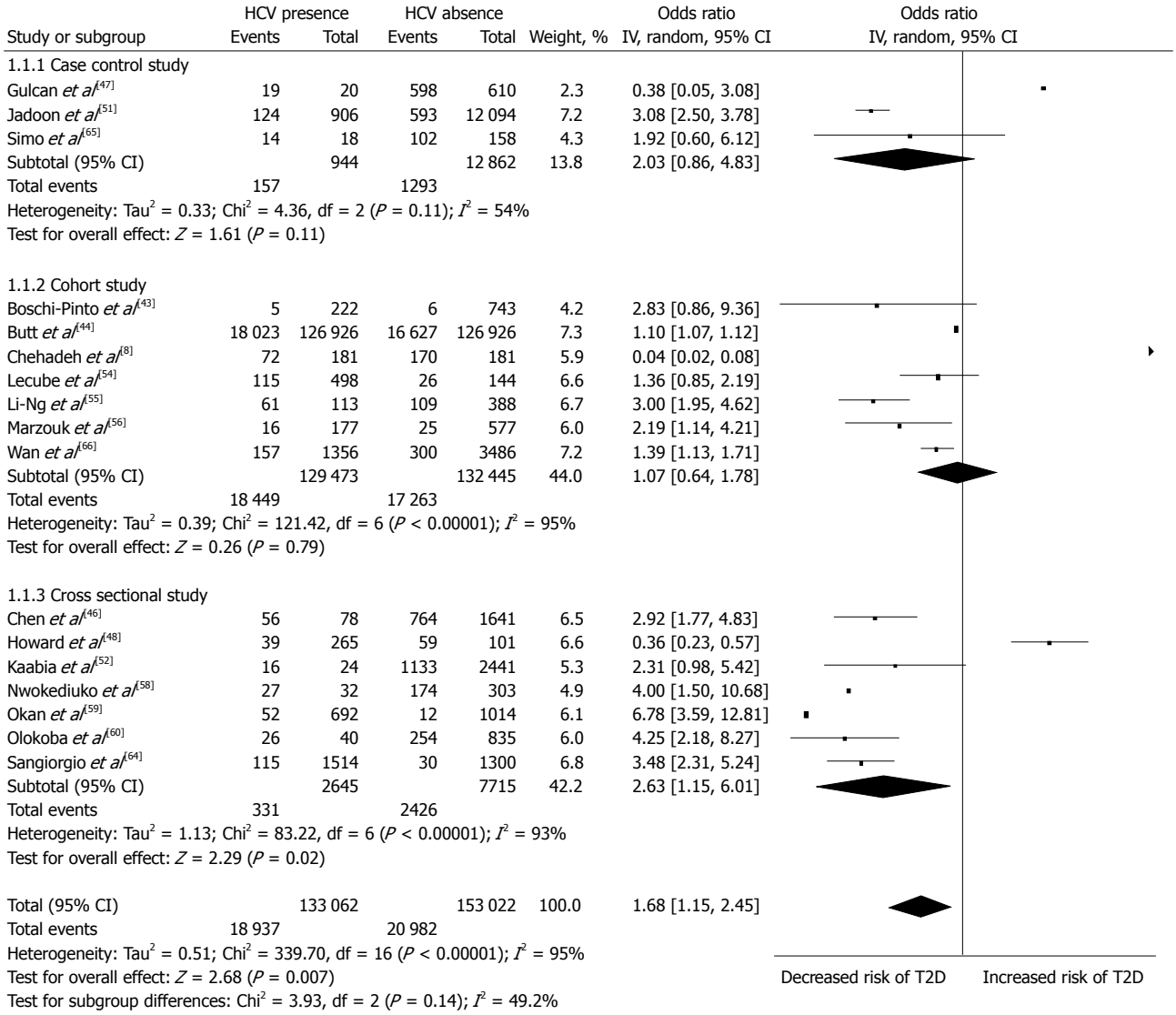

Figure 2 Forest plot of comparison: Hepatitis C virus-infected patients *vs* hepatitis C virus-noninfected patients, outcome is type 2 diabetes mellitus. HCV: Hepatitis C virus; IV: Inverse variance; T2D: Type 2 diabetes mellitus.

Table 3 Stratified analysis of type 2 diabetes mellitus in hepatitis C virus-infected participants

Description	Cases	OR	95% CI
Age (k = 3; n = 599)	455 *vs* 144	7.39	5.82-9.38
⩾ 40 yr			
< 40 yr			
BMI (k = 3; n = 190)	65 *vs* 190	0.87	0.08-9.19
⩾ 27			
< 27			
Gender (k = 8; n = 757)	401 *vs* 356	1.26	1.03-1.54
Male			
Female			
Family history of diabetes (k = 3; n = 580)	420 *vs* 164	4.64	0.57-38.04
Yes			
No			

OR: Odds ratio; BMI: Body mass index; k: Number of primary studies; n: Number of participants.

(summary OR: 1.26, 95% CI: 1.03-1.54) with age over 40 years (summary OR: 7.39, 95% CI: 5.82-9.38) had significantly increased type 2 DM prevalence (Table 3). Funnel plots of the associations between HCV and type 2 DM were investigated, providing little evidence of publication bias (Figure not shown).

For better stability of the results, sensitivity analysis with three prospective studies (n = 6449)[43,54,66] provided the pooled OR: 1.41 (95% CI: 1.17-1.7, I^2 = 0%), supporting the increased frequency of type 2 DM in HCV (Figure 4).

DISCUSSION

This review indicates that patients with HCV infections were at higher risk of developing type 2 DM compared with patients with HBV infection. Findings of this review are comparable with a previous review[14], and a large sample-individual study[56]. In the 2008 review[14] an excess risk of type 2 DM in HCV-infected cases was observed in

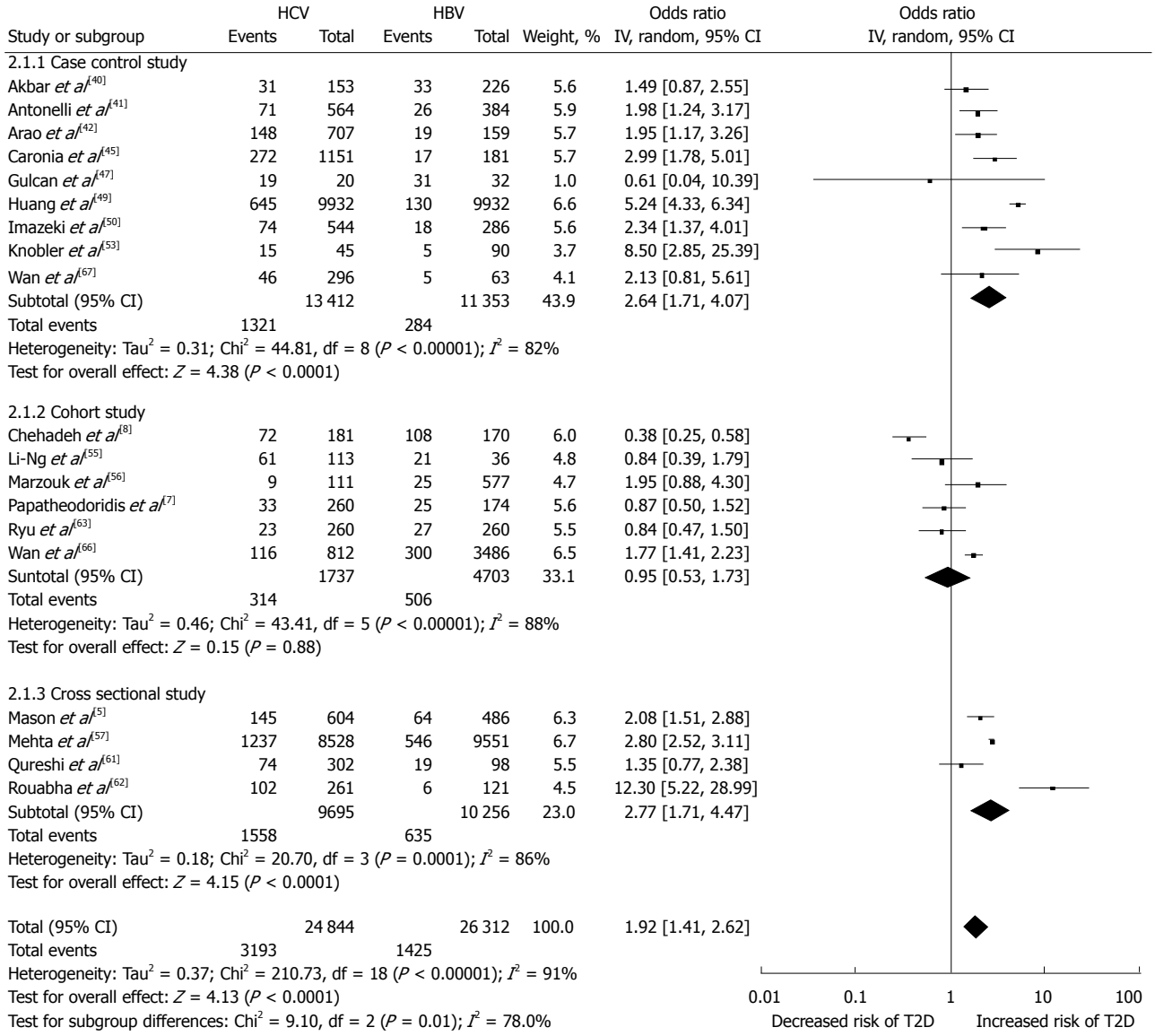

Figure 3 Forest plot of comparison: Hepatitis C virus-infected patients *vs* hepatitis B virus-infected patients, outcome is type 2 diabetes mellitus. HCV: Hepatitis C virus; HBV: Hepatitis B virus; IV: Inverse variance; T2D: Type 2 diabetes mellitus.

comparison to HBV-infected controls (summary OR: 1.63, 95% CI: 1.11-2.39). In the present analysis, this is also encountered (summary OR: 1.92, 95% CI: 1.41-2.62) and the association becomes stronger over time.

As both these viruses can replicate in extra-hepatic sites they can produce β-cell damage resulting in diabetes[10,61]. The lower risk in HBV infection could be explained by two factors: (1) Hepatitis B has been controlled in most developed countries, with active HBV vaccination programme; the occurrence of chronic HBV and its complications in these countries is very low; and (2) The disease progression is rather fast in HBV infection and therefore very few patients reach the level of cirrhosis and thus diabetes frequency is lower in this population[61].

An excess risk of type 2 DM in HCV infected cases was also observed in comparison to non-HCV infected controls in the present analysis (summary OR: 1.63, 95% CI: 1.11-2.39). The evidence of heterogeneity in these studies could be explained by variation in definition of case and control subjects and in the sample size of the primary studies.

Available studies had suggested that an expression of the HCV core protein induces hepatic insulin resistance through alterations in signaling in the insulin receptor substrate-1 pathway. This, along with other factors such as diet and obesity, can result in expression of the diabetic phenotype[38,61,68-70]. When insulin resistance reaches extents no longer compensated by the β-cell, insulin secretion declines and hyperglycemia emerges[10,68,70]. The complex interaction of chronic HCV infection with the host hepatic glucose and lipid metabolism has not been fully understood[10,65,67,70] and it remains to be determined.

In the studies identified, anti-HCV antibody was assessed only at the entry point of the study. Anti-HCV is considered as time-varying[43]. As such, there may be

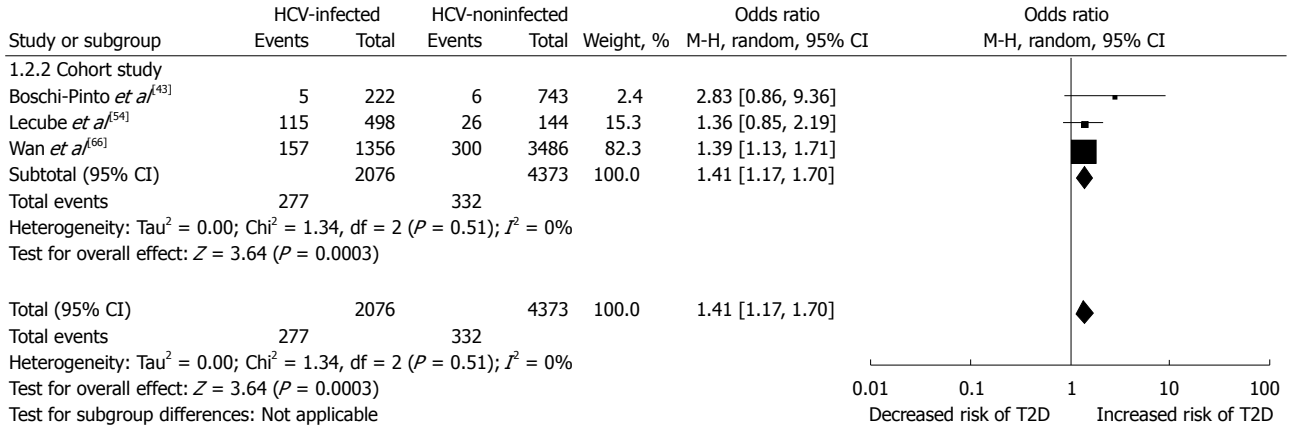

Figure 4 Sensitivity analysis with prospective studies: Hepatitis C virus-infected patients *vs* hepatitis C virus-non-infected patients, outcome is type 2 diabetes mellitus. HCV: Hepatitis C virus; M-H: Mantel-Haenszel; T2D: Type 2 diabetes mellitus.

a likelihood of changes in serological status of anti-HCV over the study period. Liver disease and endocrine disorders, both common in the general population, have a bidirectional and complex relationship[69]. In addition, it is conceivable that patients with an earlier stage of chronic HCV infection have β-cell dysfunction but that diabetes does not become established until cirrhosis has supervened. Thus, a combination of β-cell dysfunction and insulin resistance is required for overt expression of diabetes mellitus[2,10,45]. Patients in some of the primary studies were not confirmed for the absence of cirrhosis by liver biopsy which is the best predictor of disease progression[2]. As such, we were unable to rigorously exclude cirrhosis individuals from the present analysis, and including these patients in the analysis may have exaggerated the association estimated. Of interest, it has been postulated that HCV has a permissive rather than a direct effect on the development of diabetes and acts in concert with other determinants to lead to diabetes[70]. Recent animal model evidence suggests a more direct effect of HCV infection on insulin resistance in the liver[38] indicating the role of hepatic tumor necrosis factor-α in affecting insulin signaling in this animal model of HCV infection[71]. In the present review, as cross-sectional and longitudinal prospective studies both show the same evidence, an excess type 2 DM risk in HCV-infected persons suggests a direct role of HCV in inducing derangement of glucose metabolism[9,10,45]. Further, there may be other factors influencing the development of type 2 DM in HCV infected patients which is not possible to address in the present analysis.

There are limitations to the present study. Most, if not all, observational studies have the potential for ascertainment bias[10,70] particularly for the studies in which diabetes status was defined by self report. Thus, there may be biased estimates of association. Moreover, recall bias is a factor in case-control studies. Although confounding factors were addressed in many of these observational studies, it is likely that there may be unmeasured confounding factors which may introduce bias into our findings. Further, as patient level data were not available for each study, we could not make further adjustments for important factors such as genotype that were not included in most of the primary studies.

Biological plausibility

Findings of those prospective studies[42,53,66] which have measured HCV prior to diagnosis of type 2 DM support evidence for a temporal relationship between exposure and outcome. In a study[43], a significant link between viral load and diabetes was found and it supported the diabetogenic role of HCV infection. The influence of viral load on the progression rate of type 2 DM was not examined in most of the studies. More research is needed to assess a dose-response association. It is also recommended that surveillance of HCV could indicate whether trends in its incidence continue to reflect changes in the prevalence of type 2 DM in the defined group.

Public health implications

If the associations do support temporality, the early detection and provision of aggressive antiviral treatment for HCV could prevent the development of type 2 DM, particularly in patients at high risk of HCV.

Nevertheless, the findings of the current analysis, to a certain extent, represent the HCV endemic countries. The present study has significant strengths in two ways: (1) It is comprehensive, including most recent studies; and (2) It addresses traditional risk factors (age, gender, BMI, family history of diabetes) which could potentially affect the outcome. As the prevalence of obese patients obtained in the group of HCV-positive patients with type 2 DM was significantly lower than that in diabetic HCV-negative patients found in an independent study[8] and also in the present meta-analysis, it is suggesting the pathogenesis of diabetes in HCV infection could be different from that in the general population.

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COMMENTS

Background

Several observational studies assessing the association between hepatitis C virus (HCV) infection and type 2 diabetes mellitus (type 2 DM) have been published. However, these studies have provided inconclusive results, with some studies supporting the excess type 2 DM with HCV infection compared to non-infected controls, and some studies showing differently. The authors, thus, performed a meta-analysis to synthesise the available evidence on the association between HCV infections and type 2 DM.

Research frontiers

Based on the available evidence, the present study aimed to investigate the association between HCV infection and type 2 DM, and also the effect of study design and traditional risk factors on the association

Innovations and breakthroughs

Combining the electronic database and hand searches, a total of 35 observational studies (in 31 articles) were identified for the final analysis, based on the inclusion criteria set for the present analysis. The findings support the association between HCV infection and type 2 DM. However, the direction of association remained to be determined.

Applications

The results support an association between HCV infection and type 2 DM. Findings of this review are comparable with previous reviews, and a large sample-individual study. An early detection and provision of aggressive antiviral treatment for HCV could prevent the development of type 2 DM.

Peer review

The authors show the review of the association between HCV infection and diabetes by meta-analysis. This paper is an interesting and instructive manuscript.

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BRIEF ARTICLE

A population-based cohort study of symptomatic gallstone disease in diabetic patients

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Abstract

AIM: To investigate the prevalence of gallstone disease (GSD) and to evaluate the risk of symptomatic GSD among diabetic patients.

METHODS: The study was conducted by analyzing the National Health Research Institutes (NHRI) dataset of ambulatory care patients, inpatient claims, and the updated registry of beneficiaries from 2000 to 2008. A total of 615 532 diabetic patients without a prior history of hospital treatment or ambulatory care visits for symptomatic GSD were identified in the year 2000. Age- and gender-matched control individuals free from both GSD and diabetes from 1997 to 1999 were randomly selected from the NHIR database (n = 614 871). The incidence densities of symptomatic GSD were estimated according to the subjects' diabetic status. The distributions of age, gender, occupation, income, and residential area urbanization were compared between diabetic patients and control subjects using Cox proportion hazards models. Differences between the rates of selected comorbidities were also assessed in the two groups.

RESULTS: Overall, 60 734 diabetic patients and 48 116 control patients developed symptomatic GSD and underwent operations, resulting in cumulative operation rates of 9.87% and 7.83%, respectively. The age and gender distributions of both groups were similar, with a mean age of 60 years and a predominance of females. The diabetic group had a significantly higher prevalence of all comorbidities of interest. A higher incidence of symptomatic GSD was observed in females than in males in both groups. In the control group, females under the age of 64 had a significantly higher incidence of GSD than the corresponding males, but this difference was reduced with increasing age. The cumulative incidences of operations for symptomatic GSD in the diabetic and control groups were 13.06 and 9.52 cases per 1000 person-years, respectively. Diabetic men exhibited a higher incidence of operations for symptomatic GSD than did their counterparts in the control group (12.35 *vs* 8.75 cases per 1000 person-years).

CONCLUSION: The association of diabetes with increased symptomatic GSD may provide insight to the treatment or management of diabetes in clinical settings.

Key words: Gallstone disease; Diabetes; Symptomatic; Incidence density; Hazard ratio

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INTRODUCTION

Gallstone disease (GSD) is one of the most common and costly digestive diseases worldwide, and it is more prevalent in Europe and America than in Asia and Africa. Symptomatic GSD and its related complications inflict heavy economic costs and social burdens[1-3] because surgical gallbladder removal, usually by laparoscopic cholecystectomy, is often required. GSD affects 10%-15% of the United States population (over 25 million people). Approximately 25% of the patients require treatment, at a cost of several billion dollars annually[4]. The prevalence of GSD in Taiwan is 4.3%-10.7% and increases significantly with age, which is consistent with reports from other countries[5]. In addition, an increasing trend in the incidence of severe GSD among patients aged 20-39 years has been reported in Taiwan[6]. Published epidemiological studies of GSD have revealed a steady upward trend in the admission rates for treatment of gallstones since the 1990s[7]. However, the reported prevalence of GSD varies considerably depending on study design, patient ethnicity, and geographic region[8-11]. A number of factors, including old age, female gender, genetics, diet, obesity, diabetes, and the use of oral contraceptives or hormone therapy, have been associated with increased risk of GSD[12,13].

Diabetic patients appear to have an increased risk of developing gallstones[14,15]. Although previous studies reported mixed results regarding the temporal relationship between GSD and diabetes, the reciprocal relationship between GSD and diabetes suggests a common etiological or biological mechanism[16] that may be reflected in gallstone composition. Gallstones are generally classified as either cholesterol stones or pigment stones according to their morphology and composition. The composition of cholesterol stones varies widely across different populations. For example, gallstones consist of more than 50% cholesterol in Western patients and an overwhelming 95% in Germany[17-20]. A mechanistic link between diabetes and GSD was recently defined using an animal model with increased cholesterol secretion and insulin resistance[21].

The inconsistent reports of the prevalence of GSD in diabetic patients have been attributed primarily to variations in study design[22-26]. One case-control study reported an estimated GSD rate of 32.7% in patients with diabetes and 20.8% in corresponding non-diabetic controls[14]. In contrast, Persson *et al*[22] reported no differences in the prevalence of GSD between diabetic patients and controls. Another case-control study reported that diabetes increased the prevalence of gallstones in females but not in males (47% *vs* 26%)[23]. To our knowledge, few studies have investigated the incidence of GSD in diabetic and non-diabetic patients using a cohort design. Furthermore, the majority of studies have been hospital-based or community-based, which might compromise the representativeness of the study sample, thus reducing the statistical power for comparisons of GSD risk in patients with and without diabetes. A population-based follow-up study conducted in Kinmen, Taiwan, reported that the incidence of GSD was 3.56% per year among type 2 diabetics[24-27]. However, it seems to be inappropriate to generalize the results from this small sample (n = 281) to the entire Taiwanese population.

Examining the co-occurrence of medical conditions related to diabetes and GSD may shed light on a common etiology and enable the identification of common biological mechanisms or pathways, which may greatly contribute to clinical interventions for GSD. Furthermore, diabetic patients with a number of complications must be aware of the symptoms and treatment for all of these diseases[28]. Given that most patients with GSD are asymptomatic and are not aware that they have gallstones, although the assessment of GSD risk among diabetic patients may add disease burden to policy makers responsible for planning health care resources, this may draw attention to the importance of managing diabetes *per se* and its related complications in clinical settings[1,29,30].

This study aimed to examine the risks of developing GSD among diabetic patients. The presence of comorbidities associated with diabetes was also evaluated in the representative diabetic cohorts retrieved from the Taiwanese National Health Research Insurance (NHRI) database.

MATERIALS AND METHODS

Data source

The Department of Health in Taiwan created the universal National Health Insurance (NHI) system in 1995, and approximately 96% of the Taiwanese population

had been covered in the NHI program by the end of 1996[28]. The Bureau of NHI (BNHI) has contracts with 97% of hospitals and 90% of clinics across the island[31,32]. To ensure the accuracy of the claim data, the BNHI conducts expert reviews of a random sample of 50-100 ambulatory and inpatient claims from each hospital and clinic quarterly. The computerized administrative claims and datasets compiled by the NHRI are made available to investigators for research purposes after the individual health information is encrypted to ensure privacy[33]. This study was conducted using the NHRI dataset of ambulatory care claims, inpatient claims, and the updated registry of beneficiaries from 2000 to 2008.

Study cohorts and comorbidities

Diabetic ambulatory care claims record the patients with diabetes-related diagnoses (ICD-9: 250 or A-code: A181). An individual was classified as a diabetic patient if she or he had an initial diabetes-related diagnosis at any time in 2000 and then experienced one or more additional diagnoses within the subsequent 12 mo. The first and last outpatient visits within a given year must be at least 30 d to avoid the accidental inclusion of miscoded patients[34]. To detect newly diagnosed gallstone cases, we excluded patients who sought hospital or ambulatory care treatment for gallstones (ICD-9: 574) from 1997 to 1999. A total of 615 532 diabetic patients were identified in the year 2000.

Subjects in the control group were selected from all beneficiaries insured in 2000 who were free from both diabetes and GSD from 1997 to 2000. A total of 614 871 control individuals were randomly selected to generate an age- and gender-matched control population for the diabetic group.

Once the study subjects were identified, we examined the ambulatory care visits and hospitalization claims for selected comorbidities including hypertension (ICD-9: 401, 405), gout (ICD-9:274), hyperlipidemia (ICD-9: 272.0-272.9, A182), cystic fibrosis (ICD-9: 277.0), sickle cell anemia (ICD-9: 282.6), cirrhosis (ICD-9: 571.2, 571.5, 571.6), cholangitis (ICD-9: 576.1), Caroli's disease (ICD-9: 576.2), Crohn's disease (ICD-9: 555.9), and hemolytic anemia (ICD-9: 282-283). The comorbidities mentioned above were counted only when the initial diagnosis had been made during the study period (2000-2008).

Study endpoints

The study subjects from the diabetic and control groups were linked to ambulatory care visits and hospitalization claims from 2000 to 2008 for possible gallstone episodes (ICD-9-CM 574). Person-years (PYs) of follow-up were calculated for each diabetic patient from the time of his/her first diagnosis of diabetes in 2000 to the date of the first ambulatory care visit or hospitalization due to gallstones prior to the end of 2008. The PYs for control subjects were defined as the period between the first day of insurance coverage by NHI in 2000 and the date that the first gallstone symptoms developed and were diagnosed.

Statistical analysis

The age, sex, occupation, income, and residential urbanization level were compared between diabetic patients and control subjects. The differences in the rates of selected comorbidities were assessed in the two groups. We also estimated the incidence densities of symptomatic GSD according to the subjects' diabetic status.

Cox proportion hazards models were generated to assess the gender- and age-specific effects of diabetes on the risk of developing gallstones. Hazard ratios (HR) and 95% CI were calculated to estimate the relative risk of developing symptomatic GSD. All analyses were performed using SAS statistical software (version 9.1 for Windows; SAS Institute, Inc., Cary, NC), and the results were considered to be statistically significant when two-tailed *P* values were less than 0.05.

RESULTS

A total of 615 532 diabetic patients and 614 817 control participants who were initially free of symptomatic GSD were included in this study (Table 1). The two groups had similar baseline age and gender distributions, with a mean age of 60 years and a greater proportion of females. Although the average insurance premium was lower for patients in the diabetic group, the Charlson score was extraordinarily higher. The geographical distributions and urbanization scores of diabetic patients were also similar to those of the control group.

The diabetic group exhibited significantly higher baseline rates for all comorbidities of interest (Table 2). The largest discrepancy in prevalence was noted for hyperlipidemia (73.4% *vs* 37.8%), followed by hypertension (86.7% *vs* 61.8%) and gout (32.4% *vs* 23.3%).

Over the 8-year follow-up period, 60 734 diabetic patients and 48 116 controls developed symptomatic GSD and underwent operations. The cumulative operation rates for the diabetic and control groups were 9.87% and 7.83%, respectively (Table 3). A higher incidence of symptomatic GSD was also found in females than in males in both groups. Particularly, females under the age of 64 in the control group had a significantly higher incidence of GSD (20% and 22% more) than the corresponding males, but this difference decreased with increasing age. A similar but less significant pattern was also observed in the diabetic group (Table 3). Figure 1 shows that the cumulative incidence rates of operations for symptomatic GSD in the diabetic and control groups were 13.06 and 9.52 cases per 1000 PYs between 2000 and 2008. Diabetic men had a higher incidence of operations for symptomatic GSD than did their control counterparts (12.35 *vs* 8.75 cases per 1000 PYs), representing a significantly increased adjusted hazard ratio of 1.12 (95% CI: 1.07-1.16) for diabetic men. Furthermore, diabetic women also had a modest but significant additional risk of developing GSD during the 8-year follow-up period (HR = 1.05; 95% CI: 1.01-1.08).

Table 1 Characteristics of diabetic and control groups in this study, 2000-2008, Taiwan, China

Variables[1]	Control group		Diabetic group	
	n	%	*n*	%
Age, yr				
< 45	69 617	11.3	69 825	11.3
45-64	296 810	48.3	297 142	48.3
> 64	248 444	40.4	248 562	40.4
Mean age (± SD)	60.0 ± 12.8		60.1 ± 12.7	
Sex				
Female	319 308	51.9	319 310	51.9
Male	295 563	48.1	295 566	48.1
Insurance premium (NTD)[2]				
Dependent	156 296	25.4	169 761	27.6
< Median (19 200)	135 948	22.1	137 408	22.3
⩾ Median	322 627	52.5	308 363	50.1
Mean premium (± SD)[3]	20 142.6 ± 15 269.4		19 307.7 ± 14 454.7	
Charlson score				
0	551 094	89.6	0	0.0
1	49 777	8.1	444 658	72.2
⩾ 2	14 000	2.3	170 874	27.8
Mean score (± SD)	0.1 ± 0.4		1.4 ± 0.8	
Geographic area				
Northern	269 239	44.2	269 920	44.4
Central	151 693	25.0	141 321	23.2
Southern	168 995	27.8	178 627	29.4
Eastern	17 938	3.0	17 944	3.0
Urbanization status				
Metropolis	243 808	39.8	255 467	42.0
Satellite city/town	163 515	26.8	159 687	26.2
Rural area	202 343	33.2	193 949	31.8
Total	614 871	100.0	615 532	100.0

[1]The inconsistencies between the total population and the sums of the populations for individual variables are due to missing information; [2]NTD: New Taiwan Dollars; [3]Dependent insurers were not included.

Table 2 Prevalence of selected comorbidities at baseline in diabetic and control groups, 2000-2008, Taiwan, China

Variables[1]	Control group		Diabetic group		*P* value[2]
	n	%	*n*	%	
Hypertension					< 0.001
No	235 138	38.2	81 913	13.3	
Yes	379 733	61.8	533 619	86.7	
Gout					< 0.001
No	471 346	76.7	416 400	67.6	
Yes	143 525	23.3	199 132	32.4	
Hyperlipidemia					< 0.001
No	382 560	62.2	163 598	26.6	
Yes	232 311	37.8	451 934	73.4	
Cystic fibrosis					0.006
No	614 836	99.9	615 470	99.9	
Yes	35	0.1	62	0.1	
Cirrhosis					< 0.001
No	589 838	95.9	569 024	92.4	
Yes	25 033	4.1	46 508	7.6	
Cholangitis					< 0.001
No	605 197	98.4	602 604	97.9	
Yes	9674	1.6	12 928	2.1	
Caroli's disease					< 0.001
No	612 979	99.7	613 326	99.6	
Yes	1892	0.3	2206	0.4	
Crohn's disease					< 0.001
No	590 912	96.1	590 404	95.9	
Yes	23 959	3.9	25 128	4.1	
Hemolytic anemia					< 0.001
No	612 100	99.6	612 111	99.4	
Yes	2771	0.4	3421	0.6	
Total	614 871	100.0	615 532	100.0	

[1]Hypertension (ICD-9: 401-405, A260, A269), gout (ICD-9: 274), hyperlipidemia (ICD-9: 272.0–272.4, A182), cystic fibrosis (ICD-9: 277.0), cirrhosis (ICD-9: 571.2, 571.5, 571.6), cholangitis (ICD-9: 575.8, 576.1), Caroli's disease (ICD-9: 576.2), Crohn's disease (ICD-9: 555.0, 555.1, 555.9), hemolytic anemia (ICD-9: 282–283); [2]Based on χ^2 test.

DISCUSSION

This study aimed to explore the risk of developing symptomatic GSD in diabetic patients compared with the general population and to examine the risk of comorbidities related to diabetes. The study revealed a higher incidence of symptomatic GSD in patients with diabetes in all age groups. Furthermore, the cumulative incidence trends were more marked in women than in men. The prevalence of selected comorbidities was higher in the diabetic group with symptomatic gallstones than in those without gallstones.

A previous study in an Italian population reported that the cumulative incidence of GSD was 0.67% per year, and GSD was more common in females than in males[13]. Another study reported that the 5-year incidence of gallstone disease was approximately 2%-3% among Danish individuals over the age of 40[12]. Our analysis of the Taiwanese NHRI datasets revealed that the incidence and the incidence density of symptomatic GSD in non-diabetic patients were approximately 7% and 9% per year, respectively. These incidence estimates are slightly higher than those measured in Western countries. Previous evidence has shown that both incidence and prevalence increase with age[1,9-12]. Therefore, one potential explanation for this discrepancy might be that the control group in this study, which was age- and gender-matched to the diabetic group, was older than the study cohorts in previous reports.

Despite the controversies about the prevalence of GSD in diabetic *vs* non-diabetic groups, GSD is not uncommon in patients with diabetes. Previous studies have reported that roughly 14%-30% of diabetic patients develop GSD[14,22-25]. One population-based follow-up study indicated that 3.56% of type 2 diabetic patients developed GSD per year. However, no conclusions about relative risk can be made based on these estimated incidence rates because no control groups were included in these studies[27]. Our study, based on a population-based dataset in Taiwan, illustrated that approximately 9.52% of the Taiwanese population developed symptomatic GSD annually, and the incidence density was higher in diabetic group than in the general population (13.06% *vs* 9.52%). In addition, females and older individuals were more likely to develop symptomatic GSD, regardless of their diabetes status. The results were consistent with the previously described epidemiology of GSD. It is noteworthy that gender differences in symptomatic GSD in-

Table 3 Overall age- and sex-specific incidence densities and relative hazards of gallstone disease (ICD-9: 574) in diabetic and control groups, 2000-2008, Taiwan, China

Variables[1]	Control group				Diabetic group				aHR[2] (95% CI)[2,4] in association with diabetic group
	No. of patients	No. of events	ID[2] (per 1000 patient-years) (95% CI)[2,3]		No. of patients	No. of events	ID[2] (per 1000 patient-years) (95% CI)[2,3]		
Men									
< 45	40 537	1467	4.25 (4.24-4.25)		40 537	2360	7.06 (7.06-7.07)		0.80 (0.66-0.96)[4]
45-64	141 899	9184	7.59 (7.58-7.59)		141 899	12 561	11.23 (11.22-11.23)		1.03 (0.96-1.09)[4]
> 64	113 127	10 241	12.31 (12.30-12.31)		113 129	12 224	16.40 (16.39-16.40)		1.20 (1.14-1.27)[4]
Total	295 563	20 892	8.75 (8.75-8.76)		295 566	27 145	12.35 (12.34-12.35)		1.12 (1.07-1.16)[5]
Women				Increased % *vs* males				Increased % *vs* males	
< 45	29 080	1292	5.11 (5.11-5.12)	+ 20.8%	29 079	1898	7.69 (7.69-7.70)	+ 8.9%	0.75 (0.60-0.94)[4]
45-64	154 911	12 596	9.30 (9.29-9.30)	+ 22.5%	154 911	15 989	12.59 (12.58-12.59)	+ 12.1%	0.96 (0.90-1.02)[4]
> 64	135 317	13 336	12.61 (12.60-12.61)	+ 20.8%	135 318	15 685	16.81 (16.81-16.82)	+ 2.5%	1.13 (1.07-1.18)[4]
Total	319 308	27 224	10.21 (10.21-10.22)	+ 2.4%	319 310	33 572	13.70 (13.70-13.71)	+ 10.9%	1.05 (1.01-1.08)[5]
Overall	614 871	48 116	9.52 (9.52-9.53)		615 532	60 734	13.06 (13.06-13.07)		1.08 (1.05-1.10)[6]

[1]Inconsistencies between the total population and the sums of populations for individual variables are due to missing information; [2]ID: Incidence density; aHR: Adjusted hazard ratio; [3]Based on poisson assumption; [4]Based on Cox proportional hazard regression adjusted for all variables, except for age and sex; [5]Based on Cox proportional hazard regression adjusted for all variables, except for sex; [6]Based on Cox proportional hazards regression adjusted for age, sex, insurance premium, Charlson score, geographic area, urbanization status, and status of diabetes, hypertension, gout, hyperlipidemia, cystic fibrosis, cirrhosis, cholangitis, Caroli's disease, Crohn's disease and hemolytic anemia.

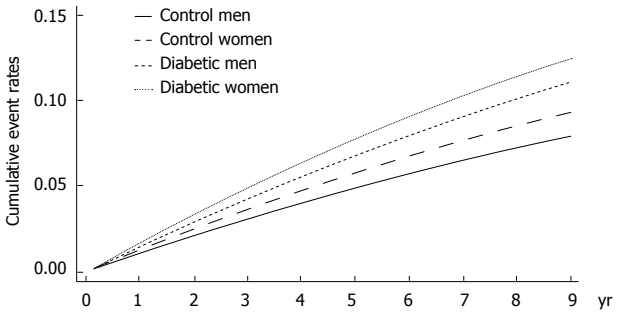

Figure 1 Cumulative incidence of gallstone disease in patients with or without diabetes over the study period.

cidence were not significant in patients over 64 years of age in either the diabetic or the control group, suggesting that age is a more important variable than gender.

Our findings indicate that subjects in the diabetic group suffer from more comorbidities than those in the control group, which was not surprising, but the significantly higher incidence rates of hypertension and hyperlipidemia were particularly noteworthy. Gallbladder function and bile acid metabolism are the two major factors associated with gallstone formation[35]. Diabetes or insulin resistance may affect gallbladder motility or contractility, further promoting the formation of gallstones[36-40]. This may be explained by the fact that diabetes tends to lower the levels of high-density-lipoprotein cholesterol and raise the triglyceride and low-density-lipoprotein levels, that may subsequently affect gallbladder dysmotility[35,37,39]. Previous evidence has shown that hypersecretion of hepatic cholesterol and altered lipid profiles derived from diabetic dyslipidemia may also be linked to the super-saturation of bile with cholesterol, thereby altering bile acid metabolism and cholesterol crystallization[40,41]. The higher incidence of symptomatic GSD in diabetes may be attributable to the higher prevalence of hypertension and hyperlipidemia among diabetic patients.

Considerable clinical evidence has indicated that an array of abdominal manifestations is likely to be associated with GSD and diabetes, including cystic fibrosis, cirrhosis, cholangitis, Caroli's disease, and Crohn's disease[5,11,42-48]. Calcium salts of unconjugated bilirubin in the enterohepatic circulation have been suggested to underlie these co-occurring manifestations. For instance, bilirubin excretion may be related to an increased risk of calcium bilirubinate precipitation, especially in chronic hemolytic disorders. Chronic bacterial infections of the bile ducts may also contribute to gallstone formation by increasing the combination of unconjugated bilirubin with calcium[41,46]. Based on the results of this study (Table 2), we strongly suggest that gallstones developed in diabetic patients were primarily cholesterol stones; this hypothesis will be examined directly in future studies.

Gallstone formation may be caused by many etiological factors, each of which may produce different clinical consequences. Most patients remain asymptomatic for a long period, frequently for life. Gallstones may traverse the cystic duct with or without symptoms of obstruction. Transient cystic duct obstruction causes periodic painful episodes, whereas persistent obstruction usually produces inflammation and acute cholecystitis, leading to the onset of symptomatic GSD. However, there is little information regarding the direct mechanisms that underlie the increased onset of GSD in diabetes. Because elderly patients are more likely to develop symptomatic GSD, it is important to diagnose GSD early in patients with diabetes. Patients may benefit from the early detection of GSD and the underlying comorbidities that may promote both GSD and diabetes, which could subsequently enhance the effectiveness of diabetes management.

Urban-rural differences were also detected in this study. Diabetic patients in metropolitan areas had a higher incidence of symptomatic GSD than patients from rural areas. The observed urbanization-level differences likely reflect the differences in the distribution of medical resources and/or treatment-seeking behaviors[49]. Future research is needed to explain the urban-rural variations and enable policy-makers to promote policies that will reduce or eliminate these differences.

There were several limitations that should be noted in this study. First, potential misclassification might arise due to our exclusive reliance on claims datasets. A previous report showed that the accuracy of diabetes diagnoses in the NHI claims data was only 74.6%[50]. In order to avoid this bias, we included and analyzed only the patients that had been diagnosed with diabetes at least twice, with the first and the last outpatient visits at least 30 d but less than one year apart. It is also possible that newly diagnosed or undiagnosed diabetic cases without records of ambulatory care visits were included in the control group. Therefore, the overall difference in incidence of GSD could have been underestimated[51]. Second, type 1 and type 2 diabetes were not differentiated in this dataset, which limited our interpretation of the study findings. However, other studies have shown that type 2 diabetes accounted for the majority of diabetic patients in Taiwan; as a result, the interpretations of our results are likely to be most relevant for type 2 diabetes[52,53]. Third, other factors that might confound our results were not available, such as body mass index, socioeconomic status, duration and treatment of diabetes, smoking, alcohol use, family history of GSD, *etc*. Fourth, the incidence of symptomatic GSD among diabetic patients with/without comorbidities was not reported; therefore, these differences in prevalence could not be analyzed.

Despite the above-mentioned limitations, this is one of very few studies to examine the risk of symptomatic GSD in patients with diabetes using a Taiwanese population-based cohort study design. Given the high coverage rate of National Health Insurance in Taiwan, the likelihood of non-response and loss to follow-up was relatively limited, ensuring the representativeness of the sample. In addition, we took advantage of the longitudinal nature of the NHI dataset to follow up the incidence rate of symptomatic GSD and related comorbidities in diabetic and control groups.

In conclusion, an increased risk of symptomatic GSD in diabetic patients over an 8-year study period was observed in this study. Diabetes and GSD may share a number of common risk factors or etiologies. A crucial link between insulin resistance and increased cholesterol predisposed the diabetic patients to gallstone formation[21]. These results may provide insight into the treatment or management of diabetes in clinical settings. Future research is needed to facilitate public health prevention or intervention programs to reduce the incidence of symptomatic GSD[54].

COMMENTS

Background

Gallstone disease (GSD) is a one of the most common of all digestive diseases worldwide. Symptomatic GSD and related complications necessitate surgical removal of gallbladder, inflicting a heavy economic costs and social burdens. Most patients with GSD are asymptomatic and unaware of having gallstones. Diabetic patients with complications particularly require an adequate awareness for care management of GSD. The apparent incongruity for GSD prevalence in diabetic patients should be attributable to varied study designs.

Research frontiers

This study aimed to investigate the incidence of GSD and examined the risks of developing symptomatic GSD among the diabetes using a cohort design and retrieving data from the National Health Insurance Research database of Taiwan.

Innovations and breakthroughs

The results showed that the cumulative operation rates for diabetes and controls were 9.87% and 7.83% among 615 532 diabetic patients and 614 817 control participants over the 8-year follow-up period. Diabetic patients also tended to have significantly higher prevalence in developing comorbidities, most notably hyperlipidemia and hypertension. Higher incidence of symptomatic GSD was found in females than in males in both groups. Females aged < 64 years in control group had a significantly higher incidence than corresponding males, but the difference reduced with increasing age. A similar but less significant pattern was also observed in diabetic group. Both diabetic men and women were characterized with higher incidence of symptomatic GSD operation than their corresponding counterparts after 8-year follow-up. The authors concluded that higher incidence of symptomatic GSD was found in patients with diabetes in all age groups and the trends of cumulative incidence were more marked for women than men. In addition, the prevalence of selected comorbidities in diabetic group with gallstone was also higher than those without symptomatic GSD.

Terminology

GSD is caused by gallstones that block the normal flow of bile if they lodge in any of the ducts that carry bile from the liver to the small intestine. Severe damage or infections affecting the gallbladder, liver, or pancreas can occur if any of these ducts remain blocked for a significant period of time, which necessitate surgical removal of gallbladder, usually by laparoscopic cholecystectomy.

Peer review

This is a good study in which authors analyzed incidence of symptomatic GSD in patients with diabetes by using a representative database over a long period of time. The results are interesting and suggest that diabetes is associated with increased risk of developing symptomatic GSD. The investigators also drew a conclusion that patients with diabetes developed more symptomatic GSD in all age groups and were more notable for women than men. This study was based on a nationwide population-based datasets, which illustrated that about 9.52% population in Taiwan developed symptomatic GSD per year and the incidence density was higher in diabetic patients (13.06%). Diabetes associated with increased risk of developing symptomatic GSD was observed over an 8-year study period. Being female and with older ages were associated with increased incidence of symptomatic GSD, however, the potential importance of gender was overridden by aging.

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BRIEF ARTICLE

Swab culture monitoring of automated endoscope reprocessors after high-level disinfection

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Abstract

AIM: To conduct a bacterial culture study for monitoring decontamination of automated endoscope reprocessors (AERs) after high-level disinfection (HLD).

METHODS: From February 2006 to January 2011, authors conducted randomized consecutive sampling each month for 7 AERs. Authors collected a total of 420 swab cultures, including 300 cultures from 5 gastroscope AERs, and 120 cultures from 2 colonoscope AERs. Swab cultures were obtained from the residual water from the AERs after a full reprocessing cycle. Samples were cultured to test for aerobic bacteria, anaerobic bacteria, and mycobacterium tuberculosis.

RESULTS: The positive culture rate of the AERs was 2.0% (6/300) for gastroscope AERs and 0.8% (1/120) for colonoscope AERs. All the positive cultures, including 6 from gastroscope and 1 from colonoscope AERs, showed monofloral colonization. Of the gastroscope AER samples, 50% (3/6) were colonized by aerobic bacterial and 50% (3/6) by fungal contaminations.

CONCLUSION: A full reprocessing cycle of an AER with HLD is adequate for disinfection of the machine. Swab culture is a useful method for monitoring AER decontamination after each reprocessing cycle. Fungal contamination of AERs after reprocessing should also be kept in mind.

Key words: Automated endoscope reprocessor; Gastrointestinal scope; High-level disinfection; Swab culture; Monitoring; Decontamination

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INTRODUCTION

Gastrointestinal (GI) scopes are complex reusable instruments that require unique consideration with respect to decontamination. Most of the guidelines with updated guidance emphasize decontamination of these scopes[1-3]. While decontamination has been reviewed by several working groups in Britain, problems related to preventing contamination of rinse water, and procedures to monitor contamination have not been addressed thus far. In a recent study, we reported that GI scope contamination might be the result of a contaminated automated

endoscope reprocessor (AER)[4]. There is currently no literature on the quality of disinfection of AERs after reprocessing with high-level disinfection (HLD). Therefore, we conducted this bacterial culture study on AERs after HLD in order to monitor the quality of disinfection.

MATERIALS AND METHODS

From February 2006 to January 2011, a 5-year prospective bacterial study was conducted with randomized consecutive sampling every month in GI scope unit, Chang Gung Memorial Hospital, Kaohsiung Medical Center. We took a total of 420 swab cultures, including 300 cultures from gastroscope AERs and 120 cultures from a colonoscope AER. The swab cultures were obtained from the dependent part of the inner surface of the AER after a full reprocessing cycle. Collected samples were cultured to test for aerobic and anaerobic bacteria and mycobacterium tuberculosis. The samples were incubated at 37 ℃ and examined for bacterial growth at 24 h and 48 h and for mycobacterium growth at 6 wk, and then the results were analyzed.

Culture results were reported as positive or negative. If a culture was positive, the specific AER was reprocessed and could only be used again for clinical use after repeated cultures were found negative according to our previous method[2]. GI scope decontamination was performed in accordance with the guidelines of the European Society of GI Endoscopy (ESGE)[3]. Manual cleaning was performed by trained GI nurses, with tap water, enzymatic soap, brushing, and irrigation, followed by AER, performed by a trained health technician. The liquid disinfectant used was 2.4% alkaline glutaraldehyde, and disinfectant-soaking duration was 20 min. If the cultures were positive, the soaking duration was prolonged to 25 min. The disinfectant was forced into the working channels and the GI scope was completely submerged. Then, the GI scopes were flushed with sterile filtered water prior to forced air-drying. The disinfectant solution, 2.4% alkaline glutaraldehyde, was stored at a temperature of 15 ℃-30 ℃ and changed every 2 wk despite overstorage[4].

Reprocessing cycle of AER

After each scope procedure, thorough manual cleaning with Endozime Premium (Ruthof Corporation, NY, United States), including brushing and flushing of all accessible endoscope channels, was performed before automatic endoscope disinfection. We used the EW-30 AER (Aizu Olympus Co., Ltd, Tokyo, Japan) for reprocessing. Manual cleaning and reprocessing was performed by a fully trained scope nurse using accredited standards of practice as defined by the Digestive Endoscopy Society of Taiwan. HLD involved total immersion of the scope in 2.4% alkaline glutaraldehyde solution (Cidex 14, Ethicon, Inc., NJ, United States) for 20 min at a preset temperature of 25 ℃ and an additional washing cycle of 30 min in each reprocessing. The disinfectant was forced into the suction channels and the scope was completely submerged. The normal relief valve pressure of the AER was 1.85 ± 0.05 kgf/cm^2, and the water supply requirements were 17 L/min. Subsequent flushing with 200 cc of 90% alcohol for 10 min, rinsing, and drying were essential steps to remove the chemical solution and prevent bacterial colonization during storage. The rinse cycle used reverse osmosis-treated water for decontamination.

Table 1 Rate of positive swab culture from the automated endoscope reprocessor after gastroscope and colonoscope reprocessing *n* (%)

Category	AER	*P* value
Gastroscope (*n* = 300)	6 (2.0)	NS
Colonoscope (*n* = 120)	1 (0.8)	NS
Total (*n* = 420)	7 (1.7)	NS

AER: Automated endoscope reprocessor; NS: Not significant.

Table 2 Organisms from swab culture of automated endoscope reprocessor after a full cycle of reprocessing with high-level disinfection

Category	Gastroscope	Colonoscope	Total
GNGN Bacteria[1]	2	1	3
Moraxella osloensis	1	-	1
Yeast-like organisms	2	-	2
Candida glabrata	1	-	1
Total positive culture	6	1	7

[1]All of the positive cultures had aerobic bacteria and mono-floral colonization. GNGN: Glucose-nonfermenting gram-negative bacteria.

Statistical analysis

The χ^2 test was used to analyze independent and paired samples. Statistical analyses were performed using the SPSS statistical software for Windows, version 19.0 (Chicago, IL, United States). *P* values less than 0.05 were considered statistically significant.

RESULTS

The overall positive culture rate was 1.7% (7/420) in swab cultures from AERs after a full reprocessing cycle with HLD. For gastroscope and colonoscope AERs, the positive swab culture rates were 2.0% (6/300) and 0.8% (1/120) respectively, without a statistically significant difference in the culture rate between the upper and lower GI scope AERs (Table 1). All 7 positive swab cultures, including 6 gastroscope reprocessing culture and 1 colonoscope reprocessing culture, showed monofloral colonization. None of the cultures was positive for mycobacterium tuberculosis, and no anaerobic bacteria were found in any swab cultures. Among the cultures from gastroscope reprocessing, 50% (3/6) were positive for aerobic bacteria, while the remaining 50% (3/6) showed fungal contamination (Table 2).

DISCUSSION

The British Society of Gastroenterology Endoscopy Committee first published recommendations on endoscope decontamination practices in 1988, and recommendations from the fourth working group were published in the journal Gut in 1998[1]. Some of these decontamination recommendations are based on microbiological studies[5-8]. Most of the decontaminating guidelines are directed towards GI scopes and associated devices, but no literature is available on AER decontamination. According to our previous report, leakage of the inflow water valve of an AER could be one of the reasons for failure of decontamination of GI scopes and associated devices, even after subjecting them to a full reprocessing cycle[4]. Therefore, in this study, we aimed to monitor proper disinfection of AERs after HLD; to the best of our knowledge, this is the first study to do so. The overall positive culture rate of swab cultures from AERs after a full reprocessing cycle with a HLD process was 1.7% (7/420). Surprisingly, the rate was lower than the previously reported 18.4%-24% contamination rate for GI scope culture[5-8]. This suggests the contamination of GI scopes is not fully caused by AER contamination. We would like to clarify that since drying has been shown to be an important component of GI scope decontamination, the same is true of AERs as well?

The importance of drying in decontamination to make this point clearer is performing in our ongoing study. On the other hand, controlled trials in the field of GI scope decontamination are lacking because of a reluctance to expose "placebo control" patients to the risk of an infection. A controlled study to clarify the relationship between AER and GI scope contamination is necessary and is ongoing in our lab. An AER should be used for all GI scope decontamination following manual cleaning. Effective disinfection is difficult to achieve due to the complex nature of the internal structures of these long and narrow diagnostic instruments[4,9,10]. Manual disinfection is unacceptable. Inflow water used in an AER should be free of particulate contamination and microorganisms. This can be achieved either by using bacteria-retaining filters or by reverse osmosis. In our GI scope units, we used water treated by reverse osmosis in AER reprocessing[10]. The final rinse water should be sampled from the AER and regularly tested for microbiological quality in accordance with the current Health Technical Memorandum (HTM)[11]. A glutaraldehyde-based disinfectant (Cidex®) that was widely used in the past has been withdrawn from the United Kingdom market by its manufacturer. This is not only because there have been advances in the development of disinfectants with superior bactericidal activity but also because glutaraldehyde is chemically related to formaldehyde and has similar toxic effects on the skin and mucous membranes as formaldehyde does. The resulting adverse effects include severe dermatitis, conjunctivitis, sinusitis, asthma, and even chemical colitis. A further problem with glutaraldehyde-based disinfectants is their potential to cross-link residual protein material. The resulting amalgam is very difficult to remove from the working channels of endoscopes that have been repeatedly flushed with aldehydes[3]. This again underscores the importance of manual pre-cleaning and brushing of all accessible internal channels and valve chambers before disinfection. Glutaraldehyde and its derivatives kill most bacteria and viruses (including human immunodeficiency virus and hepatitis B) in less than 5 min. Mycobacteria are more resistant to 2% glutaraldehyde, and earlier guidelines recommended that endoscopes be immersed in 2% glutaraldehyde for 20 min at room temperature[1]. Although we did not detect mycobacterial contamination in our study, we found that of the 1.7% positive cultures from AERs, 50% (3/6) were positive for fungal contamination. The high rate of fungal contamination is most likely due to failure to properly dry the AER after completion of reprocessing. Other than manual pre-cleaning and reprocessing disinfection, the last of the major processes of decontamination of a scope is drying before storage[3]. This step can prevent contamination by fungus or bacterial colonization on the surface of the GI scope after disinfection. It has been recommended that, before the start of each list, each scope to be used should undergo a full reprocessing cycle unless last used and decontaminated within the preceding 3 h. Many GI units are now using drying and storage chambers built purposefully for these scopes, some of which have been shown to prevent colonization of endoscope channels for up to 72 h. Therefore, all AERs should be validated and tested in accordance with guidance provided in the DoH Estates and Facilities HTM publications and relevant standards[12]. AERs should also include flow monitoring for each individual channel to detect blockages.

Furthermore, variant Creutzfeldt-Jacob disease (vCJD) is a rare and fatal condition cause by the consumption of beef contaminated by the bovine spongiform encephalopathy agent[13]. In contrast to the traditional forms of CJD, vCJD contaminated in GI tract, conventional HLD with AER was reported hard to full decontamination. ESGE guideline suggested that endoscope study is not recommended in possible patients[14]. Fortunately, there is no patient with suspicion of vCJD infection before endoscope examination, and there was no positive culture for vCJD in our seriers. The further study is necessary.

In conclusion, a full reprocessing cycle of an AER with HLD is adequate for disinfection of the machine. Swab culture is a useful method for monitoring AER decontamination after each reprocessing cycle. Fungal contamination of AER after reprocessing should be considered.

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COMMENTS

Background

Swab culture is a sample method for the detection of bacterial contamination. In the real world, it is always used to monitoring of the clearing effectiveness such

as the button of the elevator. But up to now, there are still now ideal methods to monitor the decontaminated effect of automated endoscopy reprocessor itself in clinical practices.

Research frontiers

Automated endoscopy reprocessor is a very important washing machine for the endoscopy decontamination in daily clinical practice. Authors apply the swab culture method to monitor the examinated endoscopy, which is the source of the reprocessor contamination.

Innovations and breakthroughs

In fact, decontamination of the automated endoscopy reprocessor is limited description before. Swab culture is a common method for the identification of the pathological organisms from the wound infection. For the quality of the infection control and the hospital identification with high standard monitoring, the results of the swab culture from the automated endoscopy reprocessor should be a standard score of a hospital identification and guideline in the clinical practice in the future.

Applications

The study results suggest that the method of swab culture from the inner surface of automated endoscopy reprocessor is a useful method that could be used in monitoring decontamination after a complete endoscopy reprocessing cycle.

Terminology

Automated endoscopy reprocessor: Automated endoscopy reprocessor is a automatic washing machine for the decontamination of the practically used endoscopy. Accompanist with high-level disinfection, it is effective prevention the hospital acquired microbiological infection.

Peer review

This is an interesting prospective study. Which has dealth with an important topic not properly coverly before. The authors analyze the monitoring effect of swab culture from the inner surface of automated endoscopy reprocessor after the end of daily decontamination. The results are interesting and suggest that swab culture is a potential monitoring method that could be used in preventing not complete disinfection or contamination induced by hospital acquired infectious outbreak.

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BRIEF ARTICLE

Prognostic significance of PTEN, Ki-67 and CD44s expression patterns in gastrointestinal stromal tumors

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Abstract

AIM: To develop a prognostic approach for gastrointestinal stromal tumors (GISTs) using a cluster of indicators and follow-up information.

METHODS: One hundred and four GISTs that had not been subjected to targeted therapies were collected and classified by NIH risk assessment and anatomic location. By immunohistochemistry, the expressions of PTEN, Ki-67, CD44s matrix metalloproteinase (MMP)-9 and TIMP-1 were detected on tissue microarray. Univariate and multimarker survival analyses were performed and then a COX hazard proportion model was constructed to evaluate a cluster of predictors of GIST.

RESULTS: Our data showed small intestinal GIST are more aggressive than gastric GIST. The NIH risk assessment correlated with disease-free survival for either gastric GIST or small intestinal GIST. Immunohistochemical analysis revealed that Ki-67 labeling indexes (LIs) < 5% predicted higher disease-specific survival (DSS) in gastric and small intestinal GIST. CD44s positivity and PTEN LIs ⩾ 50% correlated with higher DSS in gastric GIST. MMP-9 and TIMP-1 had no correlation with survival. Multimarker analysis revealed that the expression pattern of PTEN LIs ⩾ 50% combined with Ki-67 LIs < 5% and CD44s positivity reliably predicted favorable outcomes for gastric GIST (P = 0.009), as did the combination of PTEN LIs ⩾ 50% and Ki-67 LIs < 5% for small intestinal GIST (P = 0.011). Authors also found that high NIH risk grade was correlated with DSS in patients with gastric GIST and disease-free survival in patients with small intestinal GIST.

CONCLUSION: PTEN LIs ⩾ 50%, Ki-67 LIs < 5% and CD44s positivity provides an accurate, favorable prognosis for gastric GIST. PTEN LIs ⩾ 50% and Ki-67 LIs < 5% does the same for small intestinal GIST. Ki-67 LIs enhances the NIH assessment.

Key words: Gastrointestinal stromal tumor; Prognosis; PTEN; Ki-67; CD44s

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INTRODUCTION

Gastrointestinal stromal tumor (GIST) is the most com-

mon mesenchymal tumor in the alimentary tract. The principle pathogenesis of GIST has been identified as the continuous activation of KIT, caused by gain-of-function mutation in c-kit[1] or platelet derived growth factor α (PDGFRA)[2]. Based on this finding, the molecular targeted therapy of imatinib[3] or sunitinib[4,5] has achieved great success.

Clinicians are trying to improve the survival rate of GIST patients by prophylactic interference of imatinib or sunitinib, based on the prognosis. Because of the expense of the targeted therapy, an accurate prognosis is very important, but prognoses of GIST are highly variable. Although the NIH risk assessment is currently used as a standard guideline for localized tumors[6], many researchers consider that evaluation of large cohorts is necessary for reliable data. Some modifications have been made to the NIH assessment, to enhance prediction accuracy[7,8]. Current researchers have also found some molecular indicators, such as $p16^{INK4a}$[9], PTEN[10], p53[11,12], p27[13,14], CD44s[15,16] and some other cell-cycle regulators[17], but their utility is debated. Despite the mixed opinion, there are limitations in using one marker for a prognosis. A tumor is resulted from the accumulation of numerous molecular incidents, and one prognostic marker may only be applicable to a minority of patients. Our objective was to find a multi-marker indicator to improve prognoses for all patients. PI3K/Akt has been found to be a major signal transduction pathway in GIST[18]. Accordingly, we selected PTEN, the inhibitor of PI3K/Akt, for analysis. The markers related to adhesion and metastasis, such as CD44s, matrix metalloproteinase (MMP)-9 and TIMP-1, were also selected. Ki-67 was chosen to analyze the prognostic value of cell proliferation. All five markers were detected on tissue microarray of 104 GISTs. Follow-up survival data was collected. The prognostic values of these five markers were analyzed and compared to the NIH risk assessment.

MATERIALS AND METHODS

Case collecting

A total of 155 gastric and small intestinal GISTs were collected from the archive of the Pathology Department of Chinese PLA General Hospital. All the tumors were reassessed by the immunohistochemical panel of CD117, CD34, SMA and S-100 protein. The cases with CD117 positivity were diagnosed as GIST. One hundred and four cases were completely followed-up. One hundred and twenty one samples were obtained from these 104 cases. These tumor samples included primary, recurrent and metastatic GIST. All the tumor paraffin blocks were used to construct the tissue microarray.

Risk assessment

Formalin fixed, paraffin embedded, HE stained slides were reviewed by two experienced pathologists. The mitotic index was determined by counting 50 adjacent high-power fields in the most active areas. Using the NIH risk assessment[6], all the tumors were classified into four grades of risk: very low, low, intermediate and high.

Tissue microarray and immunohistochemistry

Three tissue cores, 1 mm in diameter, were sampled from each tumor specimen to construct the tissue microarray (TMA). The TMA blocks were sectioned at 4 μm and stained with hematoxylin-eosin sequentially. Antigen retrieval was carried out with EDTA (pH 8.0; Santa Cruz Biochemistry, Calif) for 15 min by microwave. The primary antibody was incubated for 1 h at room temperature and subsequently detected using a Two-step PicTure™ kit (PV6000, Invitrogen Co. Carlsbad California United States). The primary antibodies, sources, and dilutions were as follows: CD117, A4502, polyclonal, Dako A/S Co. Ltd., Denmark, 1:200; SMA, clone 1A4, Invitrogen Co. Carlsbad California, United States, 1:100; S-100, clone 4C4.9, Invitrogen Co. Carlsbad California, United States, 1:50; Ki-67, clone K-2, Invitrogen Co. Carlsbad California, United States, 1:50; PTEN, clone 28H6, Invitrogen Co. Carlsbad California, United States 1:100; CD44s, clone 156-3c11, Novocastra Laboratories Ltd. Newcastle, United Kingdom, 1:50; MMP-9, clone 15W2, Novocastra Laboratories Ltd. Newcastle, United Kingdom, 1:25; TIMP-1, clone 6F6a, Novocastra Laboratories Ltd. Newcastle, United Kingdom, 1:50.

The labeling indexes (LIs, %) of PTEN and Ki-67 were determined by counting 1000 cells in the most active area. For CD117, SMA, S-100, CD44s, MMP-9 and TIMP-1, staining of more than 10% of the tumor cells was considered positive.

Statistical analysis

All the patients were followed up after the resection of the tumor. The death of patients with GIST, untreated with imatinab, was selected as the end point. We analyzed follow-up data using Stata Statistical Software (Intercooled Stata 7.0, Stata Co., College Station, TX). The χ^2 test was used to analyze the expression status of the selected markers. Survival analysis was performed using Kaplan-Meier plots and the log-rank test to reveal the prognostic usefulness of the selected markers. Univariate and multivariate COX proportional hazard models with both backward and forward elimination of variables were also constructed to find the most significant factor for prognosis. Statistical significance was set at $P < 0.05$.

RESULTS

Clinicopathologic features and the follow-up data

In a total of 155 patients, having a male-to-female ratio of 2.5:1 (111 *vs* 44), there were 83 gastric and 72 small intestinal GISTs. A total of 104 cases had complete follow-up data. The median follow-up time was 33 mo, within the range of 3 to 230 mo. Fifty one cases without follow-up data were excluded from the subsequent survival analysis.

In 83 gastric patients, the male to female ratio was 2.1:1 (55 *vs* 26). The age ranged from 13 to 82 years (mean: 55.4

years; median: 57 years). The 62 followed-up cases included 42 males and 20 females. Thirteen patients died of GIST, four were alive with GIST and 45 were disease-free. The survival time of the 13 died patients ranged from 6 to 132 mo. The 3-year disease-specific survival rate (DSS) was 80.77% ± 11.5% and the 5-year DSS was 66.51% ± 17.06%.

In 72 small intestinal patients, the male to female ratio was 3.3:1 (55 *vs* 17). The age ranged from 20 to 77 years (mean: 50.6 years; median: 51.5 years). Forty-two followed-up cases included 31 males and 11 females. Fifteen patients died of GIST, four were alive with GIST and 23 were event-free. The survival time of the died patients ranged from 3 to 230 mo. The 3-year DSS was 73.65% ± 14.24% and the 5-year DSS was 61.76% ± 18.30%. There was no significant difference between the DSS of patients with gastric and small intestinal GIST ($P = 0.274$).

There were 23 patients that suffered recurrence, nine with gastric GIST and 14 with small intestinal GIST. Most of them were noted to have intra- abdominal spreading. A total of 16 patients developed metastasis, eight with gastric GIST and eight with small intestinal GIST. Small intestinal GIST presented a higher liability to recurrence and metastasis than gastric GIST (22/42 *vs* 17/60, $P = 0.013$). GIST metastasized to the liver in 15 cases, indicating that the liver was another common metastatic site. One of these 15 patients suffered multi-organic metastases to the bone, brain and lung at the same time. Beside liver metastasis and abdominal spread, one patient also suffered metastasis to subcutaneous tissue. Detailed information on the 155 GISTs is provided in Table 1.

Five patients suffered a secondary malignant tumor. Two patients with gastric GIST had early and advanced gastric adenocarcinoma, respectively. Three small intestinal GIST patients were found to have early gastric carcinoma, sigmoid colonal adenocarcinoma and bile duct adenocarcinoma, respectively.

Table 1 Clinical and pathological parameters in 155 cases of gastrointestinal stromal tumor

	In general	Gastric GIST	Small intestinal GIST
Total case	153	81	72
Followed-up cases	104	62	42
Age (yr)			
< 50	38	14	24
⩾ 50	64	46	18
Gender			
Male	72	41	31
Female	30	19	11
Tumor size (cm)			
< 2	1	1	0
2.1-5	31	20	11
5.1-10	46	29	17
> 10	24	10	14
Mitotic index			
⩽ 5/50HPF	54	31	23
5-10/50HPF	15	9	6
> 10/50HPF	33	20	13
Risk grade			
Very low risk	2	2	0
Low risk	23	14	9
Intermediate Risk	28	17	11
High risk	49	27	22
Recurrence	22	8	14
Metastasis	15	7	8
Survival rate (%)			
3-yr disease specific	76.64 ± 9.38	80.77 ± 11.50	73.65 ± 14.24
5-yr disease specific	69.05 ± 11.18	66.51 ± 17.06	61.76 ± 18.30

GIST: Gastrointestinal stromal tumor.

Comparison of selected immunohistochemical markers in gastric and small intestinal GISTs

Sixty-one gastric and 42 small intestinal GIST patients were included in the analysis of survival data based on the expressions of selected immunohistochemical markers. These results are summarized in Table 2 and displayed in Figure 1. All 103 GISTs were CD117 positive. Among these 103 cases, 83 were positive for CD34, 32 for SMA and four for S-100. The expressions of CD44s and MMP-9 were statistically predominant in small intestinal GISTs. TIMP-1 and Ki-67 had no significant difference between the gastric and the small intestinal GIST. The expression of Ki-67 was negatively correlated to PTEN ($P = 0.027$) and CD44s ($P = 0.02$). In patients that died of gastric GIST, the expressions of MMP-9 and Ki-67 were statistically higher than in those who survived. In contrast, CD44s and PTEN were significantly lower. The difference in TIMP-1 was not significant.

In patients that died of small intestinal GIST, Ki-67 LIs were much higher than in those who survived, while PTEN was significantly lower. The other markers, including CD44s, MMP-9, and TIMP-1, showed no significant difference.

Significance of PTEN, Ki-67 and CD44s on GISTs prognosis

For gastric GISTs, PTEN LIs < 50% was significantly correlated with lower specific survival rate ($P = 0.006$, Figure 2B), as was Ki-67 LIs ⩾ 5% ($P = 0.004$, Figure 2C) and CD44s negativity ($P = 0.006$, Figure 2D). In a total of 13 patients who died of GIST, there were five with PTEN LIs < 50%, six with Ki-67 LIs ⩾ 5% and eight patients who lost the expression of CD44s. For small intestinal GISTs, Ki-67 LIs ⩾ 5% significantly correlated with worse outcomes. Multivariate analysis did not reveal an independent factor for gastric or small intestinal GISTs. In a total of 15 patients who died of GIST, there were 10 with Ki-67 LIs ⩾ 5%.

No statistically significant difference was observed in disease specific survival for the patients grouped by the expressions of MMP-9 or TIMP-1. The balance between MMP-9 and TIMP-1 also did not correlate with disease specific survival. We failed to find an independent prognosis indicator after constructing the univariate and multivariate COX hazard proportional model (Table 3).

Combined analysis of PTEN, Ki-67 and CD44s

Combined analysis revealed that for gastric GISTs, the

Table 2 Expressions of 5 molecular markers in gastric and small intestinal gastrointestinal stromal tumor

Protein	Gastric GISTs			Small intestinal GISTs		
	Positive (%)		*P* value	Positive (%)		*P* value
	DOD (n = 13)	Alive (n = 48)		DOD (n = 15)	Alive (n = 27)	
CD44s	5 (38.4)	35 (72.9)	0.020	15 (100)	23 (85.2)	0.397
MMP-9	11 (84.6)	23 (47.9)	0.018	11 (73.3)	22 (81.5)	0.339
TIMP-1	7 (53.8)	22 (45.8)	0.608	3 (20.0)	14 (51.9)	0.085
PTEN						
< 50%	5 (38.5)	8 (8.3)	0.014	11 (73.3)	6 (22.2)	0.003
⩾ 50%	8 (61.5)	44 (91.7)		4 (26.7)	21 (77.8)	
Ki-67						
< 5%	7 (53.8)	41 (85.4)	0.022	5 (33.3)	24 (88.9)	0.0001
⩾ 5%	6 (46.2)	7 (14.6)		10 (66.7)	3 (11.1)	

GIST: Gastrointestinal stromal tumor; MMP: Matrix metalloproteinase.

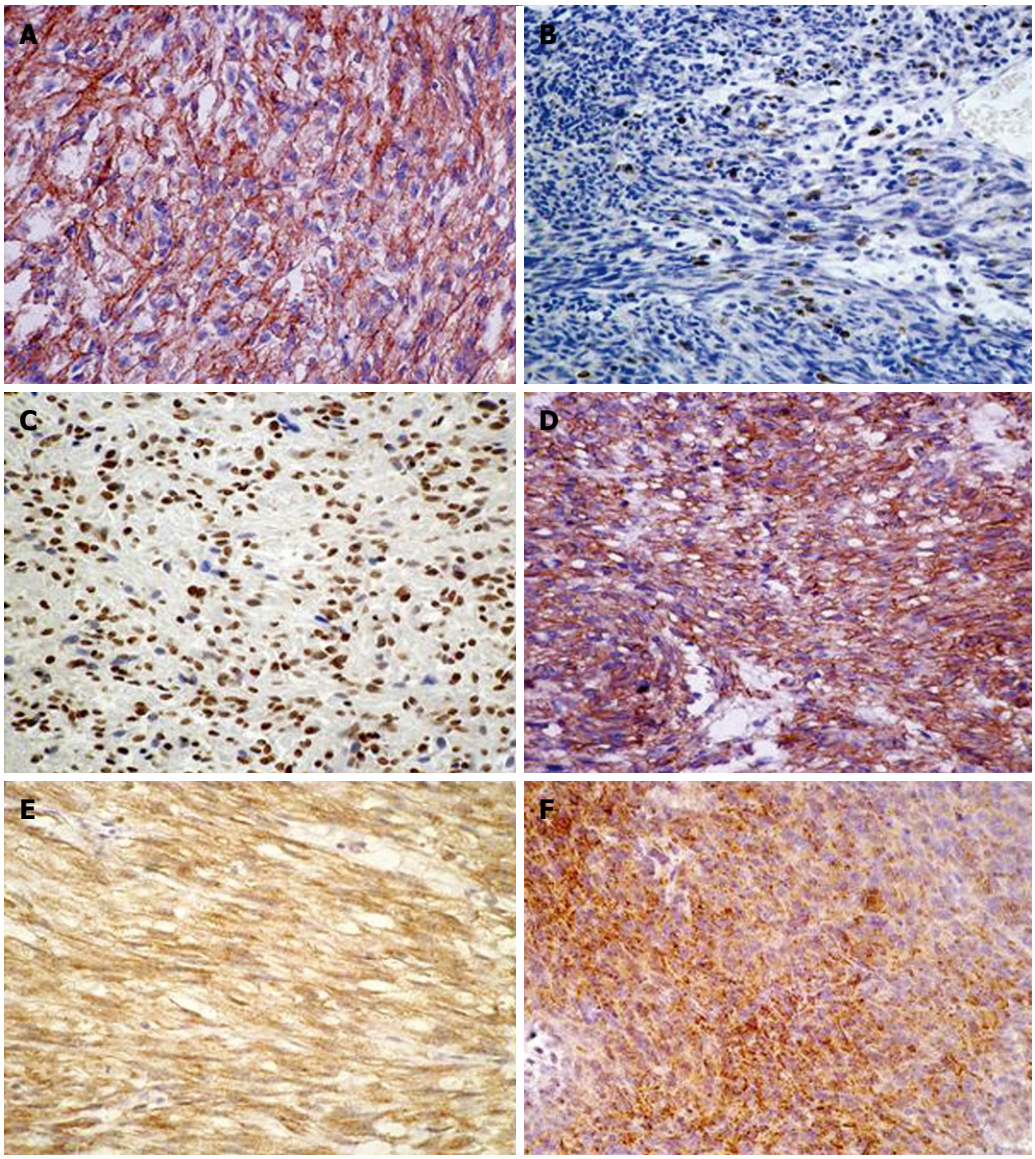

Figure 1 Examples of the selected markers expressed in gastrointestinal stromal tumor. A: The tumor cells were strongly positive for CD117 with diffuse membrane staining; B: Nuclear positivity of Ki-67 in the tumor cells; C: Nuclear positivity of PTEN in gastrointestinal stromal tumor; D: CD44s was diffusely positive in tumor cell membrane; E: Matrix metalloproteinase 9 was diffusely positive in cytoplasm; F: TIMP-1 was diffusely positive in cytoplasm. Immunostains counterstained with hematoxylin-eosin, original magnifications × 200.

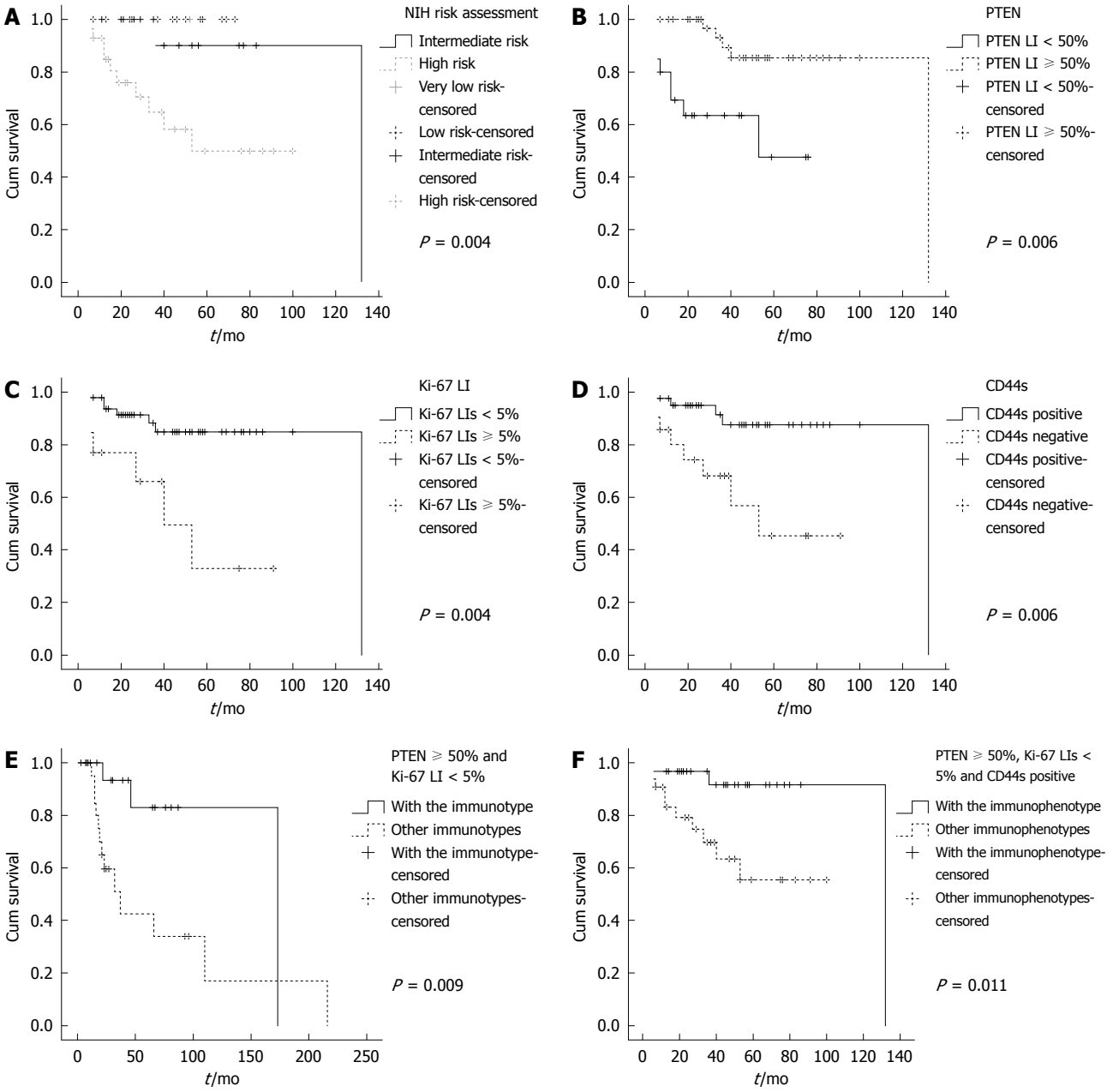

Figure 2 Kaplan-Meier cumulative survival plots of patients with gastric gastrointestinal stromal tumor. The end point was death due to gastrointestinal stromal tumor (GIST). A: High NIH risk assessment correlated significantly with worse outcomes in patients with gastric GIST (P = 0.004); B: PTEN labeling indexes (LIs) ⩾ 50% correlated significantly with favorable outcomes in patients with gastric GIST (P = 0.006); C: In patients with gastric GIST, those with Ki-67 LIs < 5% had significantly more favorable outcomes than those with Ki-67 LIs ⩾ 5% (P = 0.004); D: CD44s positivity was a significant, favorable indicator for patients with gastric GIST (P = 0.006); E: For patients with small intestinal GIST, the immunophenotype of PTEN LIs ⩾ 50% and Ki-67 LIs < 5% was a favorable indicator (P = 0.009); F: Gastric GIST patients with immunophenotype of PTEN LIs ⩾ 50%, Ki-67 LIs < 5% and CD44s positivity had significantly more favorable outcomes than those with other immunophenotypes (P = 0.011).

survival rate of the patients with the expression pattern of PTEN LIs ⩾ 50%, Ki-67 LIs < 5% and CD44s positivity was significantly higher than those with other immunophenotypes (P = 0.009, Figure 2E). In the 13 patients who died of gastric GIST, 11 did not show the above immunophenotype. Of the two patients with the phenotype, one survived over 10 years after the resection of GIST and the other survived 53 mo.

For patients with small intestinal GIST, the survival rate was significantly higher in those with the combined expressions of PTEN LIs ⩾ 50% and Ki-67 LIs < 5% relative to those with other immunophenotypes (P = 0.011, Figure 2F). In the 15 patients who died of small intestinal GIST, only two showed this favorable immunophenotype.

Prognostic impact of NIH risk assessment

The NIH risk assessment showed excellent correlation with disease-specific survival. The patients were classified as higher risk with worse prognosis (P = 0.001).

Table 3 Univariate and multivariate COX regression analyses of molecular markers in gastric gastrointestinal and small intestinal stromal tumor

	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Gastric GIST				
PTEN	0.23 (0.07-0.74)	0.013	0.35 (0.10-1.20)	0.100
Ki-67	4.54 (1.45-14.16)	0.009	2.69 (0.73-9.87)	0.130
CD44s	0.21 (0.07-0.73)	0.013	0.43 (0.83-9.98)	0.320
PTEN, Ki-67, CD44s	5.92 (1.29-27.08)	0.022	1.35 (0.15-11.80)	0.780
MMP-9	4.22 (1.92-19.31)	0.042		
TIMP-1	0.90 (0.29-2.82)	0.862		
MMP-9/TIMP-1	0.72 (0.22-2.38)	0.586		
Small intestinal GIST				
Ki-67	3.29 (1.14-9.46)	0.019		
PTEN, Ki-67	4.02 (1.25-16.09)	0.021		

MMP: Matrix metalloproteinase; GIST: Gastrointestinal stromal tumor; OR: Odds ratio.

GIST arising from gastric and small intestine showed different recurrent potential; we subsequently analyzed the prognostic value of risk assessment in these two anatomic sites. For gastric GIST, high risk stratification was correlated with significantly worse prognoses (P = 0.004, Figure 2A). But for small intestinal GIST, the risk stratification had no significant correlation with DSS (P = 0.205). Considering the disease-free survival rate, the risk stratification was significantly correlated with survival not only in small intestinal GISTs (P = 0.011), but also in gastric GISTs (P = 0.044).

Ki-67 and risk assessment

In our series, 29 gastric patients who were graded as high risk had shorter survival time than the other three grades (61.31 ± 17.28 mo *vs* 121.64 ± 17.06 mo). Fourteen gastric patients with Ki-67 LIs ≥ 5% had shorter survival time than those with Ki-67 LIs < 5% (49.75 ± 10.84 mo *vs* 111.46 ± 15.26 mo). Ten of these 14 cases were classified as high risk grade, four were not in this group but suffered relapse or metastasis.

For small intestinal GISTs, two cases with Ki-67 LIs ≥ 5% suffered relapse and one died of the GIST in 32 mo. Neither of these two cases were classified as high risk grade by NIH risk assessment.

DISCUSSION

In this study based on follow-up data, we developed a cluster of immunohistochemical markers that can facilitate the assessment of GIST prognosis in Chinese patients. The expression patterns of PTEN, Ki-67 and CD44s can help clinicians evaluate the clinical outcome of the patients with gastric GIST, as can the combination of PTEN and Ki-67 for those with small intestinal GIST.

Although PTEN, Ki-67 and CD44s can individually assist the prognosis, there are still apparent limitations because each marker just focuses on some of the patients. When tested by follow-up data, although eight patients expressed PTEN LIs ≥ 50%, seven of them died of GIST within 5 years after the surgery resection. Such a conflict phenomenon is also very common in patients with gastric GIST when the prediction is based only on Ki-67 or CD44s. This also occurs for Ki-67 or PTEN in patients with small intestinal GIST. Combining the expressions of PTEN, Ki-67 and CD44s can improve the specificity and accuracy of the prognosis.

Our results showed that Ki-67 LIs is negatively correlated with PTEN and CD44s. PTEN and Ki-67 are both involved in cell proliferation. PTEN can upregulate $p27^{kip1}$ resulting in apoptosis and cell cycle arrest, suppression of cell proliferation by a mechanism independent of Akt activity in nuclei[19]. Also, PTEN upregulates p53 activity by maintaining the high acetylation of p53[20]. In our study, PTEN was exclusively located in nuclei, and PTEN LIs < 50% was statistically correlated with a worse outcome of gastric GIST. A similar result was also reported by Ricci, regarding the correlation of decreased PTEN expression with worse outcomes[10].

Ki-67 is widely used to predict the proliferation potential of malignant tumors. Many reports have confirmed the prognostic value of Ki-67 in GIST[21-23]. The differences in these reports are the cut-off value of the Ki-67 index, which varied from 4.5% to 10%. Whether Ki-67 is one of best predictors is debatable. Nakamura proposed that Ki-67 LIs and risk assessment were useful for predicting GIST outcome[17], while Wong considered that mitotic count[24], not Ki-67 LIs, remained the best predictor of gastric GIST. Our results revealed that higher Ki-67 LIs were associated with worse outcomes. Interestingly, there were four cases of low risk gastric GISTs with high Ki-67 LIs that suffered worse outcomes. Another two cases of low risk intestinal GISTs that had high Ki-67 LIs also resulted in worse outcomes. This indicates that Ki-67 may enhance the NIH consensus criteria, especially when applied to patients of low risk. Though the absolute cut-off of Ki-67 LIs is difficult to define, Ki-67 may become one of the most robust indicators of GIST.

CD44 is now widely accepted as a stem cell marker in many kinds of carcinomas, including gastric cancer, colorectal cancer[25], and breast cancer. While only a minority of the cancer cells have the capability of car-

cinogenesis, this portion of the cell population is stem cells; in Du's series, no more than 5% of the cells were positive for CD44. In GISTs, once CD44 is positive, it is diffusely expressed by nearly all the cells. Based on this finding, CD44 may not be a suitable stem cell marker for GIST. CD117 and CD34 are stem cell markers for the haematopoietic stem cells. In GIST, CD117 is the crucial diagnostic marker; CD34 is one of the most important differential diagnostic markers. The expression of these stem cell markers in GIST may support the hypothesis that GIST originates from mesenchymal stem cells. The suitable robust stem cell markers for GIST needs further investigation. CD44 and its associated partner proteins monitor changes in the extra-cellular matrix that influences cell growth, survival and differentiation. It can be a molecular switch between growth and arrest depending on the extra-cellular conditions. As reported for CD44-deficient fibroblasts, the recruited CD44s suppress metastasis and proliferation[26]. Furthermore, CD44 can promote tumor invasion by recruiting MMP-9[27]. In small intestinal GIST, the expressions of CD44s and MMP-9 are significantly higher than in gastric GISTs. The co-expression of CD44s and MMP-9 may be responsible for the high metastatic liability of small intestinal GIST.

The anatomic site of GIST is now believed to be a prognostic factor. Some experts believe that when GIST is considered as a location-specific entity, multiple clinicopathologic parameters can be used together to define the biological behavior. In the revised version of NIH risk stratification, anatomic location was considered to be an important factor in the assessment of moderate and high risk[6]. In our series, the small intestinal GIST was more aggressive than gastric GIST. Of the patients with small intestinal GIST, 52.38% suffered recurrence or metastasis, while only 25% of gastric GIST patients did. The molecular makers expressed in gastric and small intestinal GIST were also different, as were the prognostic indicators. CD44 had no impact on the prognoses for patients with small intestinal GIST, and a similar result has been reported for rectal GIST[28]. The dissimilar anatomic locations may be responsible for these difference. Based on these facts, we believe that GIST should be sub-grouped by anatomic location first and then assessed by other factors.

In conclusion, the expression patterns of PTEN, Ki-67 and CD44s are useful for the prognosis of patients with gastric GIST, and PTEN and Ki-67 are valuable outcome indicators for patients with small intestinal GISTs, Ki-67 LIs can enhance the NIH consensus criteria.

COMMENTS

Background

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the digestive tract. Because the targeted therapy of imatinib and sunitinib has achieved great success, accurate prediction of GIST outcomes is becoming more and more important. The biological behavior of GIST is highly variable. NIH risk assessment is now widely used as a prognostic indicator, but further investigation of the prognostic factors is still needed.

Research frontiers

NIH risk assessment is widely used for GIST risk stratification; the revised version is based on tumor location, diameter and mitotic index. Many investigators have analyzed the prognostic value of other factors including stage, grade, KIT mutation and immunohistochemical markers.

Innovations and breakthroughs

Based on follow-up information on patients without targeted therapy, we comprehensively analyzed anatomic location, NIH risk assessment and a number of immunohistochemical markers including PTEN, Ki-67, CD44s, matrix metalloproteinase 9 and TIMP-1.

Applications

This study provides a new cluster of prognostic markers for GIST. PTEN LIs $\geqslant$ 50%, Ki-67 LIs < 5% and CD44s positivity correlates with favorable outcomes for gastric GISTs, as does PTEN LIs $\geqslant$ 50% and Ki-67 LIs < 5% for small intestinal GISTs. Anatomic location is a prognostic indicator of GISTs; depending on location, GISTs may have different biological features. The intrinsic nature needs further investigation.

Terminology

GIST is the most common mesenchymal tumor of digestive tract. Gain-of-function mutation of in *c-kit* or platelet derived growth factor α are hypothesized as the principle molecular pathogenesis. Based on this point, the targeted therapy of imatinib or sunitinib has achieved great success. But the biological behavior of GIST is highly variable; many experts are investigating improvements in prognostic markers.

Peer review

This is an interesting, well done and well written paper adding useful insights in the field.

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BRIEF ARTICLE

Vitamin D receptor gene polymorphisms and colorectal cancer risk: A systematic meta-analysis

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Abstract

AIM: To investigate the relationship between polymorphisms present in the vitamin D receptor (*VDR*) gene and colorectal cancer risk, a systematic meta-analysis of population-based studies was performed.

METHODS: A total of 38 relevant reports published between January 1990 and August 2010 were identified, of which only 23 qualified for this meta-analysis based on our selection criteria. Five polymorphic variants of the *VDR* gene, including *Cdx*-2 (intron 1e) and *Fok*I (exon 2) present in the 5′ region of the gene, and *Bsm*I (intron 8), *Apa*I (intron 8), and *Taq*I (exon 9) sites present in the 3′ untranslated region (UTR), were evaluated for possible associations with colorectal cancer risk. Review manager 4.2 was used to perform statistical analyses.

RESULTS: In the meta-analysis performed, only the *Bsm*I polymorphism was found to be associated with colorectal cancer risk. In particular, the *Bsm*I B genotype was found to be related to an overall decrease in the risk for colorectal cancer [*BB vs bb*: odds ratio (OR) = 0.87, 95% CI: 0.80-0.94, $P = 3 \times 10^{-4}$; *BB vs Bb* + *bb*: OR = 0.90, 95% CI: 0.84-0.97, $P = 5 \times 10^{-4}$]. Moreover, in subgroup analyses, the *Bsm*I *B* genotype was significantly associated with colon cancer, and not rectal cancer. An absence of between-study heterogeneity was also observed.

CONCLUSION: A meta-analysis of 23 published studies identified the *Bsm*I polymorphism of the *VDR* gene to be associated with an increased risk of colon cancer.

Key words: Vitamin D receptor; Polymorphism; Meta-analysis; Colorectal cancer

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INTRODUCTION

Colorectal cancer represents the third most common cancer worldwide, second only to lung cancer and gastric

cancer[1]. Furthermore, it is estimated that there are more than 370 000 cases of colon and rectal cancer diagnosed in Europe every year, with 200 000 cases resulting in death[2]. However, the underlying etiology of colorectal cancer, including cancerous growths of the colon, rectum, and appendix, remains poorly understood. It has been proposed that some categories of external agents, including physical, chemical, and biological carcinogens, may contribute to the development of this disease, and the role of these factors in carcinogenesis would depend largely on genetic factors. Correspondingly, a recent study showed that insufficient levels of vitamin D may result in colorectal cancer[3]. Furthermore, genetic variations in genes controlling vitamin D activity would be hypothesized to play an important role in determining susceptibility to colorectal cancer.

In vivo, vitamin D helps bones and muscles grow, and may also help prevent many diseases, such as prostate cancer and breast cancer. The biological activity of vitamin D is mediated by the vitamin D receptor (*VDR*)[4], which interacts with other cell signaling pathways to influence cell behavior. Expression of *VDR* has been detected in various organs and tissues of the human body, including the kidney and bone cells. *VDR* is also expressed in normal colon mucosa[5]. In the intestine, *VDR* plays an important role in regulating cell proliferation, differentiation, and the induction of apoptosis[6]. Furthermore, *VDR* may be associated with the effects of calcium on colorectal epithelial proliferation[7].

Molecular epidemiological studies have shown that polymorphisms in the *VDR* gene may be linked to biological functions of vitamin D. At the 5' end of the *VDR* gene, a *Fok*I polymorphism (rs2228570/rs10735810, exon 2) has been associated with a frameshift in the *VDR* protein[8]. Moreover, polymorphisms in the 3' untranslated region (UTR), including *Bsm*I (rs1544410, intron 8), *Apa*I (rs7975232, intron 9), and *Taq*I (rs731236, exon 9) sites, have been shown to influence gene transcription and mRNA stability[9]. Additionally, these polymorphisms have exhibited the potential for strong linkage disequilibrium (LD)[10,11], and functional differences have been associated with the associated haplotypes[11,12]. Given that polymorphisms in the *VDR* gene could potentially influence the binding of 1, 25$(OH)_2D_3$ and the anti-proliferative effects of vitamin D, *VDR* polymorphisms have been hypothesized to be associated with colorectal cancer risk.

In 2001, the first report of an association between colorectal cancer and the *VDR* gene was published by Kim and colleagues[13]. They identified a random subset of 393 cases of colorectal adenomas and 406 colonoscopy-negative controls from a clinic-based, case-control study conducted in the United States between 1991 and 1994. Based on their analysis, the *Bsm*I *BB* genotype was found to be associated with a reduced risk of colorectal adenoma when intake of calcium and vitamin D was reduced. In addition to the *Bsm*I site[12,14-23]. Other polymorphic sites present in the *VDR* gene, including *Cdx*-2[12,19,21,24,25], *Fok*I[12,15,16,18,19,21-24,26-33], *Apa*I[12,16,19,21,34], and *Taq*I[12,16,19,23,24,29,31,33-35], have been evaluated in genetic association studies. However, the results are inconsistent. Since it can be difficult for individual studies to achieve sufficient statistical power to detect associations between *VDR* polymorphisms and colorectal cancer risk, a meta-analysis that combines data from all published studies may detect genetic associations more accurately. In addition, a reduced probability of false-negatives might also be achieved[36]. Therefore, a systematic meta-analysis of population-based studies was performed to investigate the association between *VDR* polymorphisms and the risk of colorectal cancer. Based on the search strategy and criteria used, 23 studies were analyzed which identified several important polymorphic variants.

MATERIALS AND METHODS

Search strategy and data extraction

To examine the association between *VDR* polymorphisms and colorectal cancer risk, a search of the MEDLINE database (from January 1990 to August 2010) and the US National Library of Medicine's PubMed database (http://www.ncbi.nlm.nih.gov/pubmed) was performed. In addition, various scientific research tools available on the web were used to search relevant references such as Google (http://scholar.google.com/) and Scirus (http://www.scirus.com/). In particular, data relevant to five well-characterized polymorphic variants was identified, including: *Cdx*-2, *Fok*I, *Bsm*I, *Apa*I, and *Taq*I sites within *VDR*. Keywords used in searches included "vitamin D receptor" in combination with "polymorphism", "vitamin D", "genotype", "allele", "colorectal cancer", or "risk".

Papers selected for this meta-analysis included a case-control study and complete data, including the authors' names; the subjects' region/country; year of publication; numbers of cases and controls; mean age (or range) of the case/control group; diagnostic criteria used; and number of subjects with the *VDR* genotype in both case and control groups. All relevant references that met these inclusive criteria and that were published as articles or abstract containing original data, were included in this study. In contrast, case-only studies, studies with incomplete data, or studies with inadequate control groups were excluded. In addition, the data extracted needed to conform to the guidelines of MOOSE, a proposal for reporting meta-analyses of observational studies[37]. If the same or overlapping data were reported in multiple publications, the most recent publication was selected[38].

Statistical analysis

For each data set included in this study, the odds ratios (ORs) and corresponding 95% CI for the incidence of cancer in subjects with or without particular restriction sites (lowercase *vs* uppercase lettering), was compared. Furthermore, deviations from the Hardy-Weinberg equilibrium for each control group were assessed using the goodness-of-fit test. To estimate associations with colorectal cancer risk, various genotypic models were

Table 1 Characteristics of case-control studies included in the meta-analysis

First author	Year	Country	Racial descent	Mean age in cases	Mean age in controls	Cases/ controls	Genotyping method	Quality control	Adjusted	Studied polymorphisms
Ingles *et al*[11]	1998	United States	American	62.3	62.2	373/394	PCR-RFLP	Yes	Yes	*Fok*I
Kim *et al*[13]	2001	United States	American	58.0 ± 9.7	53.0 ± 11.0	393/406	TaqMan	Yes	Yes	*Bsm*I
Peters *et al*[27]	2001	United States	American	18-74	18-74	208/184	PCR-RFLP	Yes	NR	*Fok*I
Slatter *et al*[23]	2001	United States	American	NR	NR	424/366	PCR-RFLP	NR	Yes	*Fok*I, *Bsm*I, *Taq*I
Speer *et al*[14]	2001	Hungary	European	64	63	56/112	PCR-RFLP	NR	NR	*Bsm*I
Grau *et al*[29]	2003	United States	American	60.8 ± 9.0	60.9 ± 9.0	372/379	PCR-RFLP	Yes	Yes	*Fok*I, *Taq*I
Wong *et al*[28]	2003	Singapore	Asian	66	56.5	217/890	PCR-RFLP	Yes	Yes	*Fok*I
Peters *et al*[35]	2004	United States	American	62.9	62.3	763/774	PCR-RFLP	Yes	NR	*Taq*I
Slattery *et al*[15]	2004	United States	American	30-79	30-79	1936/2130	PCR-RFLP	NR	Yes	*Fok*I, *Bsm*I
Murtaugh *et al*[30]	2006	United States	American	30-79	30-79	2450/2821	PCR-RFLP	NR	Yes	*Fok*I
Park *et al*[16]	2006	South Korea	Asian	55	55	190/354	PCR-RFLP	NR	Yes	*Fok*I, *Bsm*I, *Apa*I, *Taq*I
Flügge *et al*[12]	2007	Germany	European	61.9 ± 10.0	62.2 ± 11.2	256/256	PCR-RFLP	Yes	Yes	*Cdx*-2, *Fok*I, *Bsm*I, *Apa*I, *Taq*I
Kadiyska *et al*[19]	2007	Bulgaria	European	59	59 ± 5	133/94	PCR-RFLP	NR	Yes	*Bsm*I
Slattery *et al*[18]	2007	United States	American	30-79	30-79	2380/2990	TaqMan	Yes	Yes	*Fok*I, *Bsm*I
Yaylim-Eraltan *et al*[31]	2007	Turkey	European	59.1 ± 4.0	52.0 ± 0.8	26/52	PCR-RFLP	NR	Yes	*Fok*I, *Taq*I
Grünhage *et al*[32]	2008	Germany	European	65 ± 9	63 ± 8	192/220	PCR-RFLP	NR	Yes	*Fok*I
Hubner *et al*[17]	2008	United Kingdom	European	NR	NR	137/409	TaqMan	Yes	Yes	*Cdx*-2, *Fok*I, *Bsm*I, *Apa*I, *Taq*I
Ochs-Balcom *et al*[24]	2008	United States	American	62.8 ± 10.2	58.5 ± 12.1	250/246	TaqMan	Yes	Yes	*Cdx*-2, *Fok*I, *Taq*I
Parisi *et al*[20]	2008	Spain	European	NR	NR	170/120	PCR-RFLP	NR	Yes	*Bsm*I
Theodoratou *et al*[21]	2008	United Kingdom	European	62.0 ± 10.8	62.4 ± 10.5	3005/3072	Microarray	Yes	Yes	*Cdx*-2, *Fok*I, *Bsm*I, *Apa*I
Wang *et al*[33]	2008	China	Asian	38-78	19.6 ± 1.3	69/218	PCR-RFLP	NR	Yes	*Fok*I
Jenab *et al*[22]	2009	Europe	European	NR	NR	1248/1248	TaqMan	Yes	Yes	*Fok*I, *Bsm*I
Mahmoudi *et al*[34]	2010	Iran	Asian	52.7 ± 14.0	44.4 ± 17.7	160/180	PCR-RFLP	Yes	Yes	*Apa*I, *Taq*I

NR: Not reported.

selected, including codominant, additive, recessive, and dominant. Both the Peto Mantel-Haenszel fixed-effects model and the DerSimonian Laird random-effects model (with weights based on the inverse variance) were used to calculate summary ORs, and both within- and between-study variations were considered[39]. A *P*-value less than 0.10 was considered statistically significant when comparing trials showing heterogeneity, and random-effects analysis was selected. In contrast, fixed-effects analysis was used for comparing trials exhibiting homogeneity. Inverted funnel plots were also used to examine asymmetry, in which the ORs were plotted on a logarithmic scale against the inverse of their corresponding standard errors[40]. In the presence of publication bias, the funnel plot was asymmetric and the data showed remarkable skewness. There may be many reasons for this, most notably that some studies with negative findings are not published. In contrast, the plots were symmetric when bias was absent.

All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS, version 13.0) and Review Manager (version 4.2, The Cochrane Collaboration), and all *P*-values were two-sided.

RESULTS

Characteristics of case-control studies included in the meta-analysis

According to the criteria defined above, 38 published studies relevant to the *VDR* gene and colorectal cancer risk were reviewed. Fifteen of these papers were excluded due to insufficient clarity in data presentation, repeated literature, or significant differences were present in their study design compared with the other papers identified[41]. The remaining 23 eligible case-control studies are listed in Table 1, and were included in a meta-analysis to investigate possible associations between *Cdx*-2, *Fok*I, *Bsm*I, *Apa*I, and *Taq*I polymorphisms present in the *VDR* gene and the risk of colorectal cancer.

In 21/23 studies, data regarding the 5' end of the *VDR* gene were provided. In four of these studies, 2639 cases and 2948 controls were analyzed for the *Cdx*-2 polymorphism, while 17 studies included 13 301 cases and 15 942 controls analyzed for the *Fok*I polymorphism. In addition, the 3' UTR region of the *VDR* gene has been analyzed. For example, 12 studies containing 10 083 cases and 11 242 controls analyzed the *Bsm*I polymorphism, 5 studies including 2739 cases and 3200 controls analyzed the *Apa*I polymorphism, and 9 studies including 2580 cases and 3016 controls analyzed the *Taq*I polymorphism.

Among controls, the frequency of the *c* allele at the *Cdx*-2 site ranged from 65.6% in Berlin-Bush populations of Germany, to 80.0% in a United Kingdom population[12,19,21,24,25]. In contrast, the frequency of the *f* allele of *Fok*I among controls ranged from 31.7% in Turkey, to 47.2% in a Singapore population[12,15,16,18,19,21-24,26-33]. The frequency of the *b* allele at *Bsm*I among controls ranged from 56.1% in a Bulgarian population, to 94.7% in a Korean population[12,14-23], while the frequency of the *a* allele at *Apa*I

Table 2 Summary odds ratios and 95% CI in the vitamin D receptor gene

SNP	Model	Total No. cases	Total No. controls	OR (95% CI)[1]	*P* value[2]	*P* value[3]
Cdx-2	Codominant (*CC vs cc*)	152/1561	146/1820	1.25 (0.98-1.59)	0.07	0.26
	Codominant (*Cc vs cc*)	926/2487	982/2802	1.09 (0.97-1.22)	0.15	0.64
	Codominant (*C vs C*)	1230/4048	1279/4622	1.10 (1.01-1.21)	0.03	0.47
	Dominant (*CC + Cc vs cc*)	1078/1561	1208/1820	0.98 (0.88-1.09)	0.72	< 0.001
	Recessive (*CC vs Cc + cc*)	152/2487	146/2802	1.22 (0.96-1.54)	0.10	0.23
*Fok*I	Codominant (*ff vs FF*)	1844/5068	2377/5982	0.94 (0.87-1.01)	0.09	0.001
	Codominant (*Ff vs FF*)	6189/11 257	7583/13 565	0.98 (0.93-1.03)	0.34	0.001
	Codominant (*f vs F*)	9867/16 320	12 190/19 329	0.97 (0.94-1.00)	0.07	< 0.001
	Dominant (*ff + Ff vs FF*)	8033/5068	9960/5982	0.96 (0.92-1.01)	0.15	< 0.001
	Recessive (*ff vs FF + Ff*)	1844/11 257	2377/13 565	0.95 (0.89-1.02)	0.13	0.01
*Bsm*I	Codominant (*BB vs bb*)	1512/3838	1817/4122	0.87 (0.80-0.94)	< 0.001	0.65
	Codominant (*Bb vs bb*)	4733/8571	5303/9425	0.94 (0.88-0.99)	0.03	0.64
	Codominant (*B vs b*)	7757/12 409	8937/13 577	0.93 (0.90-0.97)	< 0.001	0.85
	Dominant (*BB + Bb vs bb*)	6245/3838	7120/4122	0.92 (0.87-0.97)	0.003	0.84
	Recessive (*BB vs Bb + bb*)	1512/8571	1817/9425	0.90 (0.84-0.97)	0.006	0.28
*Apa*I	Codominant (*AA vs aa*)	748/578	1004/603	0.85 (0.73-0.99)	0.03	0.06
	Codominant (*Aa vs aa*)	1378/1956	1593/2196	0.91 (0.79-1.04)	0.18	0.39
	Codominant (*A vs a*)	2944/2534	3601/2799	0.92 (0.85-0.99)	0.02	0.04
	Dominant (*AA + Aa vs Aa*)	2161/578	2597/603	0.89 (0.78-1.01)	0.07	0.12
	Recessive (*AA vs Aa + aa*)	783/1956	1004/2196	0.89 (0.80-1.00)	0.05	0.21
*Taq*I	Codominant (*tt vs TT*)	382/1112	398/1320	1.05 (0.89-1.24)	0.58	0.07
	Codominant (*Tt vs TT*)	1086/2198	1298/2618	0.93 (0.83-1.05)	0.23	0.30
	Codominant (*t vs T*)	1850/3310	2098/3938	0.99 (0.92-1.08)	0.86	0.10
	Dominant (*tt + Tt vs TT*)	1468/1112	1696/1320	0.95 (0.85-1.07)	0.42	0.37
	Recessive (*tt vs Tt + TT*)	382/2198	398/2618	1.07 (0.92-1.25)	0.38	0.01

[1]Based on fixed effects model; [2]Test for overall effect; [3]Test for heterogeneity. OR: Odds ratios.

among controls ranged from 23.0% in a Korean population to 49.3% in a population of the United Kingdom[12,16,19,21,34]. Lastly, the frequency of the *t* allele at *Taq*I among controls ranged from 8.8% in a Korean population to 43.6% in a population of the United States[12,16,19,23,24,29,31,33-35].

Qualitative assessment of included studies

Genotyping of the *Cdx*-2, *Fok*I, *Bsm*I, *Apa*I and *Taq*I polymorphisms was performed using the polymerase chain reaction-restriction fragment length polymorphism technique in 75% of the studies included in this meta-analysis. Due to the low sensitivity of this classic technology, quality control of this genotyping was required, and included blindness to the case-control status, random repeats of samples, or validation using a different genotyping method. However, only 38.9% (7/18) of the eligible studies provided sufficient quality control. Regarding sample size, only 5/24 (20.8%) studies employed more than 1000 cases or controls. Moreover, most of these studies were associated with poor statistical power due to sample sizes that were less than 500 and in some cases contained less than 100 cases or controls.

Assessment of Hardy-Weinberg proportion is regarded as an important criterion for evaluating genetic association studies[38]. Most of the studies included in this meta-analysis reported genotype frequencies in their control groups that were consistent with Hardy-Weinberg proportions ($P > 0.05$). For example, deviations from Hardy-Weinberg proportions in controls were observed in three studies for *Fok*I[29,31,32], two studies of *Bsm*I[19,21], one study of *Apa*I[19], and two studies of *Taq*I[23,24].

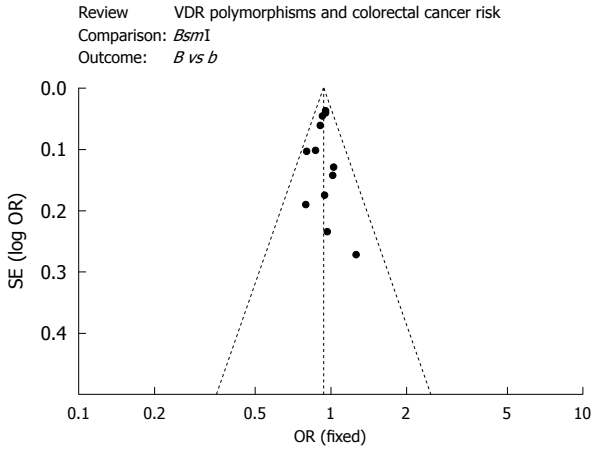

Figure 1 A funnel plot was used to estimate the publication bias of the studies included in the meta-analysis performed.

Funnel plotting was performed to evaluate whether publication bias was present in the meta-analysis performed. As shown in Figure 1, the shapes of the funnel plots obtained appear to be symmetrical in codominant, dominant, and recessive models, suggesting that publication bias is absent in the meta-analysis performed.

Cdx-2, FokI, BsmI, ApaI, TaqI polymorphisms and colorectal cancer risk

A heterogeneity test of potential associations between the *Cdx*-2, *Fok*I, *Bsm*I, *Apa*I, and *Taq*I polymorphisms and risk of colorectal cancer are presented in Tables 2 and 3.

Table 3 The *Bsm*I effect odds ratios stratified by anatomical site

Model	Colon cancer			Rectal cancer		
	Total No. cases/controls	OR (95% CI)	*P* value[1]	Total No. cases/controls	OR (95% CI)	*P* value[1]
Codominant (*BB vs bb*)	1365/1581	0.80 (0.68-0.93)	0.004/0.21	659/790	0.92 (0.73-1.15)	0.46/0.90
Codominant (*Bb vs bb*)	2257/2438	0.99 (0.88-1.12)	0.92/0.95	1029/1240	0.94 (0.80-1.11)	0.48/0.21
Codominant (*B vs b*)	5328/5972	0.91 (0.84-0.98)	0.01/0.25	2440/2966	0.95 (0.85-1.06)	0.38/0.99
Dominant (*BB + Bb vs bb*)	2664/2986	0.94 (0.84-1.05)	0.25/0.65	1220/1483	0.94 (0.77-1.16)	0.59/0.40
Recessive (*BB vs Bb + bb*)	2664/2986	0.80 (0.69-0.92)	0.002/0.19	1220/1483	0.93 (0.80-1.09)	0.40/0.51

[1]Test for overall effect and heterogeneity, respectively. OR: Odds ratios.

***Cdx*-2 polymorphism:** Currently, only four studies have investigated the relationship between the *VDR Cdx*-2 polymorphism and colorectal cancer risk, and all of these studies were in Hardy-Weinberg equilibrium[12,19,21,24,25]. Furthermore, in the overall and subgroup analyses performed, the *Cdx*-2 polymorphism did not appear to be linked to colorectal cancer risk.

***Fok*I polymorphism:** Seventeen studies included in the meta-analysis performed found the *Fok*I polymorphism to be statistically heterogeneous in all genetic models ($P \leqslant 0.01$)[12,15,16,18,19,21-24,26-33]. Moreover, no significant association was found between *Fok*I and colorectal cancer risk in overall and subgroup analyses.

***Bsm*I polymorphism:** A total of 12 studies examined the association between colorectal cancer and the *Bsm*I polymorphism, and there was little statistical evidence of heterogeneity among the studies ($P \geqslant 0.28$)[12,14-23]. Individuals with the *BB* genotype (OR = 0.87; 95% CI = 0.80-0.94, $P = 3 \times 10^{-4}$; $P = 0.65$ for heterogeneity), or the *Bb* genotype (OR = 0.94; 95% CI: 0.88-0.99, $P = 0.03$; $P = 0.48$ for heterogeneity), were associated with a significant decrease in colorectal cancer risk compared with patients carrying the *bb* genotype. The Dominant model (*BB + Bb vs bb*) and the recessive model (*BB vs Bb + bb*) also showed a significant association with colorectal cancer risk, with the associated ORs being 0.92 (95% CI: 0.87-0.97, $P = 0.003$; $P = 0.84$ for heterogeneity) and 0.90 (95% CI: 0.84-0.97, $P = 0.006$; $P = 0.28$ for heterogeneity), respectively (Figure 2). Although two of these studies were not consistent with Hardy-Weinberg proportions[17,21], the effect was negligible. In addition, the *BB* genotype showed a decreased risk for colon cancer compared with the *bb* (OR = 0.80, 95% CI: 0.68-0.93, $P = 0.004$; $P = 0.21$ for heterogeneity), or *Bb + bb* genotypes (OR = 0.80, 95% CI: 0.69-0.92, $P = 0.002$; $P = 0.19$ for heterogeneity). However, no significant differences were observed between these polymorphisms and rectal cancer risk (Table 3).

***Apa*I polymorphism:** The association between the *Apa*I polymorphism and colorectal cancer was investigated in five studies, of which only one study was not consistent with Hardy-Weinberg proportions[12,16,19,21,34]. Moreover, although the codominant model (*AA vs aa*, OR = 0.83; 95% CI: 0.71-0.97, $P = 0.02$) showed a significant association with colorectal cancer risk, the *P* value of 0.05 suggested that this genetic model was statistically heterogeneous.

*Taq*I polymorphism. Except for the recessive model (*tt vs Tt + TT*, $P = 0.06$), there was little evidence of statistical heterogeneity among the nine studies that investigated an association between the *Taq*I polymorphism and colorectal cancer risk ($P \geqslant 0.35$)[12,16,19,23,24,29,31,33-35]. When the two studies in which controls were not in Hardy-Weinberg equilibrium were excluded, the pooled ORs for all genetic models for *Taq*I were shifted, yet the results remained null[23,24].

DISCUSSION

This study was undertaken to assess whether *VDR* polymorphisms in both the 5' (*Cdx-2* and *Fok*I) and 3' (*Bsm*I, *Apa*I and *Taq*I) regions of the *VDR* gene are associated with colorectal cancer risk. A total of 38 reports had previously evaluated a possible genetic association, and only 23 of these were eligible for this study based on the selection criteria employed. The pooled ORs (95% CI) for these studies were identical according to both fixed- and random-effects models. Moreover, only the polymorphic variant, *Bsm*I, was found to be associated with increased risk for colorectal cancer. The meta-analysis performed also showed that the *Bsm*I *B* genotype was related to a significant decrease in overall risk for colorectal cancer, with the co-dominant *BB* and the *BB + Bb vs bb* dominant model exhibiting 0.87- and 0.92-fold increases in the risk for disease, respectively. The *BB vs Bb + bb* recessive model also had a 90% decreased risk for colorectal cancer.

In addition, subgroup analyses by anatomical site identified the *Bsm*I polymorphism to be significantly associated with colon cancer, and not rectal cancer. Furthermore, compared with the *bb* or *Bb + bb* genotypes, the *BB* genotype was associated with a 90% decrease in the risk for colon cancer. However, it remains unclear why the *Bsm*I site is related to the risk of colon cancer, and not rectal cancer. It is possible that the difference in epithelial cells between the two cancers plays a role, with ciliated columnar epithelial cells being present in the lining of the colon, while squamous epithelial cells are present in the rectum. The role of the micro-environment may also be a contributing factor, since physical damage as a result of oxygen or poisonous food residues is more likely to influence the rectum than the colon. In addition,

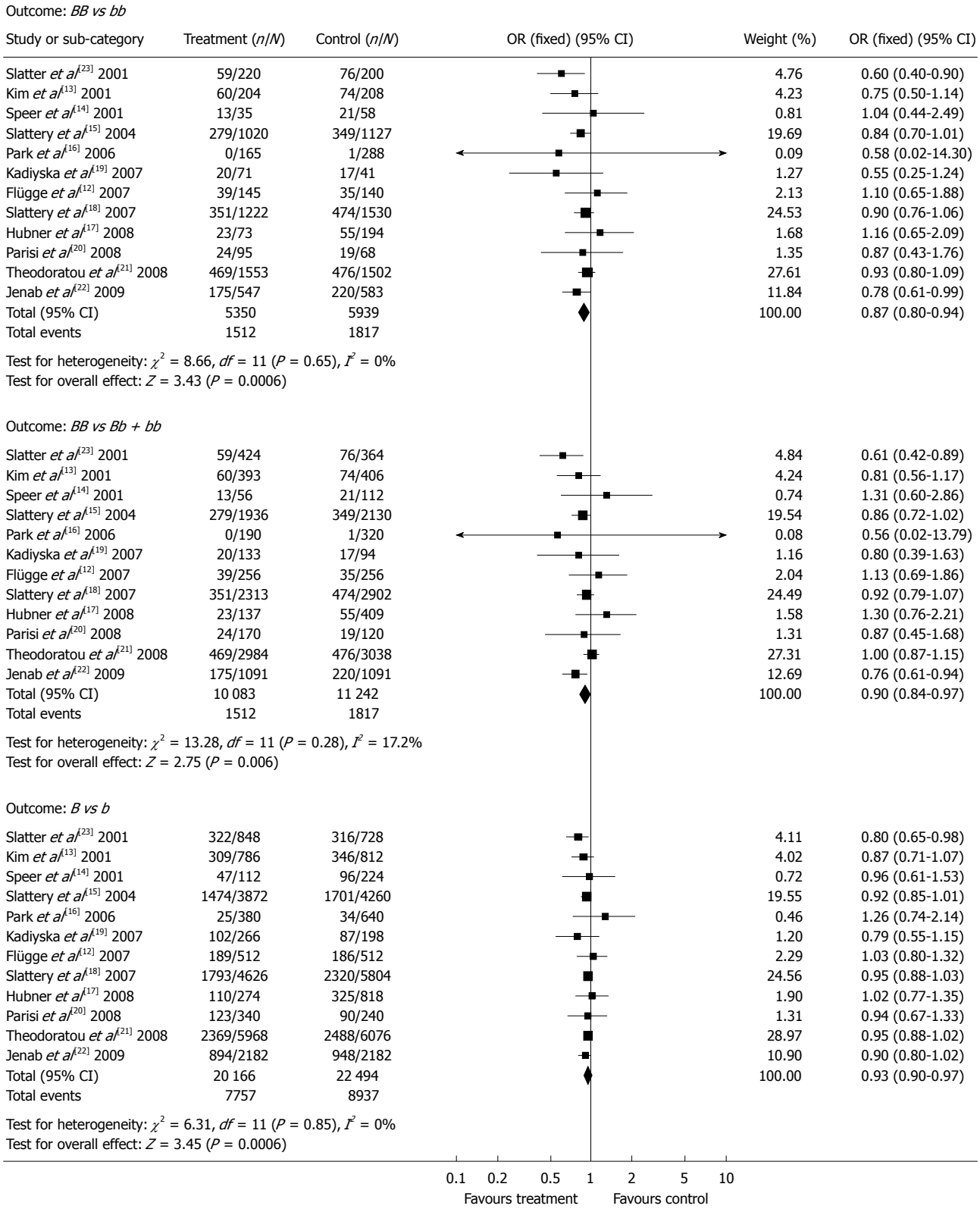

Figure 2 Forest plot of the meta-analysis performed to investigate the association between the *BsmI* polymorphism of the vitamin D receptor gene and colorectal cancer risk (fixed-effects model).

genetic factors may have a more important role in colon cancer than rectal cancer.

Based on the distribution differences observed between cases of colorectal cancer and controls, we hypothesized that the *Bsm*I *B* allele might have a protective effect against tumorigenesis. Correspondingly, of the 12 relevant reports reviewed, 9 studies supported this hypothesis. In these studies, the populations analyzed were from Asia (n = 1), Europe (n = 4), and the United States (n = 4). In addition, Jenab *et al*[22] evaluated different Cau-

casian populations from 23 centers in Denmark, France, Greece, Germany, Italy, the Netherlands, Norway, Spain, Sweden, and the United Kingdom. In this study, the *BB* genotype, rather than the wild-type *bb* genotype, was associated with a reduced risk of colorectal cancer. The *Bsm*I *BB* genotype was also found to be associated with a reduced risk of colorectal cancer among non-aspirin/NSAID users[42]. Thus, multiple lines of evidence support the hypothesis that the *Bsm*I *B* allele mediates a protective effect against the development of cancers in the digestive tract, especially colon cancer.

The *Bsm*I polymorphism is located in the 3' UTR of the *VDR* gene, and does not alter the amino acid sequence of the *VDR* protein. Thus, for a single *Bsm*I polymorphism, there is a low probability that it directly influences *VDR* function[9]. The *Bsm*I polymorphism also does not affect mRNA or protein levels of *VDR*[20], or levels of $25(OH)_2D_3$ or $125(OH)_2D_3$[43]. However, the *Bsm*I site does exhibit strong LD with other *VDR* polymorphisms, including e*Tru9*I, *Apa*I, *Taq*I, and *Poly(A)* microsatellites. Based on these results, it appears that the *Bsm*I polymorphism affects some type of biological function, and these could potentially include regulation of *VDR* transcription, translation, or RNA processing[9]. Other unidentified SNPs in the *VDR* gene, as well as SNPs in other genes such as CYP3A5[44], may also affect the function of the *Bsm*I. Furthermore, patients carrying the *Bsm*I allele have also been shown to have significantly higher levels of *erbB-2* expression, suggesting other tumor-related molecules may also be involved in the function of the *Bsm*I polymorphism[14].

Although the *Bsm*I *B* allele has been associated with a protective effect, the frequency of this effect has been found to be lower in Asian populations than in Caucasian populations. However, this is inconsistent with the incidence of colorectal cancer identified in recent epidemiological data[2]. Moreover, in the meta-analysis performed in the present study, no significant association was found between *VDR* genotypes and the risk of colorectal cancer in group analyses (not shown). In combination, these results suggest that other factors may be involved. For example, environment, food, and lifestyle may play a more significant role, in combination with genetic factors, in the occurrence and development of colorectal cancer than previously thought, which would potentially account for the inconsistent results obtained from previous studies.

COMMENTS

Background

Colorectal cancer is one of the most common cancers worldwide, and its incidence is increasing with each year. However, the underlying etiology of colorectal cancer remains unclear. Several epidemiologic studies have reported that 1, $25(OH)_2D_3$ can reduce the risk of colorectal cancer, and thus, vitamin D receptor (*VDR*), a crucial mediator of the cellular effects of 1, $25(OH)_2D_3$, may play an important role in the occurrence and development of colorectal cancer.

Research frontiers

Recently, several polymorphic variants of the *VDR* gene have been reported to be associated with the risk of colorectal cancer. However, the published findings remain inconsistent. In this study, the authors conducted a systematic meta-analysis to evaluate the evidence regarding this association.

Innovations and breakthroughs

In the present study, all relevant reports published between January 1990 and August 2010 were reviewed, with a focus on five well-characterized polymorphic variants of *VDR*: *Cdx*-2, *Fok*I, *Bsm*I, *Apa*I, and *Taq*I. In the meta-analysis performed, *Bsm*I was found to be associated with colorectal cancer, while the *Cdx*-2, *Fok*I, *Apa*I, and *Taq*I sites did not exhibit any significant association. Moreover, the *Bsm*I '*B*' genotype was associated with a significant decrease in the risk of colorectal cancer, especially colon cancer. Based on these results, it is hypothesized that *Bsm*I may mediate a protective effect on tumorigenesis.

Applications

The results of this meta-analysis have the potential to partly explain the genetics that influence the pathogenesis of colorectal cancer. This study also helps provide a basis for clinical diagnosis and methods for early intervention.

Peer review

The authors performed a systematic meta-analysis of population-based studies to investigate the association between *VDR* polymorphisms and colorectal cancer risk. The authors found a *Bsm*I site in the 3' UTR of the *VDR* gene to be associated with colon cancer risk, and then analyzed the underlying mechanism. The results are interesting and may help explain the genetic mechanism of colorectal carcinogenesis.

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BRIEF ARTICLE

Heme oxygenase-1 prevents liver fibrosis in rats by regulating the expression of PPARγ and NF-κB

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Abstract

AIM: To investigate the effects of heme oxygenase (HO)-1 on liver fibrosis and the expression of peroxisome proliferator-activated receptor gamma (PPARγ) and nuclear factor-kappa B (NF-κB) in rats.

METHODS: Sixty Wistar rats were used to construct liver fibrosis models and were randomly divided into 5 groups: group A (normal, untreated), group B (model for 4 wk, untreated), group C (model for 6 wk, untreated), group D [model for 6 wk, treated with zinc protoporphyrin Ⅸ (ZnPP-Ⅸ) from week 4 to week 6], group E (model for 6 wk, treated with hemin from week 4 to week 6). Next, liver injury was assessed by measuring serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and albumin levels. The degree of hepatic fibrosis was evaluated by measuring serum hyaluronate acid (HA), type Ⅳ collagen (Ⅳ-C) and by histological examination. Hydroxyproline (Hyp) content in the liver homogenate was determined. The expression levels of alpha-smooth muscle actin (α-SMA) in liver tissue were measured by real-time quantitative polymerase chain reaction (RT-PCR). The expression levels of PPARγ and NF-κB were determined by RT-PCR and Western blotting.

RESULTS: The expression of HO-1 increased with the development of fibrosis. Induction of HO-1 by hemin significantly attenuated the severity of liver injury and the levels of liver fibrosis as compared with inhibition of HO-1 by ZnPP-Ⅸ. The concentrations of serum ALT, AST, HA and Ⅳ-C in group E decreased compared with group C and group D ($P < 0.01$). Amount of Hyp and α-SMA in the liver tissues in group E decreased compared with group C (0.62 ± 0.14 *vs* 0.84 ± 0.07, 1.42 ± 0.17 *vs* 1.84 ± 0.17, respectively, $P < 0.01$) and group D (0.62 ± 0.14 *vs* 1.11 ± 0.16, 1.42 ± 0.17 *vs* 2.56 ± 0.37, respectively, $P < 0.01$). The expression of PPARγ at levels of transcription and translation decreased with the development of fibrosis especially in group D; and it increased in group E compared with groups C and D (0.88 ± 0.15 *vs* 0.56 ± 0.19, 0.88 ± 0.15 *vs* 0.41 ± 0.11, respectively, $P < 0.01$). The expression of NF-κB increased with the development of fibrosis especially in group D; and it decreased in group E compared with groups C and D (1.43 ± 0.31 *vs* 1.89 ± 0.29, 1.43 ± 0.31 *vs* 2.53 ± 0.54, respectively, $P < 0.01$).

CONCLUSION: Our data demonstrate a potential mechanism that HO-1 can prevent liver fibrosis by enhancing the expression of PPARγ and decreasing the expression of NF-κB in liver tissues.

Key words: Heme oxygenase-1; Peroxisome proliferator-activated receptor gamma; Nuclear factor-kappa B; Liver fibrosis; Hemin

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INTRODUCTION

Liver fibrosis is the mechanism of compensation and reparation after chronic hepatic injury, which is a necessary pathological stage for the development of chronic hepatitis. Heme oxygenase-1 (HO-1), also known as heat shock protein 32, is a microsomal enzyme and rate-limiting enzyme that catalyzes the degradation of heme into biliverdin, iron atoms and carbon monoxide (CO)[1]. HO-1 and its breakdown products play vital physiological roles in anti-inflammation, anti-oxidation and regulation of apoptosis according to reports[2,3]. Many researchers have recently confirmed that HO-1 has protective effects on liver cells under such conditions as acute liver injury, alcoholic liver disease, liver transplantation and ischemia/reperfusion injury[4-8]. In chronic liver disease, induction of HO-1 is important to prevent the development of liver fibrosis[9]. However, the underlying molecular mechanisms are still unknown.

Peroxisome proliferator-activated receptor (PPAR) is a ligand-activated transcription factor which is widely distributed in tissues[10]. Three PPAR subtypes have been identified, namely α, β and γ. PPARγ is mainly expressed in hepatic stellate cells (HSC). Studies have shown that the expression of PPARγ benefits the maintenance of HSC static phenotype[11]. Up-regulation of PPARγ resulted in a significant reduction of HSC activation, and reversed the development of liver fibrosis.

Nuclear factor-kappa B (NF-κB) is an important nuclear transcription factor, which plays an important role in the regulation of gene transcription such as cytokines, chemokines, adhesion molecules and other inflammatory mediators[12]. Up-regulating the activation of NF-κB promotes HSC proliferation and decreases HSC apoptosis. Therefore, inhibiting the activation of NF-κB resulted in reduction of HSC activation, promoting HSC apoptosis and decreasing extracellular matrix production[13,14].

In the present study, we have evaluated the role of HO-1 in liver fibrosis caused by carbon tetrachloride (CCl_4) in rat models, then observed the expression of PPARγ and NF-κB in liver after up-regulation of HO-1 by ferriprotoporphyrin Ⅸ chloride (hemin) or inhibition of HO-1 by zinc protoporphyrin Ⅸ (ZnPP-Ⅸ) pretreatment, and we finally hypothesize about a potential mechanism for the cytoprotection by HO-1.

MATERIALS AND METHODS

Reagents

ZnPP-Ⅸ is a selective HO inhibitor which can suppress the activity of HO-1 by blocking CO production and restricting the transformation of heme to biliverdin[15]. Hemin is a well-known physiological substrate and potent inducer of HO activity[16]. Both ZnPP-Ⅸ and hemin were purchased from Sigma Chemical Co. (St. Louis, MO, United States). Polyclonal antibodies HO-1 and PPARγ were purchased from Cell Signaling (Danver, MA, United States). Anti-phospho-NF-κB p65 monoclonal antibody was purchased from Santa Cruz (Santa Cruz, CA, United States). Anti-bete actin antibody was purchased from Biogenesis (Bournemouth, United Kingdom). All other chemicals were of analytical grade and commercially available.

Animals and experimental design

Male Wistar rats (Medical University Laboratory Animal Center, Shanxi, China) weighing 220 g-250 g were used to establish fibrogenesis models[17-19]. All procedures used in this study were approved by the Ethics Committee for the use of experimental animals at Shanxi Medical University. All rats were kept at 21 ℃-25 ℃ under a 12 h dark/light cycle, drank normal water and were fed with 79.5% corn meal, 20% lard and 0.5% cholesterol for the first two weeks, then 99.5% corn meal and 0.5% cholesterol thereafter. Sixty rats were randomly divided into five groups (twelve rats/group); groups B, C, D and E received subcutaneous injections of 40% CCL_4 (a mixture of pure CCl_4 and olive oil, 0.3 mL/100 g) every four days for six weeks. The rats in group A were fed with normal diet and received a 0.9% NaCl subcutaneous injection. In the fourth week, Group B and some of group A were killed, while group D and group E began to be peritoneally injected with ZnPP-Ⅸ (20 μmol/kg) or hemin (30 μmol/kg) every other day until the sixth week when they were killed with group C and the remnants of group A. The dose and preparation of ZnPP and hemin solution were based on our preliminary studies and references[20-22]. The numbers of rats were reduced to 11, 9, 9 and 10 in groups B, C, D, and E, respectively, due to deaths during the process. A small portion of the liver was removed for histological analysis by fixation with 10% formalin and subsequent embedding in paraffin. The remaining liver was cut into pieces and frozen in liquid nitrogen and kept at -80 ℃ until it was used for extraction of total RNA and proteins.

Serum biochemical and liver fibrosis indicator measurements

Markers of hepatic damage such as serum alanine aminotransferase (ALT), aspartate transaminase (AST) and albumin (ALB) levels were measured by using an automated biochemistry clinical analyzer (Hitachi, Japan) according to an automated procedure. The levels of serum hyaluronic acid (HA) and type Ⅳ collagen (Ⅳ-C) were determined using Chemiluminescence Quantitative Immunoassay Kit (Beijing Yuande Bio-Medical Engineering Co., Ltd.).

Quantification of hydroxyproline assay

Hydroxyproline (Hyp) content in the liver specimens represented the total amount of collagen in livers, which

was quantified to evaluate the degree of liver fibrosis by using a colorimetric method[23]. Kits for the measurement were purchased from Nanjing Jiancheng Bioengineering. In brief, 100 mg of freeze-dried liver specimens were weighed and tested according to the manufacturer's directions. At the end of the experiment, absorbance of each sample was read at 550 nm using a spectrophotometer. The content was obtained according to a formula and expressed as micrograms Hyp/milligram liver. Each sample was analyzed in triplicate.

Histologic evaluation

Liver tissues were fixed in 10% neutral formalin solution overnight, embedded in paraffin blocks, and then were sectioned at 4 μm thickness for staining with hematoxylin and eosin (HE) or Masson by using standard techniques. The results were analyzed by light microscopy. Representative views of liver sections are shown.

RNA isolation and real-time polymerase chain reaction

Total RNA was extracted from liver tissue using the RNA Trizol isolation reagent kit (Invitrogen, United States). cDNA was obtained by reverse transcription of RNA by using random primer and Moloney murine leukemia virus reverse transcriptase (Gibco BRL, Merelbeke, Belgium). The conditions were 25 ℃ for 5 min, 42 ℃ for 60 min and 70 ℃ for 5 min, finally cooling at 5 ℃ for use. Amplification reactions were performed with a SYBR green polymerase chain reaction (PCR) master mix (Applied Biosystems). Three microliters of diluted cDNA samples were used for quantitative analysis. The cycle conditions were 95Mul for 10 min for denaturation, followed by 50 cycles of 15 s at 95Mul, 30 s at 55Mul and 45 s at 72Mul. Each sample was analyzed in triplicate. The primers were as follows: HO-1, (forward) 5'-CAC GCA TAT ACC CGC TAC CT-3' and (reverse) 5'-AAG GCG GTC TTA GCC TCT TC-3'; PPARγ, (forward) 5'-CCC TGG CAA AGC ATT TGT AT-3' and (reverse) 5'-ACT GGC ACC CTT GAA AAA TG-3'; NF-κB, (forward) 5'-AAC ACT GCC GAG CTC AAG AT-3' and (reverse) 5'-CAT CGG CTT GAG AAA AGG AG-3'; alpha-smooth muscle actin (α-SMA), (forward) 5'-TGT GCT GGA CTC TGG AGA TG-3' and (reverse) 5'-GAA GGA ATA GCC ACG CTC AG-3'; Beta-actin, (forward) 5'-GTC AGG TCA TCA CTA TCG GCA AT-3' and (reverse) 5'-AGA GGT CTT TAC GGA TGT CAA CGT-3'. Beta-actin was used as an internal control.

Western blotting analysis

Proteins were extracted from frozen liver samples with radioimmuno-precipitation buffer containing 50 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, 1% Nonidet P-40, 2 μg/mL aprotinin, 1 mmol/L phenyl-methylsulphonyl fluoride, 1% sodium dodecyl sulfate (SDS) and 0.5% deoxycholate, then centrifuged at 10 000 *g* for 10 min at 4 ℃. Samples were subjected to 12% SDS-polyacrylamide gel electrophoresis and proteins were transferred to nitrocellulose membranes. The membranes were blocked overnight in 5% bovine serum albumin in tris buffer saline Tween20 buffer at 4 ℃, and then incubated with a 1:1000 dilution of anti-HO-1 polyclonal antibody, a 1:1000 dilution of anti-PPARγ polyclonal antibody, a 1:1000 dilution of anti-NF-κB monoclonal antibody or anti-beta-actin monoclonal antibody overnight at 4 ℃. Then the membranes were treated with horseradish peroxidase-conjugated secondary antibody. Blots were visualized using the Super ECL detection kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Relative densities of the bands were analyzed using the Kodak Digital Science Imaging System.

Statistical analysis

Data were expressed as mean ± SE and were analyzed with SPSS Version 13.0. Differences between experimental groups were analyzed using one-way analysis of variance or Student's *t* test. All differences were considered statistically significant when $P < 0.05$.

RESULTS

Expression of HO-1 in the liver of rats in different groups

Long-term application of CCl_4 can induce hepatic fibrogenesis not only in humans but also in rats. We established CCl_4 rat models to evaluate the effect of HO-1 expression on liver fibrogenesis (Figure 1). The process lasted for 6 wk. In this study, we used two opposite reagents, i.e., hemin (induction of HO-1) and ZnPP-IX (inhibition of HO-1) from week 4 to week 6 to observe the regulation and mechanism of HO-1 in rat liver fibrosis. The mRNA and protein expressions of HO-1 in groups B and C were significantly higher than group A and increased with the severity of fibrosis, but all values were lower than in group E ($P < 0.01$); while those in group D were lower than in group C ($P < 0.01$), but still higher than in group A ($P < 0.01$).

Effects of HO-1 expression on rat model of hepatic fibrogenesis

The rat model for group B exhibited inflammatory infiltration, hepatic steatosis and slight fibrosis (Figure 2B and G), while group C showed obvious fibrosis (Figure 2C and H). Treatment with hemin from week 4 to week 6 markedly reduced the severity of hepatic inflammatory infiltration and fibrosis (Figure 2E and J), whereas hepatic steatosis, inflammatory infiltration, especially fibrosis in hepatic portal areas, varying degrees of fibrosis around the central vein and extension to the hepatic lobule were aggravated in groups treated with ZnPP-IX (Figure 2D and I). These results indicate that HO-1 induction could protect rats from CCl_4-induced liver injury and fibrosis.

Effects of HO-1 on the levels of serum ALT, AST and ALB

The levels of serum ALT and AST increased with the development of fibrosis, and were higher in group C than in group B (Table 1). The increase in serum ALT and AST was markedly augmented by ZnPP-IX in group D ($P < 0.01$), but attenuated by hemin in group E com-

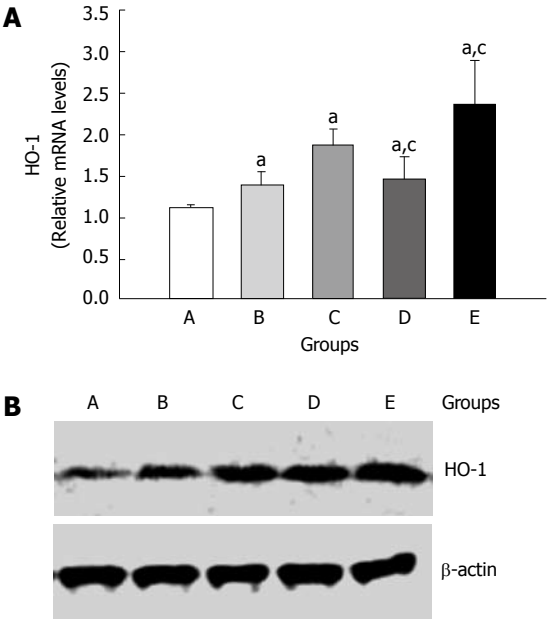

Figure 1 Effects of hemin and zinc protoporphyrin on the expression of heme oxygenase-1 in the liver of rats with fibrosis caused by CCl_4. Group A (normal, untreated); group B (model for 4 wk, untreated); group C (model for 6 wk, untreated); group D (model for 6 wk, treated with zinc protoporphyrin Ⅸ from week 4 to week 6); group E (model for 6 wk, treated with hemin from week 4 to week 6). A: Heme oxygenase-1 (HO-1) mRNA levels, determined by real-time quantitative polymerase chain reaction; B: HO-1 protein levels, detected by Western blotting. Data are expressed as the mean ± SE (n = 4 per group). $^{a}P < 0.01$ *vs* the levels in group A; $^{c}P < 0.01$ *vs* the levels in group C.

pared to group C ($P < 0.01$). The levels of ALB in group E increased significantly compared to group C ($P < 0.01$). Meanwhile, ALB levels decreased in group D but did not differ significantly from group C.

Effects of HO-1 on the expression of fibrosis-related indicators

To evaluate the effect of HO-1 induction on fibrosis, we assessed the expression levels of hepatic fibrosis-related indicators, i.e., HA, Ⅳ-C, Hyp and α-SMA. Rats in group D injected with ZnPP-Ⅸ showed enhanced expression of hepatic α-SMA and Hyp, which correlated with the levels of serum HA and Ⅳ-C. Meanwhile mice in group E injected with hemin exhibited depressed expression of these compared with group C ($P < 0.01$), which still did not recover to the levels of group B ($P > 0.05$) (Figure 3).

Role of HO-1 in the expression of PPARγ and NF-κB at mRNA and protein levels

Studies have shown that up-regulating the activation of PPARγ resulted in a significant reduction of type I collagen and α-SMA expression, inhibited HSC proliferation and even reversed the development of liver fibrosis[2-4]. Studies also have found that up-regulating the activation of NF-κB inhibited HSC apoptosis and promoted the release of inflammatory response factors[24]. To evaluate the mechanism of the effect of HO-1 on fibrosis, we explored the expression of PPARγ and NF-κB at levels of transcription and translation. Unlike the trends of HO-1, real-time PCR and Western blotting showed that the expression of PPARγ decreased with the development of liver fibrosis (Figure 4A). The expression of PPARγ decreased more obviously after application of HO-1 inhibitor in group D as compared with group C ($P < 0.05$). On the contrary, PPARγ increased significantly after pretreatment with hemin in group E, and was higher than group C ($P < 0.01$). However, the expression of NF-κB gradually increased with the development of liver fibrosis, which was consistent with the change of HO-1 (Figure 4B). After using the inhibitor of HO-1 in group D, the expression of NF-κB increased as compared with group C, whereas expression decreased more significantly than group D and even group C when HO-1 was induced in group E ($P < 0.01$).

Table 1 Quantitation of alanine aminotransferase, aspartate aminotransferase and albumin in the serum from different groups of rats (mean ± SE)

Groups	*n*	ALT (U/L)	AST (U/L)	ALB (g/ L)
A	12	20.80 ± 5.49	29.40 ± 4.45	40.2 ± 1.789
B	11	102.00 ± 24.54[b]	122.00 ± 31.43[b]	37.33 ± 2.42
C	9	166.50 ± 19.47[b]	211.83 ± 29.16[b]	28.50 ± 2.59[b]
D	9	265.67 ± 43.61[b,d]	323.83 ± 40.22[b,d]	26.17 ± 2.93[b]
E	10	114.40 ± 22.99[b,d]	152.20 ± 25.65[b,d]	32.80 ± 2.68[b,d]

$^{b}P < 0.01$ *vs* the levels in group A; $^{d}P < 0.01$ *vs* the levels in group C. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALB: Albumin.

DISCUSSION

Liver fibrosis is the mechanism of compensation and reparation after chronic hepatic injury, which is a necessary pathologic stage from chronic hepatitis to cirrhosis. Previous studies have found that 25%-40% of liver fibrosis will eventually develop to cirrhosis and even liver cancer[25]. Therefore, it is essential to further clarify the mechanism of liver fibrosis in order to block and reverse the process of liver disease. We constructed liver fibrosis models in rats by using composite factors which had been confirmed successfully in the Department of Pathophysiology, Shanxi Medical University[18,19]. At the fourth week we observed inflammatory infiltration, hepatic steatosis and slight fibrosis in livers, and obvious liver cirrhosis could be seen at the sixth week.

HO-1 is the rate-limiting enzyme for heme degradation in a wide range of human and mammalian tissues. Prior clinical and animal research has confirmed that an external irritant could up-regulate the expression of HO-1 with increasing levels of oxygen-derived free radicals in cells[26]. It has previously been reported that induction of HO-1 is an important defense mechanism against many kinds of liver injuries. In chronic liver disease, especially liver fibrosis, induction of HO-1 can reduce the secretion of type I collagen, thus effectively preventing the development of liver fibrosis[27-30]. In this study, we also examined the effects of HO-1 inhibitor or inducer, and found that with the development of liver fibrosis, the expression of HO-1 was significantly

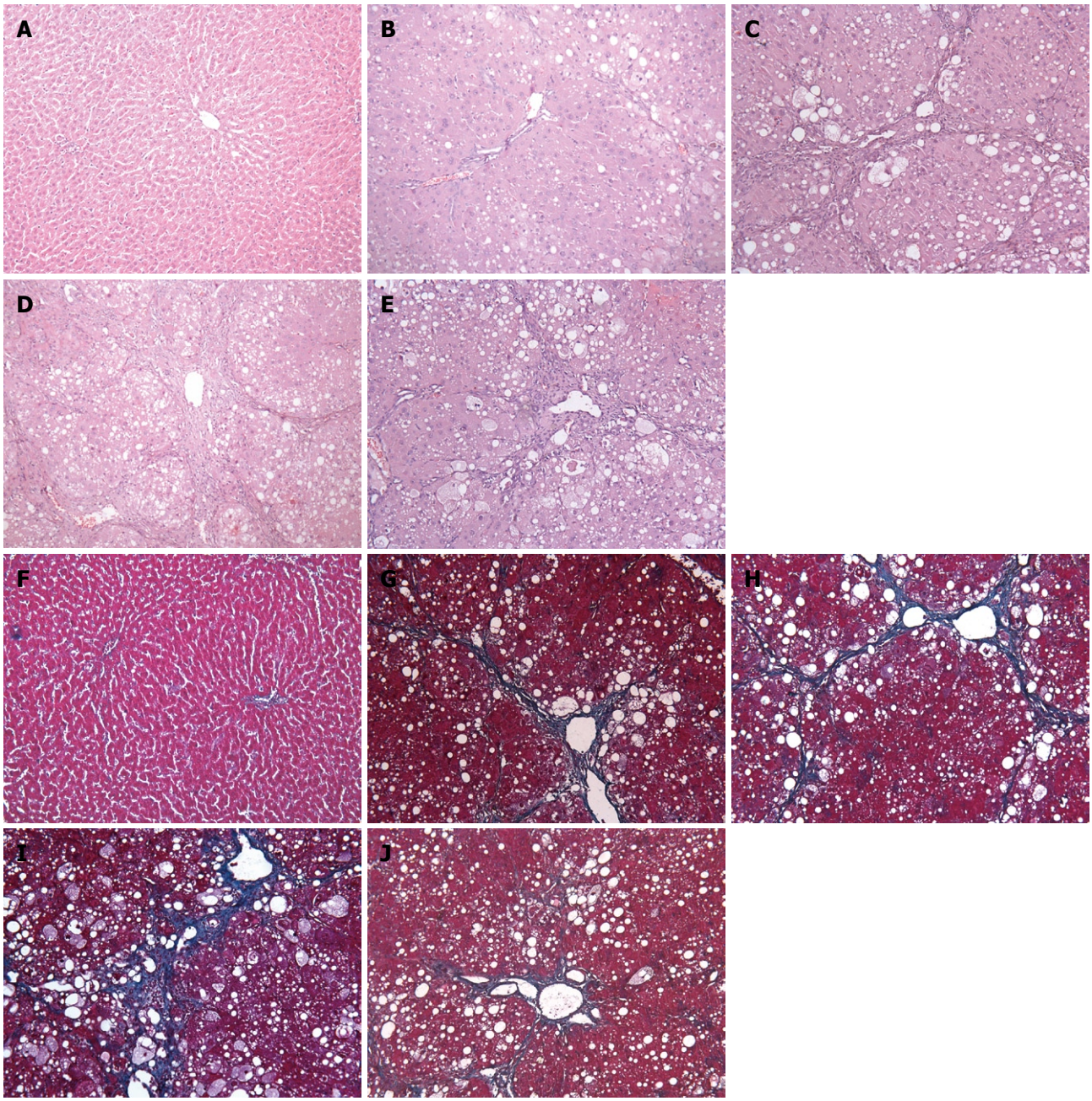

Figure 2 Effects of heme oxygenase-1 on the histopathology of rat liver fibrosis induced by CCl_4. A, F: Group A (normal, untreated); B, G: Group B (model for 4 wk, untreated); C, H: Group C (model for 6 wk, untreated); D, I: Group D (model for 6 wk, treated with zinc protoporphyrin Ⅸ from week 4 to week 6); E, J: Group E (model for 6 wk, treated with hemin from week 4 to week 6). A-E: Hematoxylin and eosin staining (magnification × 200); F-J: Masson staining of collagens (magnification × 200).

enhanced in liver of rats, whereas hemin pretreatment made this induction more prominent. We analyzed the biochemical parameters reflecting liver damage related to function and structure, such as serum ALT and AST levels, which indicated a remarkable decrease after HO-1 induction. Liver histopathology also clearly showed that HO-1 induction markedly reduced the severity of hepatic inflammatory infiltration and fibrosis. To further validate the protection by HO-1, we pretreated rats with concomitant ZnPP-Ⅸ (a competitive HO-1 inhibitor) and observed that the liver damage was more serious than in the control group and hemin-treated group. Then we assessed the levels of HA and Ⅳ-C in serum and detected the content of Hyp and α-SMA mRNA in rat liver tissues of the different groups, in order to determine the proliferation levels of fibrosis. Results showed that the induction of HO-1 could reduce all the biochemical indicators of fibrosis and attenuate the degree of fibrosis detected pathologically, while the inhibitor of HO-1 caused an opposite result. Taken together, we concluded that induction of HO-1 in hepatic tissues could produce anti-inflammatory effects and slow the process of liver fibrosis effectively; however the inhibition of HO-1 could enhance the liver fibrosis.

PPAR, including α, β and γ subtypes, are new steroid hormone receptors and ligand-activated transcription

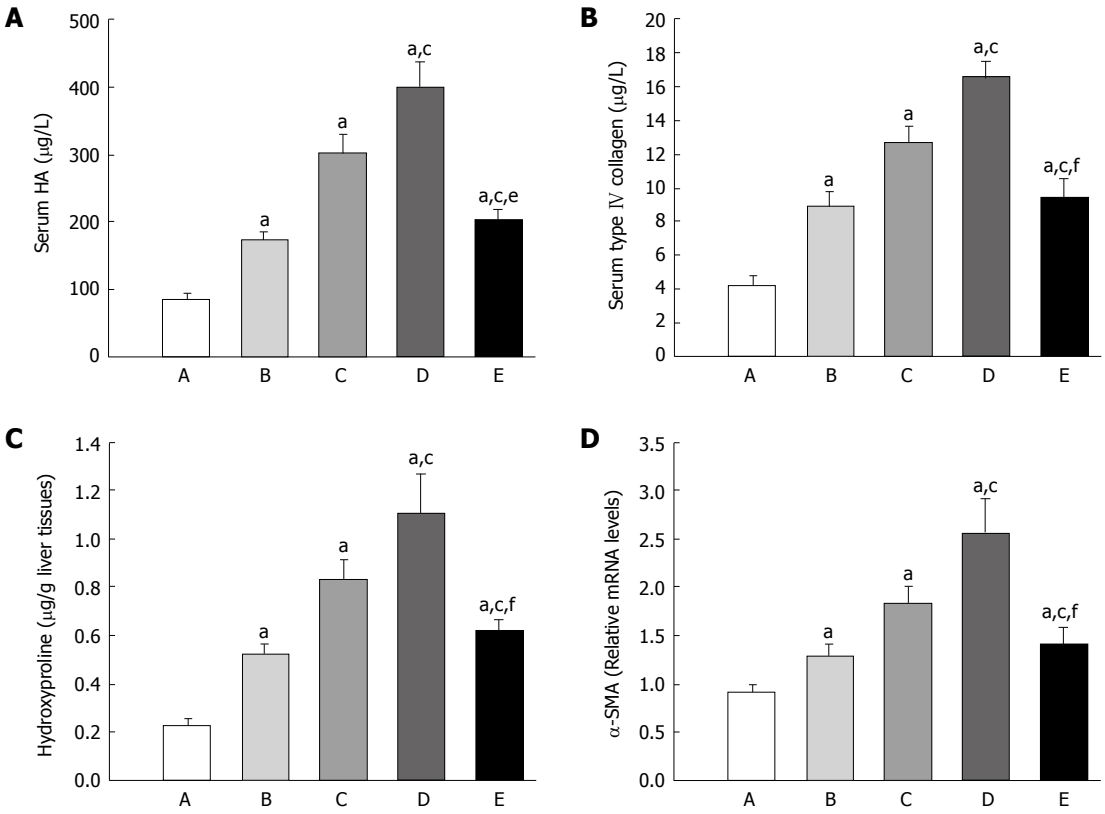

Figure 3 Effects of heme oxygenase-1 on hyaluronate acid, type Ⅳ collagen, hydroxyproline and α-smooth muscle actin expression. A: Levels of serum hyaluronate acid (HA); B: Levels of serum type Ⅳ collagen (Ⅳ-C); C: Quantity of liver hydroxyproline; D: Levels of liver alpha-smooth muscle actin (α-SMA) mRNA. Values are expressed as mean ± SE. $^{a}P < 0.01$ *vs* the levels in group A; $^{c}P < 0.01$ *vs* the levels in group C; $^{e}P < 0.05$ *vs* the levels in group B; $^{f}P > 0.05$ *vs* the levels in group B.

factors. PPARγ plays an important role in many biological processes, including adipogenesis, inflammatory reaction, cell growth regulation and cell differentiation[31]. The expression of PPARγ is beneficial in maintaining the quiescent phenotype of HSC; however the inhibition of PPARγ may be an early event in HSC transformation from quiescent to activated state[11]. Studies found that the PPARγ agonist rosiglitazone could be used to increase the expression of PPARγ in activated HSC, which could reduce oxidative stress, decrease the expression of α-SMA and the synthesis of type I collagen, inhibit cell proliferation and promote cell apoptosis[32]. Recent studies also found that PPARγ activation reduced TGF-β1-induced CTGF expression at both transcriptional and posttranscriptional levels in HSC[33]. Enhancement of PPARγ activity might interrupt the signaling pathways for platelet-derived growth factor and epidermal growth factor, and then suppress hepatic fibrogenesis[34].

NF-κB is a nuclear transcriptional activator that plays a central role in stress response and inflammation[35]. Activation of NF-κB can promote HSC proliferation, reduce HSC apoptosis and increase the production of collagen and inflammatory chemokines in the process of liver fibrosis. But inhibiting the activation of NF-κB can induce apoptosis of HSC. Studies have found that a decrease of PPARγ was accompanied with an increase of NF-κB in lung tissues, which played an important role in the development of lung fibrosis[36]. PPARγ can inhibit the transcription and DNA synthesis of NF-κB by binding p50/p65 subunits to form transcriptional repressor complexes directly or by binding p300 and CBP co-activating factors to inhibit the transcription and expression of NF-κB competitively[37]. Other research showed that a specific inductor of PPARγ such as troglitazone could interfere with NF-κB signaling pathway by activating PPARγ[38].

Studies in other areas have shown that co-regulation exists between HO-1 and PPARγ. Research regarding the interaction of HO-1 and PPARγ in human vascular endothelial cells demonstrated that HO-1 enzymatic activity mediated antiinflammatory and antiproliferative effects exerted by PPAR ligands, and that a clinically relevant (GT)n dinucleotide length polymorphism within the human HO-1 promoter significantly influenced the transcriptional regulation of HO-1 by both PPARα and PPARγ[39]. Li *et al*[40] reported that induction of HO-1 could mediate the effect of PPARγ in suppressing the proliferation of rat pulmonary artery smooth muscle cells, but that this effect was blocked by knockdown of HO-1 through siRNA transfection. Recent studies demonstrated that HO-1, as an identifier of novel trophoblast invasion-related genes, controlled motility *via* up-regulation of PPARγ; researchers found that up-regulation of PPARγ protein and activity by HO-1 was required to down-regulate cell motility, but blocking of PPARγ largely abolished the effect of HO-1[41]. Studies also found that there was an NF-κB binding site in the HO-1 promoter region. The activity of HO-1 was directly related to NF-κB[42]. HO-1 played an important

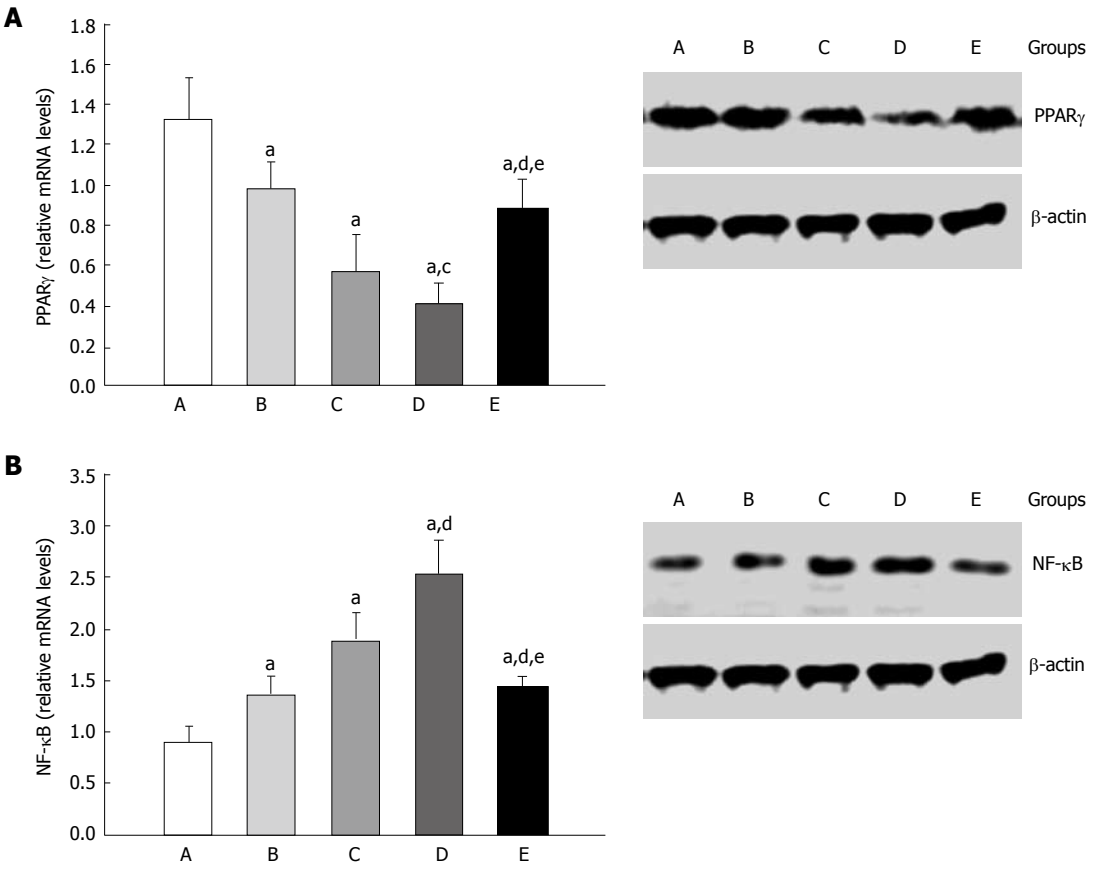

Figure 4 Levels of peroxisome proliferator-activated receptor gamma mRNA and nuclear factor-kappa B mRNA in rat liver analyzed by real-time polymerase chain reaction assay and associated protein assessed by Western blotting. A: Peroxisome proliferator-activated receptor gamma (PPARγ) mRNA and protein. The molecular weight of PPARγ is 55 kD; B: Nuclear factor-kappa B (NF-κB) mRNA and protein. The molecular weight of NF-κB is 50 kD. β-Actin was used as an invariant control. Values are expressed as mean ± SE (n = 4 per group). $^{a}P < 0.01$ *vs* the levels in group A; $^{c}P < 0.05$ *vs* the levels in group C; $^{d}P < 0.01$ *vs* the levels in group C; $^{e}P < 0.01$ *vs* the levels in group D. Control for equal loading.

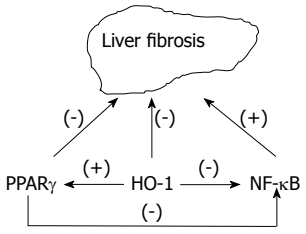

Figure 5 The regulatory pathway between heme oxygenase-1 and peroxisome proliferator-activated receptor gamma or nuclear factor-kappa B in liver fibrosis of rats. (+) indicate promoting; (-) indicate inhibiting. PPARγ: Peroxisome proliferator-activated receptor gamma; HO-1: Heme oxygenase-1; NF-κB: Nuclear factor-kappa B.

role in the down-regulation of NF-κB activation. Yeh *et al*[43] showed that HO-1 activation could attenuate the surge of inflammation-related cytokines and decrease the occurrence of cardiomyocytic apoptosis *via* inhibition of NF-κB and AP-1 translocation. Liu *et al*[44] indicated that up-regulation of HO-1 could alleviate severe acute pancreatitis-associated lung injury in rats by decreasing NF-κB activity drastically and inhibiting the serum levels of tumour necrosis factor alpha (TNF-α) and interleukin-6 significantly. Overexpression of HO-1 could protect against TNF-α-mediated airway inflammation by diminishing NF-κB activation in both cultured human tracheal smooth muscle cells and the airways of mice[45].

In this study, we found that the expression of PPARγ was decreased in group D, which was treated with ZnPP-IX to inhibit the expression of HO-1, whereas the expression of NF-κB was increased. But PPARγ was overexpressed in group E by treating with hemin to induce the expression of HO-1, whereas the expression of NF-κB was reduced. By examining the HE stained liver histology, we found that the degree of liver fibrosis was significantly higher in group D than in groups C and E. Masson staining for collagen showed the same results: that the collagen content in group D was significantly increased compared to group C, but was markedly reduced in group E compared with groups D and C. Thus we hypothesize that induction of HO-1 could alleviate the liver injury and reverse the process of liver fibrosis by up-regulating the expression of PPARγ and down-regulating the expression of NF-κB, and then affect the releasing of inflammatory cytokines such as TNF-α in NF-κB-related signal pathways or induce HSC apoptosis. Among these, the inhibition of NF-κB may be regulated directly by HO-1 on the one hand, or on the other hand be regulated by the expression levels of PPARγ which are associated with the expression of HO-1 (Figure 5). In conclusion, our data

demonstrate that the ability of HO-1 to alleviate liver fibrosis is correlated with the regulation of PPARγ and NF-κB, which construct a complex network system. Further study with regard to this mechanism will help us to form new strategies for the effective treatment of liver fibrosis.

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COMMENTS

Background

Heme oxygenase-1 (HO-1) is a microsomal enzyme and rate-limiting enzyme. HO-1 and degradation products are important defense mechanisms against many kinds of liver injuries. In chronic liver disease, induction of HO-1 is important to prevent the development of liver fibrosis effectively. However, the underlying molecular mechanisms are still unknown.

Research frontiers

Peroxisome proliferator-activated receptor gamma (PPARγ) is a ligand-activated transcription factor which benefits the maintenance of the hepatic stellate cell (HSC) static phenotype. Nuclear factor-kappa B (NF-κB) plays an important role in the regulation of gene transcription which can promote HSC proliferation and decrease HSC apoptosis, then aggravate liver fibrosis. Studies have shown that co-regulation exists between HO-1 and PPARγ. HO-1 can mediate the effect of PPARγ. Studies have also found that there is an NF-κB binding site in the HO-1 promoter region. HO-1 plays an important role in the down-regulation of NF-κB activation.

Innovations and breakthroughs

In this study, by establishing a model of liver fibrosis in rats, the authors attempted to investigate the effects of HO-1 on liver fibrosis, and to evaluate whether the role of HO-1 in liver protection was achieved by regulating the expression of PPARγ and NF-κB, which are both important in the process of liver fibrosis.

Applications

This study further clarified one of the mechanisms of HO-1 in liver protection, which could help provide a new strategy for treating liver fibrosis.

Terminology

HO-1, also known as heat shock protein 32, is a microsomal enzyme and rate-limiting enzyme that catalyzes heme degradation into biliverdin, iron atoms and carbon monoxide. HO-1 and its breakdown products play vital physiological roles in anti-inflammation, anti-oxidation and regulation of apoptosis according to reports. PPAR is a ligand-activated transcription factor which is widely distributed in the tissues. Three PPAR subtypes have been identified, namely α, β and γ. NF-κB is an important nuclear transcription factor, which plays an important role in the regulation of gene transcription such as cytokines, chemokines, adhesion molecules and other inflammatory mediators. Zinc protoporphyrin-Ⅸ is a selective HO inhibitor which can suppress the activity of HO-1 by blocking carbon monoxide production and restricting the transformation of heme to biliverdin. Hemin is a well-known physiological substrate and potent inducer of HO activity.

Peer review

The authors explored the protective effect of HO-1 in the CCl_4 rat model of liver fibrosis. In order to determine the mechanism of such protection, the authors evaluated the expression of two important transcription factors, PPARγ and NF-κB. These transcription factors are involved in regulation of hepatic stellate cell activation, the primary cell responsible for liver fibrosis. This study proposes a pathway for the protective action of HO-1 in liver fibrosis. If reproduced by other investigators, this pathway could provide information that helps in designing new therapies for prevention and treatment of liver fibrosis.

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BRIEF ARTICLE

Treatment of cholecystitis with Chinese herbal medicines: A systematic review of the literature

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Abstract

AIM: To analyze the literature on the use of Chinese herbal medicines for the treatment of cholecystitis.

METHODS: The literature on treatment of cholecystitis with traditional Chinese medicines (TCM) was analyzed based on the principles and methods described by evidence-based medicine (EBM). Eight databases including MEDLINE, EMbase, Cochrane Central (CCTR), four Chinese databases (China Biological Medicine Database, Chinese National Knowledge Infrastructure Database, Database of Chinese Science and Technology Periodicals, Database of Chinese Ministry of Science and Technology) and Chinese Clinical Registry Center, were searched. Full text articles or abstracts concerning TCM treatment of cholecystitis were selected, categorized according to study design, the strength of evidence, the first author's hospital type, and analyzed statistically.

RESULTS: A search of the literature published from 1977 through 2009 yielded 1468 articles in Chinese and 9 in other languages; and 93.92% of the articles focused on clinical studies. No article was of level Ⅰ evidence, and 9.26% were of level Ⅱ evidence. The literature cited by Science Citation Index (SCI), MEDLINE and core Chinese medical journals accounted for 0.41%, 0.68% and 7.29%, respectively. Typically, the articles featured in case reports of illness, examined from the perspective of EBM, were weak in both quality and evidence level, which inconsistently conflicted with the fact that most of the papers were by authors from Level-3 hospitals, the highest possible level evaluated based on their comprehensive quality and academic authenticity in China.

CONCLUSION: The published literature on TCM treatment of cholecystitis is of low quality and based on low evidence, and cognitive medicine may functions as a useful supplementary framework for the evaluation.

Key words: Cholecystitis; Traditional Chinese medicine; Literature analysis; Randomized controlled trials; Cognition-based medicine

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INTRODUCTION

Cholecystitis, defined as a type of acute or chronic inflammation occurring in the gallbladder caused by infection, bile stimulus, reflux of pancreatic juice to the biliary passage, as well as bilirubin and lipoid metabolic disorders *etc*. Cholecystitis is often secondary to previously asymptomatic gallstone disease[1]. Around 90%-95% of cholecystitis cases are claimed to be caused by gallstone disease, the incidence of which is 8%-10% in America and 3%-11% in China[2,3]. Recent epidemiological studies have shown that the incidence of cholelithiasis has been continuously rising, and the rate is doubling every 10 years[4]. The incidence rate of cholelithiasis grows steadily with age, varies by race, and occurs more frequently in female patients than in male patients[2].

For symptomatic cholecystitis, antibiotics and antispasmodic treatment are conventional therapy while cholecystectomy or laparoscopic cholecystectomy are also appropriate modalities of treatment[5]. However gallstone disease of this type may recur within several months. Gallstones may also recur in the biliary tract after cholecystectomy[2]. Therefore, it is important to identify effective treatment options and adjuvant therapeutic methods for cholecystitis. Traditional Chinese medicines (TCM) has a long history of use for treating cholecystitis and has developed an intecrate system of medical examination and treatment. Classic TCM works such as Huang Di Nei Jing and Shang Han Za Bing Lun have both expounded on this disease in depth. In TCM, cholecystitis is categorized as a type of illness with symptoms such as aching over the lateral torso, jaundice, hepatic distention, gallbladder distention and abdominal pain, *etc*[6,7]. Cholecystitis is considered by TCM to be caused mainly by unrestrained food and drink, exogenous heat and moisture, chronic illness and/or injury[8].

The large quantity of research literature on the TCM treatment of cholecystitis in China stimulates the development of innovative and improved therapeutic methods for the treatment of the disease. However, even basic information about the literature such as the level of evidence, quantity, trends in publication, and existence of research institutes remains unclear since they have not been sufficiently studied or evaluated outside of China due to barriers by language and access. Thus, a comprehensive analysis of this large quantity of literature is urgently required.

Based on the principles and methods described by evidence-based medicine (EBM), this study conducted an examination and statistical analysis of current literature on the treatment of cholecystitis with TCM, aiming to discuss the necessity for a systematic review as well as producing a reference to enable better research of TCM.

MATERIALS AND METHODS

Literature search

Electronic literature searches were conducted on the following databases: China Biological Medicine Database (CBM), Chinese National Knowledge Infrastructure Database (CNKI), Database of Chinese Science and Technology Periodicals (VIP), Database of Chinese Ministry of Science and Technology (Wanfang), The Cochrane Central Register of Controlled Trials (CENTRAL), MEDLINE (*via* PubMed) and EMbase. The databases were searched from the earliest possible date until June 1, 2009. The search terms included ("Cholecystitis" or "Acalculous Cholecystitis" or " Emphysematous Cholecystitis" or "Cholecystitis, Acute" or "Cholecystitis, Chronic") and ("Chinese herbs" or "TCM" or "Chinese medicine" or "Integrated TCM WM" or "herb" or "herbs" or "traditional Chinese medicine" or" Drugs, Chinese Herbal"). The search terms were adjusted depending on the database being searched. Titles and abstracts of all citations were screened independently by two reviewers (Dong ZY and Wang GL).

Literature selection criteria

Articles on TCM treatment of cholecystitis were included; and articles on cholecystitis treated by integrated traditional Chinese and modern medicine were included.

Data acquisition and quality assessment

The full-text of articles that met all the selection criteria was retrieved. The data were screened independently by two reviewers (Dong ZY and Wang GL) using a self-made data extraction form which collected the following information: year of publication, first author, the organization of the first author, the hospital level of the first author, titles of authors, study design, type of article, journal name, and indexed/citation situation by medical indexing databases. The first author of each article was contacted if there were any missing data. Articles that did not meet the inclusion criteria were excluded by reading the titles and summaries. Disagreements whether a paper was to be included were resolved by discussion.

Methodology of data classification

Methodology of data classification were listed below. (1) Classified by types of study[9]; (2) Classified according to indexed/citation situation (evaluated according to 2008 edition of "Guide to the Core Journals", and the list of MEDLINE contains Chinese journals 2008)[10,11]; (3) Classified according to the first authors' hospital-level[12]: Level-3 hospital - The national, provincial, municipal large hospitals and affiliated hospitals of medical colleges; Level-2 hospital - General hospitals of cities, counties, districts, hospitals affiliated to factories, mining enterprises and institutions; Level-1 hospital - Country-

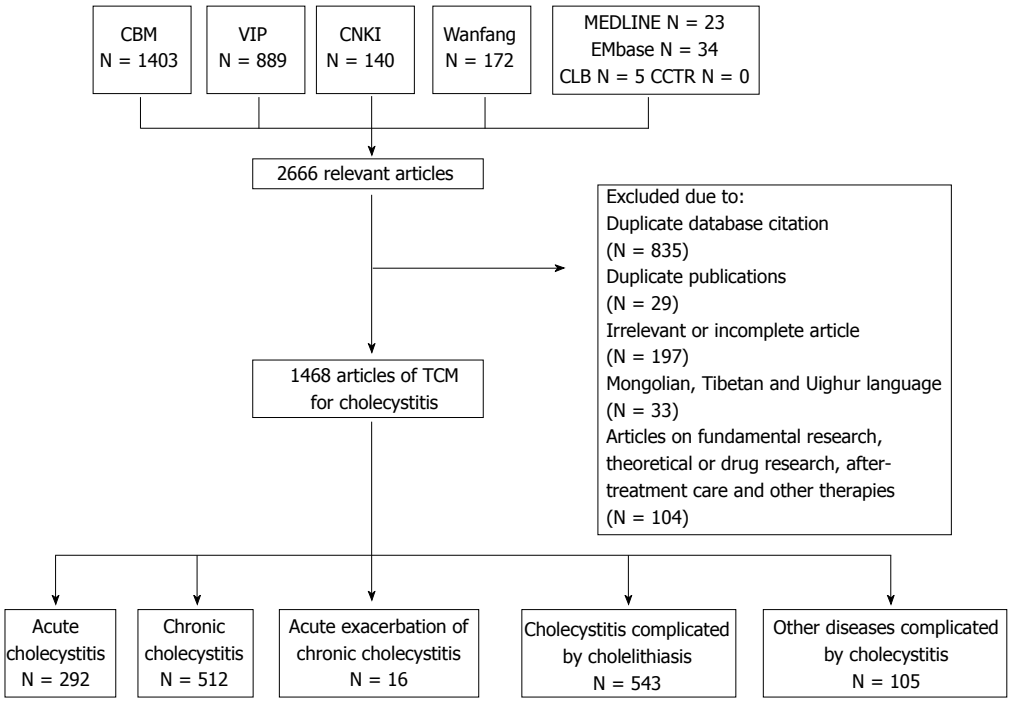

Figure 1 Prism flow diagram. CBM: China Biological Medicine Database; VIP: Database of Chinese Science and Technology Periodicals; CNKI: Chinese National Knowledge Infrastructure Database; CLB: The Cochrane Library; CCTR: Cochrane Central; TCM: Traditional Chinese medicines; N: Number.

side primary hospitals, townships or neighborhood community and private clinics and hospitals; and (4) Classified according to the strength of evidence (Grading quality of evidence and strength of recommendations, strength grading standards by Cochrane Collaboration)[13].

Statistical analysis

Statistical data were collected and recorded in Excel and SPSS 17.0. Percentages, percentage bar charts and trends lines were produced to analyze the situation and trends, while the constituent ratios were expressed in terms of percentages in order to perform a descriptive analysis.

RESULTS

Results of literature screening

Altogether 2666 potentially relevant articles were retrieved from eight databases, among which 1822 were written in Chinese and 835 were duplicate citations between databases. Twenty-nine duplicate articles were excluded; there were 197 irrelevant or incomplete articles; 33 articles were written in Mongolian, Tibetan and Uighur; and 104 articles were on fundamental research, theoretical or drug research, after-treatment care and other therapies. Thus, there were 1468 articles on the TCM treatment of cholecystitis in total. Among these articles, there were 292 concerning cholecystitis, 512 regarding chronic cholecystitis, 16 regarding acute exacerbation of chronic cholecystitis, 543 regarding cholecystitis complicated by cholelithiasis, and 105 on other diseases complicated by cholecystitis. Among the retrieved articles, 9 were foreign articles, including 2 in Bulgarian, 1 in Russian and 6 in English (Figure 1).

We also searched Evidence Based Complementary and Alternative Medicine (eCAM), The American Journal of Chinese Medicine (AJCM), Journal of Chinese Integrative Medicine (J Chin Integr Med), Chinese Journal of Integrated Traditional and Western Medicine and Alternative Medicine Review (Altern Med Rev). No relevant articles were found in any of these sources.

Literature type

In total, 1468 articles on TCM treatment of cholecystitis were retrieved: 15 articles on treatment and care, 24 on animal and fundamental experimental research, 25 on theoretical research, 9 on relevant drug research and 22 on other therapies including massage, ear points, diet, infrared ray, acupuncture and ultrasonic therapy, *etc*. The data revealed that researches on clinical treatment covered the majority of the relevant literature, with the percentage as high as 93.92%, and that TCM was applied to treat almost all types of cholecystitis (Table 1).

Evidence level of the literature

The 1468 articles were categorized according to the Cochrane collaboration criteria as shown in Table 2. No article was included into the category of the highest evidence strength, namely level Ⅰ, while 136 (9.26%) articles were categorized into level Ⅱ, 101 articles (6.88%) into level Ⅲ, 961 articles (65.46%) into level Ⅳ; and 270 articles (18.39%) into level Ⅴ. This revealed that, with randomized controlled trial (RCT) forming a low percentage, the evidence level of the research literature on TCM treatment of cholecystitis appears to be relatively low, requiring a further systematic evaluation of RCT in

Table 1 Literature type

	Acute cholecystitis	Chronic cholecystitis	Acute exacerbation of chronic cholecystitis	Cholecystitis complicated by cholelithiasis	Other diseases complicated by cholecystitis	Total
Clinical trial study	292	512	16	543	105	1468
Treatment and care	3	0	0	12	0	15
Animal and fundamental experimental research	3	3	0	17	1	24
Theoretical research	3	10	0	10	2	25
Other therapies	3	12	0	7	0	22
Relevant drug research	2	4	1	2	0	9
Total	306	541	17	591	108	1563

Table 2 Evidence strength analysis

	Acute cholecystitis (H/M)	Chronic cholecystitis (H/M)	Acute exacerbation of chronic cholecystitis	Cholecystitis complicated by cholelithiasis (H/M)	Other diseases complicated by cholecystitis (H/M)	Total	Evidence level
Systemic review	0	0	0	0	0	0	I
RCT	35 (2/2)	62 (6/0)	6 (1/0)	28 (1/1)	5 (1/0)	136	II
Case-control	19 (1/0)	40 (2/1)	1	37 (4/1)	4 (1/0)	101	III
Case report	203 (9/3)	336 (26/0)	7 (1/0)	344 (27/1)	71 (7/0)	961	IV
Experience reports/ Masters experience	31 (2/0)	54 (4/0)	2	109 (7/2)	20 (1/0)	216	V
Review	5 (1/0)	19	0	25 (2/0)	5 (1/0)	54	V
Total	293	511	16	543	105	1468	

H: Core Chinese Journals; M: MEDLINE; Foreign literatures number = 9: 2 pieces of Literatures in Bulgarian, 1 in Russian, 6 in English (SCI), 1 RCT Chronic Cholecystitis, 2 case reports, 1 case comparison, 1 review (cholecystitis), 1 case report (other complicated cholecystitis). RCT: Randomized controlled trial.

Table 3 Distribution of the first authors' hospital level

	Acute cholecystitis	Chronic cholecystitis	Acute exacerbation of chronic cholecystitis	Cholecystitis complicated by cholelithiasis	Other diseases complicated by cholecystitis	Total
Level-3 hospitals	107	149	10	163	35	464
Level-2 hospitals	106	197	5	193	37	538
Level-1 hospitals and others	76	132	1	163	28	400
Medical schools and universities	4	26	0	16	5	51
Research institutes	0	7	0	6	2	15

order to determine the efficacy and safety of the TCM treatment of cholecystitis.

General status of the literature included by databases

Among the 1477 articles on TCM treatment of cholecystitis both in Chinese and English, 107 (7.24%) were included by core Chinese journals. The 10 articles included in MEDLINE and the 6 in SCI represented 0.68% and 0.41% of the articles, respectively. This reflects a seemingly inadequate writing quality, low research level of the literature, and a generally low international recognition.

Distribution of the first authors' hospitals

The 1468 Chinese articles on TCM treatment of cholecystitis were categorized according to the first authors' hospital levels as shown in Table 3. Among the literature, authors from level-3 hospitals contributed 464 articles (31.61%); level-2 hospitals 538 (36.65%); medical schools and universities 51 (3.47%); research institutes 15 (1.02%); and level-1 hospitals, township hospitals and private clinic/hospitals contributed 400 (27.25%). This shows that the authors of the research literature in this study mainly came from level-3 and level-2 hospitals, which accounted for 68.26% of the total. The RCT distributed as such: level-3 hospitals contributed 62 articles (45.59%); level-2 hospitals 43 (31.62%), level-1 hospitals and others 25 (18.38%); medical schools and universities 6 (4.41%) (Figure 2). Literature with a higher evidence level was also mainly contributed by authors from level-3 hospitals and medical schools/universities.

DISCUSSION

From the above analysis, two major weaknesses were revealed by the selected literature on TCM treatment of cholecystitis. The first one is that only few papers were

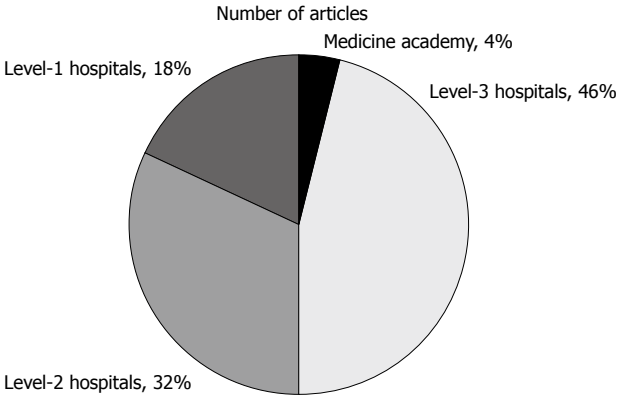

Figure 2 Distribution of the hospitals where the first authors of the randomized controlled trial research on traditional Chinese medicines treatment of cholecystitis come from.

included by SCI and MEDLINE. Although it is not appropriate to evaluate the quality of papers merely according to whether they are included by SCI, MEDLINE and other core medical databases, it is an objective index to certain degrees, based on the strict selection rules and expert evaluation system of SCI and MEDLINE[14].

The second weakness of the literature in general lies in its low evidence level. The concept of evidence levels originated from the emergence and development of EBM, and was first proposed by the clinical epidemiologist Dave Sackett from McMaster University of Canada in 1990[15-17]. High-quality papers and RCTs require an adequate high-quality research platform. Is the large quantity of papers with low evidence level given rise to by an inadequate platform for the research of TCM? To answer this question, after analyzing the distribution of the hospitals, institutions or universities of the first authors, we discovered that 35.08% of the papers were contributed by authors from level-3 hospitals and universities. This accounts for all the articles included by SCI or published in core journals not included in SCI, while 64.92% of the articles were from level-1 and level-2 hospitals with a relatively low research capacity, which indicates a close correlation between the quality of published papers and the research platform. We also found from the data that RCTs hold only less than 10% of all TCM clinical studies, which is an outstanding difference compared to the 70% share of RCTs in the clinical study of modern medicine. Why such a low percentage TCM clinical study is from RCTs is a significant question that deserves further consideration[18].

EBM is a young discipline; the first International EBM Research Center was established in the United Kingdom in 1993, and the first EBM Research Center in China was set up three years later and quickly motivated the spread of EBM theory within China. Although the preference of Chinese TCM researchers for clinical study models somewhat limits the strict RCT research to a certain degree, TCM has its own unique treatment models and standards which deserves proper recognition. For example, it advocates individualized treatment and medication. Namely, different treatment methods and medication might be applied to the same disease and even the same syndrome according to different physical conditions of the individual patient or even according to the different time and place that the illness occurs. This makes it hard for TCM to conduct a high-quality RCT which might be the major cause resulting in the low evidence level of these Chinese papers.

By examining the literature on the treatment of cholecystitis with TCM according to the principles of EBM, we hope to expose the problems and weakness in current TCM clinical studies so as to raise the quality of TCM research.

Here arises the question, how to evaluate the therapeutic effectiveness of TCM more scientifically? This is the right and urgent question that not only TCM but also the entire alternative and complementary medicine should address. EBM experts are trying to further perfect the research standard of RCTs and drafting research guidelines that can better meet the characteristics of TCM. Besides, more scholars are trying to improve the present frame of EBM. Professor Keine[18] from the Institute for Applied Epistemology and Medical Methodology in Germany has conducted a study of cognition-based medicine. Cognition-based medicine is a newly-developed methodological system of scientific medicine. Its primary element is the criteria-based assessment of therapeutic causality at the level of the individual patient[19]. Principles and criteria of single-case causality assessment have been analyzed and explained. Cognition-based medicine enables a methodological professionalization of clinical judgment as well as the explication of physician experience and expertise. Cognition-based medicine study design expands the current range of clinical research, extending from criteria-based causality assessment in single cases to new forms of cohort evaluations. Though cognition-based medicine studies only started recently, this trend is inspiring and promising. It will not only facilitate the evaluation of TCM, which greatly emphasize individualized medical treatment solution, but also accord with the trend of medical development which stresses the significance of individualized treatment, and cognition-based medicine, a beneficial complement to EBM, may play a significant role in clinical research[20,21].

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COMMENTS

Background

Epidemiological studies have shown that the incidence of cholelithiasis in recent years has been continuously rising and doubling every 10 years. For symptomatic cholecystitis, antibiotics and antispasmodic treatment are adopted clinically as conventional therapy while cholecystectomy or laparoscopic cholecystectomy are also considered as surgical modalities. However, gallstone disease of this type may recur within several months. Gallstones may also recur in the biliary tract after cholecystectomy. Therefore, it is important to identify effective treatment options and adjuvant therapeutic methods for cholecystitis.

Research frontiers

By analyzing, from the perspective of evidence-based medicine, the substantial amount of Chinese literature over the past 10 years concerning the use of traditional Chinese medicine in the treatment of cholecystitis, the authors discovered the problems existing in these relevant studies, and proposed that cognitive medicine could provide supplementary methodology for future research.

Innovations and breakthroughs

When reviewing the large amount of relevant Chinese literature according to the evaluation standards provided by evidence-based medicine, most of the articles appear to be of poor design, quality and evidence level. However, many of these studies showed that traditional Chinese medicine functions effectively in treating cholecystitis. Cognition-based medicine, a beneficial complement to evidence-based medicine, may play a significant role in clinical research.

Applications

More appropriate randomized controlled trials with large samples should be designed and conducted in order to reasonably evaluate the efficacy of traditional Chinese medicine in the treatment of cholecystitis, and cognitive medicine also functions as a useful supplementary framework for the evaluation.

Terminology

Evidence-based Medicine (EBM) aims to apply the best available evidence gained from the scientific method to clinical decision making. It seeks to assess the strength of evidence of the risks and benefits of treatments and diagnostic tests. Cognition-based medicine is a newly-developed methodological system of scientific medicine. Its primary element is the criteria-based assessment of therapeutic causality at the level of the individual patient. Principles and criteria of single-case causality assessment have been analyzed and explicated. Cognition-based medicine enables a methodological professionalization of clinical judgment as well as the explication of physician experience and expertise. Cognition-based medicine study designs expand the current range of clinical research, extending from criteria-based causality assessment in single cases to new forms of cohort evaluations. Though cognition-based medicine study only started in recent years, this trend is inspiring and promising.

Peer review

The main stay of cholecystitis treatment is either laparoscopic cholecystectomy while it is a "hot" or conservative management with antibiotics and analgesia followed by laparoscopic cholecystectomy approximately 8 wk later. However, alternative medical therapies are used when the patient is unfit for surgical intervention. The article is suitable for publication in WJG.

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CASE REPORT

High resolution impedance manometric findings in dysphagia of Huntington's disease

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Abstract

Conventional manometry presents significant challenges, especially in assessment of pharyngeal swallowing, because of the asymmetry and deglutitive movements of oropharyngeal structures. It only provides information about intraluminal pressure and thus it is difficult to study functional details of esophageal motility disorders. New technology of solid high resolution impedance manometry (HRIM), with 32 pressure sensors and 6 impedance sensors, is likely to provide better assessment of pharyngeal swallowing as well as more information about esophageal motility disorders. However, the clinical usefulness of application of HRIM in patients with oropharyngeal dysphagia or esophageal dysphagia is not known. We experienced a case of Huntington's disease presenting with both oropharyngeal and esophageal dysphagia, in which HRIM revealed the mechanism of oropharyngeal dysphagia and provided comprehensive information about esophageal dysphagia.

Key words: Dysphagia; Esophagus; High resolution impedance manometry; Huntington's disease; Oropharynx

INTRODUCTION

Conventional manometry presents considerable challenges in evaluation of oropharyngeal dysphagia because of the asymmetry and deglutitive movements of oropharyngeal structures[1]. Introduction of high resolution manometry (HRM), which employs pressure sensors at 1 cm intervals across the entire anatomic region from the oropharynx to the stomach represented a significant improvement in data recording and diagnostic yield, especially in cases of functional dysphagia over conventional manometry[1,2]. However identifying a manometric abnormality does not equate to identifying a disease and thus achalasia and (perhaps) diffuse esophageal spasm are relevant to manometric findings, which have a functional correlate and can cause dysphagia[3]. Most recently, high resolution impedance manometry (HRIM) has been introduced to combine the benefits of HRM and impedance-based bolus transit assessment. Actually patients with normal manometry can have abnormal bolus transit, and patients with abnormal manometry can have normal bolus transit[4]. Koya *et al*[5] reported that abnormal impedance even in patients with normal manometry may be a sensitive indicator of esophageal functional abnormality as represented by the symptom of dysphagia in these patients.

To our knowledge, little is known with regard to in-

vestigation of dysphagia using the HRIM technique. We experienced a case of Huntington's disease presenting with both oropharyngeal and esophageal dysphagia, in which HRIM revealed the mechanism of oropharyngeal dysphagia, and provided comprehensive information about esophageal dysphagia.

CASE REPORT

Present illness

A 65-year-old male was admitted to the hospital because of a 5-year history of progressive dysphagia. Five years before admission, he began to have difficulty in swallowing liquids such as tea and thin soup. Three years later he complained of difficulty in eating liquids as well as solid foods, pain extended up to the manubrium, and he had lost 12 kg in weight. Ten years before admission, the patient became aware of involuntary movements of all his limbs and face. In his family history, his mother and younger brother complained of the same symptoms.

Physical study in general and neurological study

The patient had a history of pulmonary tuberculosis, which had been cured 30 years previously. He had smoked cigarettes for 40 years. He had drunk moderate amounts of alcohol for 40 years, but he had stopped drinking 3 years before admission. His body temperature was 36.5 ℃, pulse was 85 bpm, and respirations were 18 breaths/min. Blood pressure was 125/85 mmHg. Physical examination revealed mild dysarthria, chorea, and limitation of down gaze in eye movement. No muscle rigidity, muscle atrophy or pathologic reflexes were noted. Brain magnetic resonance imaging exhibited ventriculomegaly and mild atrophy in all regions of his brain. The patient was finally diagnosed with Huntington's disease by the result that the CAG repeat numbers in the Huntington gene were 44 in comparison with the normal numbers of 10-35.

Endoscopic study

The whole mucosa of the hypopharynx and esophagus was normal except for a mucosal break less than 5 mm from the esophagogastric junction. Endoscopy revealed relatively normal opening of the upper esophageal sphincter (UES) and low esophageal sphincter, and no presence of residual food in the esophagus. However, spastic contraction of the mid esophagus was noticed.

Esophagogram study

Esophagography demonstrated barium retention with significant delay of contrast passage into the stomach. There were frequent irregular contractions between the mid and distal esophagus. There were no typical signs of primary esophageal motility disorder such as bird-beak appearance or corkscrew appearance.

Video fluoroscopic swallowing study

On swallowing of a spoonful of pudding mixed with barium powder, the patient had a tendency to eat rapidly. Labial closure was normal but disorganized tongue movement, as well as postural instability induced by chorea resulted in residual bolus in the vallecula and pyriform sinuses.

HRIM study

A solid-state HRIM manometry assembly (Sandhill Scientific Instruments Inc. United States) was used to evaluate dysphagia in the patient. A HRIM study was also performed in 26 healthy persons to compare the characteristics of pharyngeal motility. The HRIM catheter was 4.0 mm diameter with 32 solid pressure sensors and 6 impedance sensors. The 4 active impedance channels were located in the traditional locations for analysis, i.e., 5, 10, 15 and 20 cm above the high pressure zone of the lower esophageal sphincter (LES). There were 32 pressure sensors which spanned the esophagus from UES to the LES to allow for evaluation of swallows from initiation of the swallow to closure of the LES. The zero mark on the probe was located at the channel used for LES analysis. The study was performed in a sitting position after at least 6-h fasting. The HRIM assembly was passed transnasally and positioned to record from the hypopharynx to the stomach with about 3-5 intragastric sensors. The catheter was fixed in place by taping it to the nose. The manometric protocol included a 5-min period to assess basal sphincter pressure, 10 5-mL saline swallows and 10 5-mL viscous swallows (so called standard method). Manometric data were acquired and stored using software (Sandhill Scientific Instruments Inc. United States). The HRIM catheter was pulled back by 10 cm and the same sessions were repeated because of inability to assess all the pharyngeal manometric information and bolus transit of pharyngoesophageal segment (so called modified method). Takasaki *et al*[6] reported that vocalizing "kakakaka" in investigation of pharyngeal swallow using high resolution manometry easily identified the locations of the velopharyngeal swallowing pressure. Therefore vocalizing "kakakaka" was added to the HRIM study using modified method.

Initial HRIM findings by the standard method

The results of HRIM by the standard method are summarized in Table 1. Impedance results demonstrated 100% swallowing with incomplete bolus transit of both the liquid and the viscous solution. Manometric results revealed high LES pressure with incomplete relaxation (Figure 1A). For liquid bolus, distal esophageal high pressure simultaneous repetitive contraction was observed in 70% of swallows on the manometric topographic view and abnormal liquid transit was noted on the impedance contour view. For viscous bolus, contractile pressures were higher, and simultaneous repetitive contractions were noted on the manometric topographic view, which was associated with the sensation of retrosternal bolus hold-up, chest pain, and abnormal viscous transit (Figure 1B).

Table 1 High resolution impedance manometric findings by the standard method

	Initial finding	After botulinum toxin injection
Manometry		
LES pressure (mmHg)	44.2	37.2
Relaxation of LES		
Residual pressure (mmHg)	12.1	10.8
Duration (s)	10.1	9.3
Relaxation percent (%)	74	71
Body peristalsis		
Simultaneous contraction (%)	70	60
Peristaltic contraction (%)	30	40
Aperistalsis (%)	0	0
Amplitude of lower esophagus (mean, mmHg)	441	382
Impedance		
Liquid swallow		
Incomplete bolus transit (%)	100	70
Complete bolus transit (%)	0	30
Viscous swallow		
Incomplete bolus transit (%)	100	70
Complete bolus transit (%)	0	30

LES: Lower esophageal sphincter.

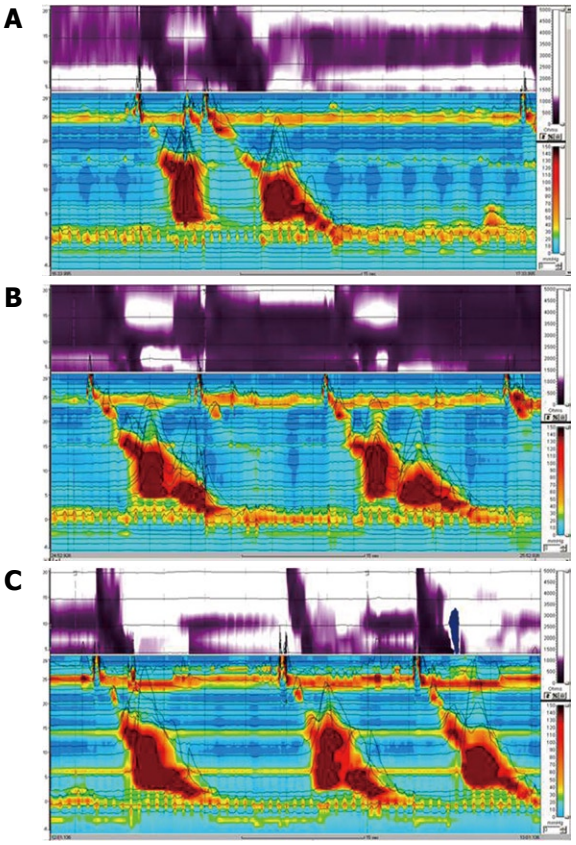

Figure 1 High resolution impedance manometry findings using the standard protocol. A: Liquid swallowing at admission reveals high amplitude, simultaneous contractions of esophageal body with incomplete lower esophageal sphincter relaxation, and incomplete bolus transit; B: Viscous swallowing at admission demonstrates higher amplitude, repetitive contractions and incomplete bolus transit; C: After injection of botulinum toxin, the isocontour of impedance shows considerably improved bolus transit compared with those at admission during saline swallows (Figure 1A), but spasms are still seen on the isocontour of manometry.

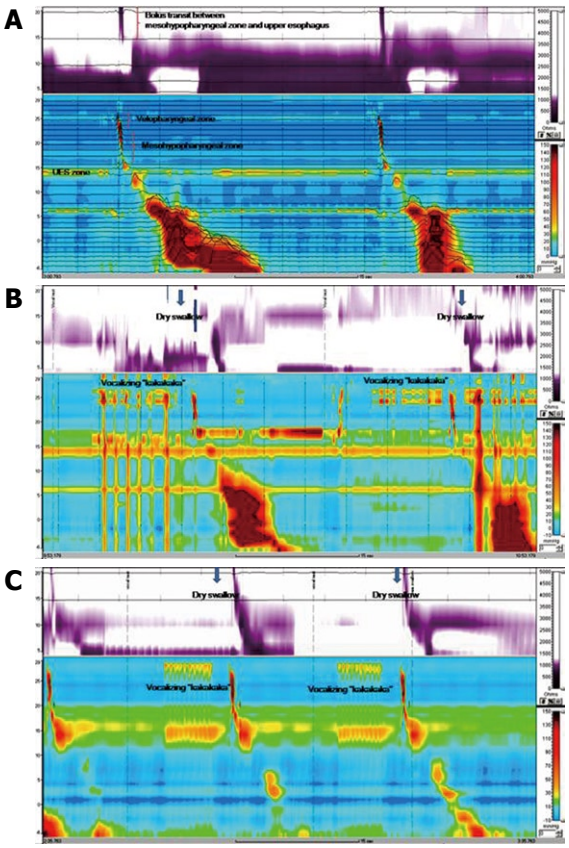

Figure 2 High resolution impedance manometry findings using modified protocol. A: Patient; unremarkable peristaltic contraction and bolus transit between the hypopharynx and upper esophagus are seen after pull back of the catheter; B: Patient; irregular contractions are seen at the velopharyngeal zone, and simultaneous contraction between velopharyngeal and mesohypo- pharyngeal zone accompanying impaired bolus transit of pharyngo-upper esophageal segment after vocalizing "kakakaka"; C: Healthy subject without dysphagia; regular contractions are noted at the velopharyngeal zone, and peristaltic contractions of pharyngo-upper esophageal segment are normal as well as bolus transit after vocalizing "kakakaka".

HRIM findings by the standard method after post-botulinum toxin injection

Given the esophageal dysmotility with a spastic component, subsequent trials of a proton pump inhibitor, a nitrate, a calcium channel blocker, and a phosphodiesterase inhibitor were made for 1 mo. There was no response to these drugs in the patient. Treatment with 100 U botulinum toxin injected around the lower esophagus showed considerable improvement in the impedance results and in symptoms such as retrosternal bolus hold-up and chest pain despite no change in the manometry results (Figure 1C).

Initial HRIM findings by the modified method

HRIM using the standard method demonstrated normal UES relaxation and peristaltic pharyngeal pressure (UES pressure 41.1 mmHg, residual pressure -1.1 mmHg, duration 0.6 s, 100% relaxation). After withdrawal of the catheter by 10 cm, HRIM revealed unremarkable bolus transit and peristalsis between the meso hypopharynx and upper esophagus (Figure 2A). However, HRIM using the modified method and vocalizing "kakakaka"

revealed irregular contractions of the velopharyngeal zone, simultaneous contraction between the velopharyngeal and mesohypopharyngeal zone, and impaired bolus transit of the pharyngo-upper esophageal segment (Figure 2B). In contrast to the HRIM findings of our case, HRIM findings using the modified method from all healthy persons showed regular contractions of the velopharyngeal zone and normal bolus transit between mesohypopharyngeal zone and the upper esophagus (Figure 2C).

DISCUSSION

To our knowledge, this report is the first study of Huntington's disease using HRIM, which indicated the combined oropharyngeal and esophageal dysphagia. The patient had difficulty in initiating swallowing and had retrosternal bolus hold-up. Insidious onset of dysphagia associated with some neurologic symptoms such as chorea and dysarthria suggested oropharyngeal dysphagia from a neurologic basis. A video fluoroscopic swallowing study provided the diagnosis of oropharyngeal dysphagia which resulted from a lack of coordination between the oral and pharyngeal stage. Unexpected involuntary movement in the oral cavity induced oropharyngeal incoordination. Oropharyngeal dysphagia in Huntington's disease results from tachyphagia, or rapid uncontrolled swallowing, secondary to impaired sensory and cognitive function[7]. Furthermore, it is caused by buccolingual chorea resulting in food being transferred impulsively[7]. Respiratory chorea, marked by involuntary respiratory movements, occurs in approximately 40% of patients, and interrupts the normal respiratory-deglutition cycle[8]. Given the anatomical structures involved in vocalization, the HRIM findings using the modified method may reflect the mechanism of oropharyngeal dysphagia in the Huntington's disease patient. In other words, the modified test may disclose oropharyngeal incoordination related to buccolingual chorea, which cannot be detected even with HRM or HRIM using the usual protocol such as the liquid and viscous swallow test. Further investigation should be carried out to confirm the role of the vocal test in either HRM or HRIM studies for assessing oropharyngeal dysphagia related to chorea.

HRIM results concerning esophageal dysphagia appeared to indicate diffuse esophageal spasm (DES) such as simultaneous contraction associated with > 10% of wet swallows, mean simultaneous contraction amplitude > 30 mmHg, and repetitive contractions. Esophageal dysmotility of the patient, however, can be better classified as an atypical disorder of LES relaxation because there was incomplete relaxation of the LES[9]. The esophageal dysmotility in this case may be an intermediate form between DES and achalasia because a few case reports have suggested a transition from DES to achalasia in some patients[10-12].

Kagel *et al*[13] reported esophageal dysphagia was relatively uncommon in Huntington's disease. It was most likely secondary to the disruptive effects of chorea in the aerodigestive tract. To our knowledge there has been no report regarding spastic esophageal dysmotility in Huntington's disease. The cause of the spastic esophageal motility disorder in the patient was unknown. There are a few studies documenting acid reflux-induced esophageal spasm[12,14,15]. Simultaneous contractions from gastroesophageal reflux should be treated first by a proton pump inhibitor[16]. Reflux esophagitis in this case may be considered the cause of the esophageal dysmotility with a spastic component. However, there was no improvement under therapy with a proton pump inhibitor.

Considering dysphagia associated with chest pain attributable to esophageal spasm, subsequent trials of a nitrate, a calcium channel blocker, and a phosphodiesterase inhibitor were performed. There was no response to these drugs in the patient. Actually current treatments for esophageal spasm, including calcium channel blockers and nitrate donors, are limited by poor efficacy and side effects[17]. Recently botulinum toxin injections in the lower esophagus and at the level of the gastroesophageal junction have been reported to have a beneficial effect in patients suffering from DES[18-20]. These beneficial effects of botulinum toxin are perhaps related to the improved manometric results. Interestingly, injection of botulinum toxin in this case showed improvement in impedance results and symptoms despite no improved manometry results. It suggests that the esophageal symptoms are more closely related to disturbed bolus transport and impaired clearance than esophageal dysmotility *per se*[4]. It may also suggest that esophageal dysmotility in terms of abnormal contractility and impaired bolus transit is limited in explaining the dysphagia. Dysphagia symptoms are related to factors other than esophageal dysmotility, e.g., esophageal sensitivity disorder.

In conclusion, HRIM enabled us to obtain more detailed and important information in the Huntington's disease patient with both oropharyngeal dysphasia and esophageal spastic dysmotility. Further investigation of HRIM will be needed to assess its role in either oropharyngeal or esophageal dysphagia.

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Rifaximin for the prevention of spontaneous bacterial peritonitis

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Abstract

According to a review article by Biecker *et al* published in a previous issue of *World Journal of Gastroenterology* in March 2011, intestinal decontamination with norfloxacin remains the mainstay of primary prophylaxis of spontaneous bacterial peritonitis (SBP) at the expense of development of quinolone-resistant bacteria after long-term use. In our research, the administration of a 4-wk regimen with rifaximin 1200 mg/d reduced significantly the ascitic neutrophil count in cirrhotic patients with sterile ascites in line with a significant decrease in plasma endotoxin levels. Our observations concur with recent findings, showing a significantly reduced 5-year probability of SBP in cirrhotic patients taking rifaximin.

Key words: Rifaximin; Cirrhosis; Ascites; Spontaneous bacterial peritonitis

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TO THE EDITOR

We read with great interest the article by Biecker *et al*[1] regarding management of ascites published on *World J Gastroenterol* 2011; **17**: 1237-1248. Development of spontaneous bacterial peritonitis (SBP) is a major complication of ascites and possibly the final step in a series of events, including intestinal bacterial overgrowth (IBO), bacterial translocation (BT) resulting in bacteremia, endotoxemia, and colonization of mesenteric lymph nodes, and finally seeding of bacteria into the ascitic fluid (AF). Indeed, SBP in non-hospitalized patients is mostly caused by gram-negative bacteria of intestinal origin. It has been hypothesized that patients at risk of SBP have probably sustained multiple episodes of colonization and resolution before they present with the first clinically apparent infection. In this respect, a previous study showed that higher neutrophil counts in sterile AF are associated with higher risk of subsequent development of SBP[2]. The high mortality of SBP warrants its prevention with administration of antibiotics aimed at decreasing the burden of gut bacteria, thus interrupting the sequence of events leading to AF infection. Norfloxacin is widely used for primary prophylaxis of SBP; however its extensive long-term use has increased the incidence of quinolone-resistant and gram-positive SBP[1].

Rifaximin is an antibiotic with a broad-spectrum activity against gram-positive and gram-negative microorganisms within the gastrointestinal tract. The main

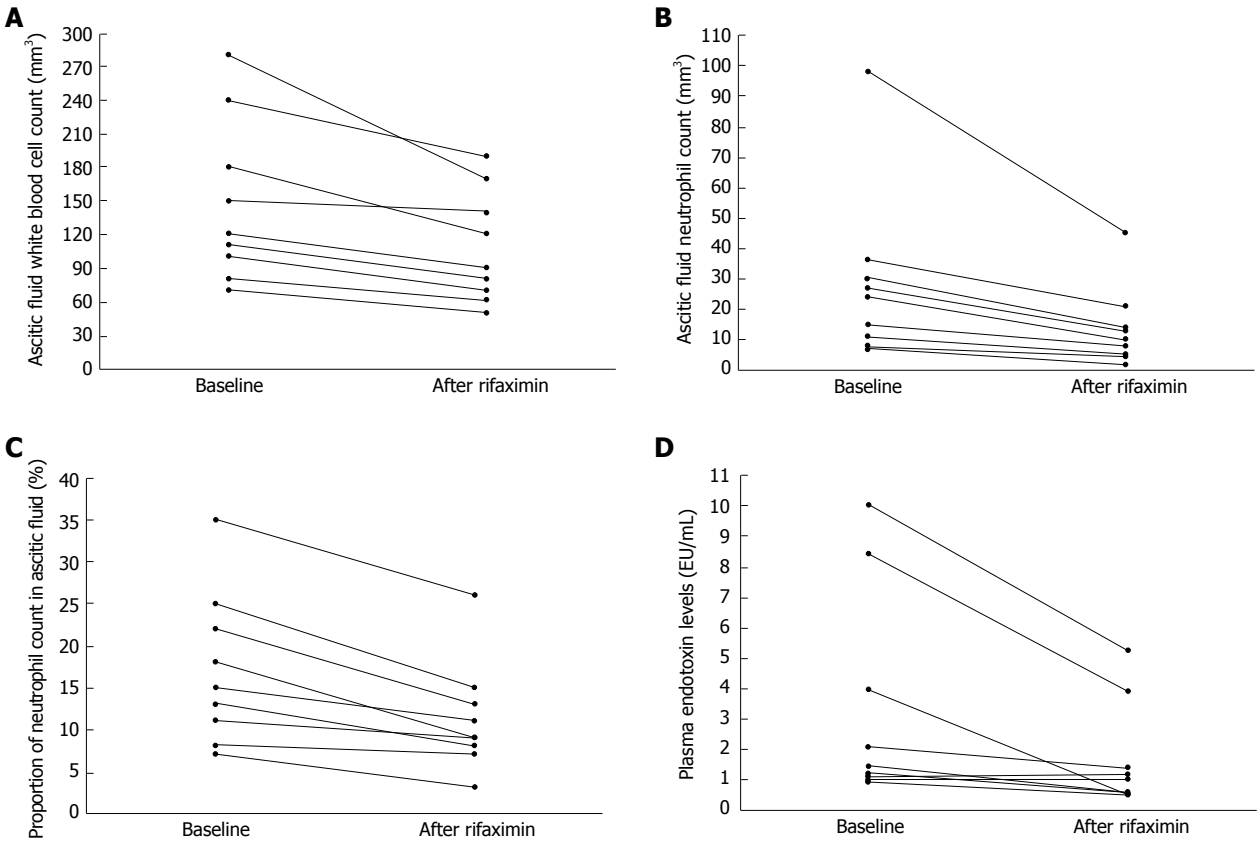

Figure 1 Individual changes in ascitic fluid white blood cell (A), neutrophil count (B) and proportion of neutrophils (C), and plasma endotoxin levels (D) after administration of rifaximin for 4 wk.

advantage of rifaximin is that it is virtually unabsorbable, which minimizes the antimicrobial resistance and adverse events and renders the drug safe in all patient populations. In addition, rifaximin has a better activity against gram-positive organisms than norfloxacin[3].

We investigated whether rifaximin can reduce the burden of gut flora and BT, which are the requisite effects of a drug used for SBP prophylaxis, by studying its effects on circulating endotoxin levels and AF neutrophil counts in cirrhotic patients with sterile ascites.

Sixteen cirrhotic patients with ascites with no history of previous SBP episodes who required regularly a large-volume paracentesis were included in our study. Cirrhosis was established by non-invasive and/or histological criteria; all patients were Child Pugh class C. The patients were studied at baseline and after a 4-wk regimen with rifaximin 1200 mg/d (Group 1, n = 9; alcohol/viral etiology: 7/2) or an observational period (Group 2, n = 7; alcohol/viral etiology: 5/2). Exploratory paracentesis was performed in association with each therapeutic paracentesis to exclude ascitic fluid infection. All patients were included after written informed consent was obtained from them and the local scientific-ethical committee approved the study. Criteria for inclusion were: (1) Abstinence from alcohol for at least 6 mo before inclusion; (2) Absence of clinical and laboratory signs of bacterial infections; (3) No history of variceal bleeding within the 2 wk preceding the study; and (4) No treatment with antibiotics during the last 8 wk before inclusion. For ethical reasons, only patients with AF total protein concentration >1 g/dL were studied. AF white blood cell (WBC) and neutrophil count, the proportion of neutrophils in AF (AF% neutrophils), and plasma endotoxin levels were measured at baseline and at the end of observational or treatment period. For the detection of plasma endotoxin levels, the Limulus amebocyte lusate chromogenic endpoint assay (Hycult biotech, Uden, The Netherlands) was used as instructed by the manufacturer. The Wilcoxon matched pairs test was used for comparing variations within the same group. Results were expressed as mean ± SE. Statistical significance was designated as $P < 0.05$.

Rifaximin caused significant reductions in AF WBC, neutrophil count, AF% neutrophil count, and plasma endotoxin levels (Table 1); the values of the abovementioned parameters decreased uniformly in all patients (Figure 1). No significant changes in the AF cytological characteristics or plasma endotoxin levels were noted in Group 2. No patient developed AF infection during the study period and no side-effects were noted by the use of rifaximin.

Our findings strongly suggest that rifaximin suppresses IBO, which in turn reduces BT and the subclinical activation of AF defence mechanisms from prior silent colonisations with bacteria in cirrhotic patients with sterile ascites. The reduction of endotoxemia by ri-

Table 1 Ascites cytological characteristics and plasma endotoxin levels in patients after rifaximin treatment or in observational period

	Group 1 (n = 9)			Group 2 (n = 7)		
	Baseline	4 wk	P value	Baseline	4 wk	P value
WBC count (per mm^3)	147.7 ± 24.1	107.7 ± 16.6	0.004	164.5 ± 30.2	175 ± 20.8	NS
Neutrophil count (per mm^3)	28.4 ± 9.3	13.5 ± 4.3	0.01	34.6 ± 6.4	37.9 ± 7.2	NS
AF% neutrophils	17.1 ± 3	11.2 ± 2.1	0.0008	21.6 ± 3.5	22.1 ± 2.5	NS
Plasma endotoxin (EU/mL)	3.3 ± 1.1	1.6 ± 0.5	0.03	2.9 ± 0.9	3 ± 0.8	NS

Data are expressed as mean ± SE. WBC: White blood cell; NS: Not significant; AF: Ascitic fluid. Group 1: 9 cirrhotic patients with refractory ascites at baseline and after 4-wk rifaximin treatment; Group 2: 7 patients in the observational period.

faximin may further reduce BT by causing a fall in portal pressures[4] considering that portal hypertension induces structural abnormalities in intestinal mucosa leading to an enhanced permeability[1]. Overall, the effects of rifaximin on IBO and BT in our study are consistent with recent findings, showing a significantly reduced 5-year probability of SBP in cirrhotic patients taking rifaximin[5]. In conclusion, the role of rifaximin as an alternative mean of preventing SBP deserves further attention in prospective studies.

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American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
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London, United Kingdom

February 16-17, 2012
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London, United Kingdom

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Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom

February 24-27, 2012
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Montreal, Canada

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International Conference on Nutrition and Growth 2012
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26th Annual New Treatments in Chronic Liver Disease
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San Antonio, TX 78249, United States

March 31-April 1, 2012
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April 20-22, 2012
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Acknowledgments

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ic effect of Jianpi Yishen decoction in treatment of Pixudiarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.00000 35706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

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No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

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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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