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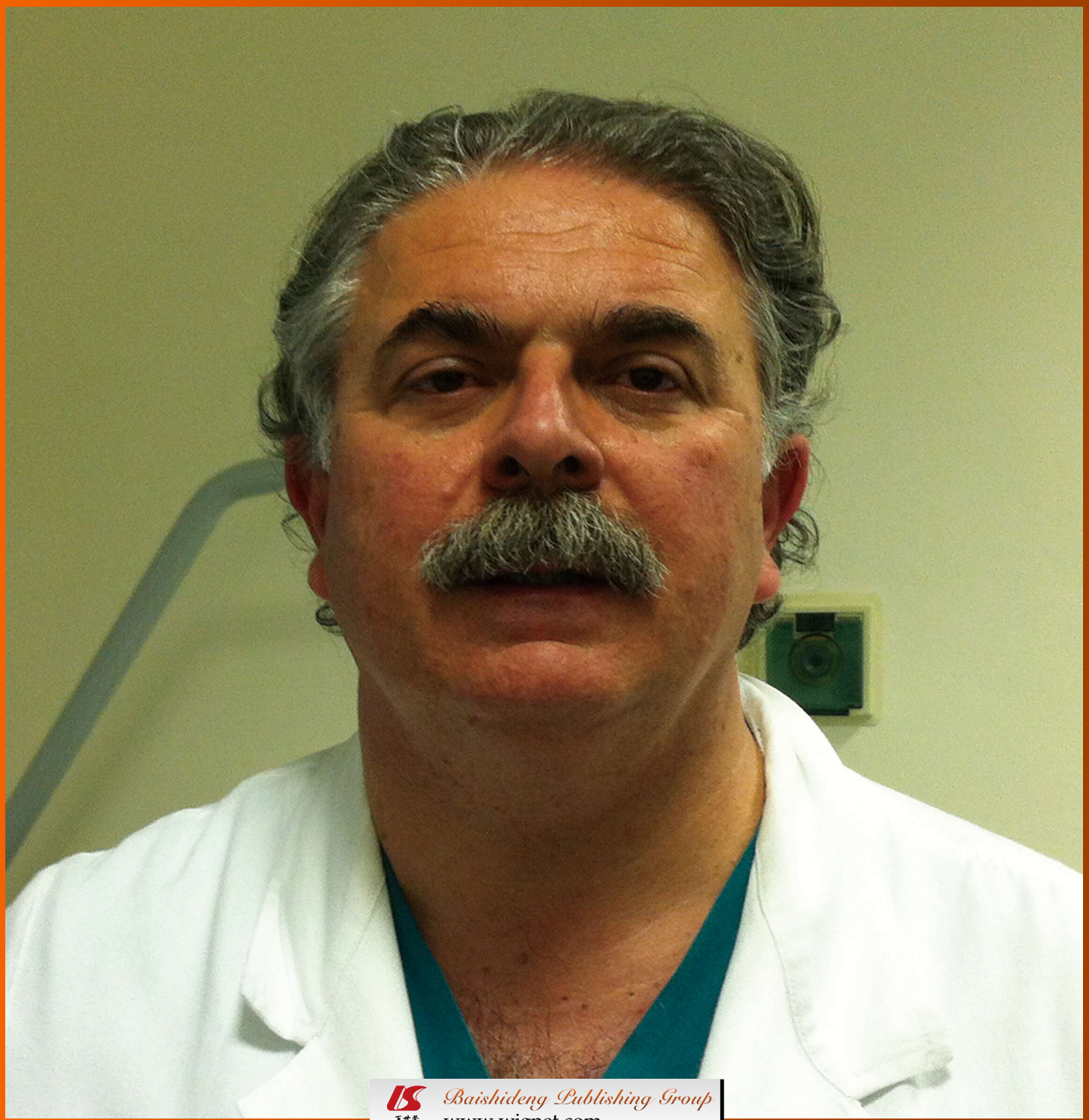

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CASE REPORT

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EDITORIAL

Cellular and molecular basis of chronic constipation: Taking the functional/idiopathic label out

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Abstract

In recent years, the improvement of technology and the increase in knowledge have shifted several strongly held paradigms. This is particularly true in gastroenterology, and specifically in the field of the so-called "functional" or "idiopathic" disease, where conditions thought for decades to be based mainly on alterations of visceral perception or aberrant psychosomatic mechanisms have, in fact, be reconducted to an organic basis (or, at the very least, have shown one or more demonstrable abnormalities). This is particularly true, for instance, for irritable bowel syndrome, the prototype entity of "functional" gastrointestinal disorders, where low-grade inflammation of both mucosa and myenteric plexus has been repeatedly demonstrated. Thus, researchers have also investigated other functional/idiopathic gastrointestinal disorders, and found that some organic ground is present, such as abnormal neurotransmission and myenteric plexitis in esophageal achalasia and mucosal immune activation and mild eosinophilia in functional dyspepsia. Here we show evidence, based on our own and other authors' work, that chronic constipation has several abnormalities reconductable to alterations in the enteric nervous system, abnormalities mainly characterized by a constant decrease of enteric glial cells and interstitial cells of Cajal (and, sometimes, of enteric neurons). Thus, we feel that (at least some forms of) chronic constipation should no more be considered as a functional/idiopathic gastrointestinal disorder, but instead as a true enteric neuropathic abnormality.

Key words: Constipation; Enteric glia; Enteric nervous system; Enteric neurons; Interstitial cells of Cajap; Neurogastroenterology

Core tip: Concerning gut motility, in the last years the basic/clinical interplay between gastroenterology and neurology has become stricter, and many pathologic conditions, among which constipation, related to abnormal gastrointestinal motility are now considered and studied by a neurogastroenterological point of view. However, the fact that these conditions are still labelled as "functional" or "idiopathic" is puzzling. We examined the evidence for taking these labels out from constipation, that should be considered as a true neurenteric dysfunction.

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INTRODUCTION

The field of gastrointestinal motor activity has always attracted the researchers' interest; however, in the time course it became apparent that most gut motor disorders are attributable to disordered neural control mechanisms. Thus, by the encounter of gastroenterologists and neurologists a common branch emerged, *i.e.*, neurogastroenterology[1], in which an equal partnership had been recognized concerning gut motility. Recently, ultrastructural morphologists joined and try to bring innovative perceptions on control mechanisms of digestive motility. This had led to interesting and exciting new perspectives in the pathophysiology of some frequent disorders, such as constipation.

Chronic constipation is a frequent symptom in the general population, where is present in 2%-30% of subjects[2]. However, apart from secondary forms, associated to an underlying disease (*e.g.,* neurological[3,4]), commonly used (at least for scientific purposes) classifications still label most cases of constipation as "idiopathic" or "functional"[5-9].

It is worth noting that the concept of functional diseases has been somewhat questioned in the last years[10], since several studies conducted on prototypic functional entities, such as irritable bowel syndrome (IBS) and functional dyspepsia (FD) have revealed that these condition may actually harbor an organic basis[11,12]. In fact, inflammation and neuronal degeneration have been reported in IBS patients[13] and duodenal mastocytosis, eosinophilia and intraepithelial lymphocytosis have been described in both IBS and FD patients[14].

Chronic constipation may be subdivided in two main subtypes, obstructed defecation (OD) and slow transit constipation (STC), that may also co-exist in the same patient[15,16], and it is generally thought (by data originating from both experimental animal models and humans) that colonic sensorimotor dysfunction and abnormal motility play a pivotal pathogenetic role[17-19]. Thus, abnormal colonic and anorectal function had been repeatedly demonstrated in these patients[20-22], and pharmacologic stimulation may help in addressing more targeted therapeutic approaches[23,24]. However, etiological factors are still poorly known[25].

This article will deal with the available evidence for neurobiological abnormalities in chronic constipation, that suggests how this symptom often underlie a true organic enteric disorder.

NEUROENTERIC ABNORMALITIES IN CONSTIPATION

To date, there is mounting evidence that colonic neuromuscular abnormalities may be of paramount importance in this setting[26,27], and there are numerous studies showing that (at least) severely constipated patients may have one or more abnormal features mainly (although not exclusively) linked to elements of the enteric nervous system (ENS)[28].

The ENS, considered as the brain of the gut, integrates secretion and motility into homeostatic patterns of behavior susceptible to disorder[29]. Thus, it is not surprising that some of the enteric circuitries responsible for these activities may be involved in the dysfunction of their basic control mechanisms. The resulting abnormalities are summarized below.

Abnormal colonic neurochemistry

This has been repeatedly shown in constipated patients: several studies showed a decreased content of vasoactive intestinal peptide (VIP) and substance P in tissues obtained from these subjects[30-34]. Moreover, *in vitro* studies confirmed that the diminished contractile response to these substances plays an important role in the impaired motility observed in the colon of constipated patients[35,36]. Of note, these abnormalities seem not to be related to chronic laxative use, since anthranoids cause a reduction in the levels of inhibitory neurotransmitters (VIP, somatostatin), but not of substance P, in the rat colon[37]. Other studies showed that excitatory nerve fibres are present in the circular muscle in STC but they are deficient in tachykinins and enkephalin[38-40]. In addition, investigations conducted on colonic strips showed that a decrease of cholinergic innervation and an increase of non-adrenergic non-cholinergic (NANC) inhibitory innervation play an important role in the impaired motility observed in the colon of patients with slow transit constipation[41]; these effects are mediated by an increase of nitric oxide and a decrease of neurotensin[42-44], as also confirmed by immunohistochemical methods in surgically resected specimens[45].

Enteric nervous system

Earlier studies addressing the ENS have shown the presence of several heterogeneous abnormalities in patients with severe constipation (especially those with STC), including reduced number of argyrophilic neurons[46] and of intraganglionic neurofilaments[47], myenteric plexus hypoganglionosis[48]. More recent studies, with the increasing use of immunohistochemical techniques[49,50], have demonstrated more consistent findings on the main elements of the ENS, such as the decrease of interstitial cells of Cajal (ICC)[51,52] (up to their complete absence in colonic inertia[53]), often associated to a reduced number of enteric neurons[54,55] and/or of enteric glial cells (EGC)[56,57]. Of interest, the expression of c-kit mRNA and c-kit protein was also found to be significantly decreased in the colon of severely constipated patients, suggesting that the c-kit signal pathway may play an important role in ICC reduction in these patients[58].

Colonic smooth muscle

Only a few studies have addressed this issue, often with

discordant findings, probably due to the heterogeneous cohorts of patients evaluated. Some authors reported that the ratio of the thickness of circular to longitudinal muscle was significantly lower in the left colon in constipated subjects[59], whereas other authors described a decreased circular muscle layer thickness in constipated patients[60], but no abnormalities of the colonic muscular layers were described in both studies. Another investigation showed the presence of amphophilic inclusion bodies in the muscularis externa of STC patients[61], even though these findings were found in about half of the patients. Normal actin expression was found in both adults and children with severe constipation[56,62], whereas the use of novel and nonconventional smooth muscle markers may reveal abnormalities linked to the smooth muscle contractile apparatus unnoticed by both routine stainings and alpha-actin, suggesting specific defects of smooth muscle cells involved in the pathogenesis of gastrointestinal motility disorders[63].

SIGNIFICANCE OF NEUROENTERIC ABNORMALITIES IN CONSTIPATION

There are few doubts that the ENS abnormalities repeatedly found in constipated patients play a pivotal role in the genesis of symptoms. In fact, the consistent finding of a significant decrease of ICC, enteric neurons, and (especially) EGC, variously associated each other, justifies the abnormal motor behavior of the large bowel in these patients.

In fact, looking at the physiological properties of these cell populations, it is obvious that the disruption of their number/connections/relationship leads to an impairment of the complex regulation of the well-coordinated colonic motor patterns[64], thus affecting the viscus' motility, due to the strict interplay between ICC, enteric neurons and EGC, with the latter acting as a physiologic bridge (not only by a simple mechanic point of view, but also by means of their neurotransmitter, immunologic, and trophic properties[65]) between the other two cell types.

Unfortunately, to date data are lacking on the possible factors causing neurenteric abnormalities in constipated patients. The current hypothesized mechanisms (often originating from experimental animal models) imply abnormalities in glial trophic factors leading to neural degeneration, and enteric localization of infective agents (bacteria, virus, prions) causing more or less selective degeneration of specific neurenteric cell populations (particularly EGC)[66], whereas genetic factors[67] or neurodegenerative changes due to aging seem to play a lesser role[68].

NEW CELLULAR PLAYERS IN NEUROMOTILITY DISORDERS

In biological sciences, interstitial tissue is seen as the connective tissue that surrounds the cells of a certain tissue while the extracellular matrix elements are known for its great capacity to retain water. However, except for a few described interstitial diseases (*e.g.,* inflammatory bowel disease, interstitial cystitis, tubulointerstitial nephropathy, interstitial lung disease) its role is easily overlooked, as well as the significance of its cellular elements. Recent studies related to biological and histological data revealed, among the known resident (fibroblasts/fibrocytes, adipose cells) and non-resident cells (mast cells, plasma cells, eosinophils, macrophages, *etc.*) of interstitial space, a novel cell type-the telocyte[69,70].

Morphologically, telocytes represent interstitial cells with telopodes-the longest cellular extensions described besides the axons of neurons[71]. This rather unique cell type, difficult to visualize by routine microscopy, displays a particular morphology by electron microscopy: (1) a small cell body (9-15 μm) with scarce cytoplasmic organelles surrounding a moderately euchromatic nucleus; and (2) telopodes are usually tortuous and organized in a 3D network by overlapping and/or by homocellular interactions[72,73]. Telopodes are very long (10-1000 μm), thin (0.1 ± 0.5 μm) and moniliform cytoplasmic extensions; the moniliform aspect is created by the alternation of thin segments-podomers with dilated segments-podoms; the latter accomodate functional units consisting of caveolae, mitocondria and endoplasmic reticulum[74] and occupy a strategic position in relation to stem cell niches, blood capillaries, and/or nerve bundles[75,76]. Telopodes also establish stromal contacts with other cells, such as mast cells, basophils, lymphocytes, eosinophils, plasma cells, or macrophages[77] and non-cellular elements (*e.g.*, collagen and elastic fibers)[78,79].

Telocytes have been described in human and mammalian cavitary and parenchymatous organs, as well as in serous membranes and other tissues (for details see www.telocytes.com). In the last two years telocytes were also described in the gut[77,80,81].

In modern times the significance of the information that could be achieved by signaling molecules found in intercellular fluids is overlooked. There is scarce information on the usefulness of the extracellular organelles (exosomes and shedding microvesicles) released in the extracellular space as mediators of cell-to-cell communication[82]. Such vesicles were recently demonstrated in the proximity of telopodes and even emerging from them in heart[83], lungs[76], skeletal muscle[76], pancreas[73], parotid gland[84] and human uterus[74].

Telocytes are supposed to be involved: (1) in intercellular signaling[72,74,77]; (2) as stem cell adjutants involved in tissue renewal[79,85]; (3) as sensors for steroid hormones[86]; (4) in the guidance of immune cells[77]; (5) as stretch sensors[87]; and (6) as contractility modulators[88]. Even though telocytes seem to be implicated in many important physiological and pathological processes[89,90], their exact functions still remain controversial. Although telocytes have not yet been described at colonic level, their possible involvement in pathophysiological mechanisms

of chronic constipation cannot be overlooked. In favor of this hypothesis there is a possible correlation between the fact that telocytes express receptors for estrogen and progesterone[91,92] and the fact that chronic constipation is linked to sex hormones[93] and is higher in women of reproductive age[94].

CONCLUSION

The improvement of scientific knowledge and the constant, increasing ability to recognize previously unknown pathophysiologic mechanisms is of paramount importance. Thus, labels such as "idiopathic" or "functional", that basically conceal the fact that too little is known of a specific pathologic entity[10], should be hopefully replaced when more knowledge is available, as pointed out several years ago[95]. As such, the recent recognition of neurenteric abnormalities in many patients complaining of constipation should point to reconsidering at least some of these forms (especially STC) as true enteric neuropathies, and to drop the "idiopathic"/"functional" label.

Besides semantic considerations, we feel that a better understanding of possible basic abnormalities in these patients is important, and may have therapeutic implications, addressing the researchers' interest for new options toward more targeted approaches[16].

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REVIEW

Systematic review of surgical resection *vs* radiofrequency ablation for hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) represents one of the most common neoplasms worldwide. Surgical resection and local ablative therapies represent the most frequent first lines therapies adopted when liver transplantation can not be offered or is not immediately accessible. Hepatic resection (HR) is currently considered the most curative strategy, but in the last decade local ablative therapies have started to obtain satisfactory results in term of efficacy and, of them, radiofrequency ablation (RFA) is considered the reference standard. An extensive literature review, from the year 2000, was performed, focusing on results coming from studies that directly compared HR and RFA. Qualities of the studies, characteristics of patients included, and patient survival and recurrence rates were analyzed. Except for three randomized controlled trials (RCT), most studies are affected by uncertain methodological approaches since surgical and ablated patients represent different populations as regards clinical and tumor features that are known to affect prognosis. Unfortunately, even the available RCTs report conflicting results. Until further evidences become available, it seems reasonable to offer RFA to very small HCC (< 2 cm) with no technical contraindications, since in this instance complete necrosis is most likely to be achieved. In larger nodules, namely > 2 cm and especially if > 3 cm, and/or in tumor locations in which ablation is not expected to be effective or safe, surgical removal is to be preferred.

Key words: Hepatocellular carcinoma; Hepatic resection; Surgical therapy; Ablation techniques; Survival; Liver failure

Core tip: The present review shows the lights and shadows of the comparative literature regarding hepatic resection and radiofrequency ablation for hepatocellular carcinoma. Nineteen studies that directly compared these two therapies were found through an extensive literature review; of them, three randomized controlled trial were available for comparison whereas the remaining studies were represented by retrospective observational studies. Results are often conflicting and further randomized controlled trial are warranted; otherwise, retrospective observational studies should include in their analyses statistical approaches aimed at reduce possible confounding sources at a minimum.

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INTRODUCTION

Hepatocellular carcinoma (HCC) represents one of the most common primary malignancies of the liver worldwide, with an incidence that varies in the different geographic areas as a consequence of the regional variations in exposure to risk factors for this tumor[1,2]. The increasing use of surveillance in clinical practice, and the advancements in diagnostic and therapeutic abilities achieved in the last decades have greatly improved patient survival[3-5]. Liver resection and radiofrequency ablation represent the most common first-line therapies adopted when HCC is diagnosed at early stages[6]. Liver resection still remains a mainstay of HCC treatment, and thanks to the considerable improvements in surgical techniques and peri-operative care, the rates of death and complications after liver resection have remarkably decreased over time, giving the procedure added value[7,8]. However, surgery can negatively impact on the already compromised function of cirrhotic livers and, on the other hand, radiofrequency ablation seems safer but its ability to achieve complete and sustained tumor necrosis can be less predictable, and technical feasibility may be sub-optimal. For these reasons, the choice between hepatic resection and radiofrequency ablation for HCC is still a matter of debate. The aim of the present review is to examine the available literature that directly compares these two therapeutic strategies. The qualities and flaws of each included study were highlighted in the attempt to reach conclusions regarding the effectiveness of one treatment with respect to the other and to make suggestions for future research on this debated topic.

LITERATURE STRATEGY SEARCH

A systematic search within the Medline and Embase databases, in the period between 1 January 2000 and 1 December 2012, was performed with the MeSH terms "hepatocellular carcinoma" and ("hepatectomy" or "surgical therapy") and "ablation techniques". The keywords "hepatocellular carcinoma", "partial hepatectomy", "hepatic resection", "radiofrequency ablation" or "percutaneous ablation" and "survival" were used to supplement the literature search. The reference lists of retrieved publications were reviewed for other relevant papers. Only articles involving human subjects and that directly compared radiofrequency ablation *vs* hepatic resection for HCC were considered for the present review. The quality of the selected articles was attributed on the basis of their level of evidence and by means of the Newcastle-Ottawa quality assessment scale for observational studies[9]. The Newcastle-Ottawa Scale (NOS) is a score system that was developed to assess the quality of non-randomized studies, in which a study is judged on three broad perspectives: (1) the selection of the study groups; (2) the comparability of the groups; and (3) the ascertainment of either the exposure or outcome of interest for case-control or cohort studies, respectively.

WHAT GUIDELINES RECOMMEND

Clinical practice guidelines should be evidence-based and should represent the consensus of expert committees. However, it is often very difficult to reach a consensus in the field of HCC, especially as regards the therapeutic approach, given the extremely limited availability of high quality trials. Table 1 reports a summary of the levels of evidence and the strength of recommendations from three published guidelines, namely, the European Association for the Study of the Liver (EASL-EORTC), updated in 2012[10], the American Association for the Study of Liver Diseases (AASLD), updated in 2010[11], and the Asian Pacific Association for the Study of the Liver (APASL), updated in 2010[12]. The EASL and AASLD guidelines are mainly based on the Barcelona Clinic Liver Cancer (BCLC) algorithm for staging and treatment of HCC and represent the most popular treatment algorithms in Western countries[13], however, the BCLC algorithm is not very popular in Asia. There are two important aspects of these guidelines that deserve attention and that are strictly related to each other. The first is represented by the role of the "alternative strategy" of ablation, with respect to resection, and the second is the recommended selection criteria for surgery. It can be immediately noted that radiofrequency ablation is always considered as a strategy alternative, and not competitive, to resection: the EASL recommends ablative therapies "for patients with BCLC 0-A tumours not suitable for surgery", the AASLD suggests that ablative therapy is "effective for patients who cannot undergo resection" and the APASL recommends local ablation as "an acceptable alternative to resection". These recommendations mainly derive from indirect comparisons of the results from the two treatments. In brief, modern standards of HCC resection in cirrhotic patients call for a peri-operative mortality < 3% and an expected 5-year survival rate above 60%[10,14-18], whereas, on the other hand, mortality after RFA has been reported to range between 0.9% and 7.9% and the 5-year survival rate to range between 40% and 70%[19-25]. Most of the uncertainties are related to the efficacy of ablation techniques, since response to ablative therapies is strongly influenced by tumor size and location[19,26-29]. In addition, patients allocated to ablation tend to suffer from a more advanced degree of liver dysfunction in comparison to those undergoing surgery, and this feature can negatively impact the observed results. On the other hand, strict selection criteria for hepatic resection can ameliorate patient survival after surgery and this is especially true as regards liver reserve. These two features are obviously related to each other, since at varying criteria for resection, different patients will be shifted to ablation techniques and this represents the second aspect that deserves attention. For example, a selection of candidates for hepatic resection strictly based on the hepatic vein pressure gradient (HVPG), as recommended by the EASL[10], could exclude several patients from surgery, shifting them to RFA. Specifically, HVPG should be <

Table 1 Proposed evidences and recommendations from international guidelines

Guidelines	Hepatic resection	Radiofrequency ablation
EASL	Resection is the first-line treatment option for patients with solitary tumors and very well-preserved liver function, defined as normal bilirubin with either hepatic venous pressure gradient ⩽ 10 mmHg or platelet count ⩾ 100000 (evidence 2A; recommendation 1B)	Local ablation with radiofrequency or percutaneous ethanol injection is considered the standard of care for patients with BCLC 0-A tumors not suitable for surgery (evidence 2A; recommendation 1B)
EORTC[9]	Additional indications for patients with multifocal tumors meeting Milan criteria (⩽ 3 nodules ⩽ 3 cm) or with mild portal hypertension not suitable for liver transplantation require prospective comparisons with loco-regional treatments. (evidence 3A; recommendation 2C)	In tumors < 2 cm, BCLC 0, Ethanol injection and radio-frequency ablation achieve complete responses in more than 90% of cases with good long-term outcome [evidence 1(i)A; recommendation 1C]
AASLD[10]	Patients who have a single lesion can be offered surgical resection if they are non-cirrhotic or have cirrhosis but still have well preserved liver function, normal bilirubin and hepatic vein pressure gradient < 10 mmHg (recommendation 2)	Local ablation is safe and effective therapy for patients who cannot undergo resection, or as a bridge to transplantation (recommendation 2); Alcohol injection and radiofrequency are equally effective for tumors < 2 cm. However, the necrotic effect of radiofrequency ablation is more predictable in all tumor sizes and its efficacy is clearly superior to that of alcohol injection in larger tumors (recommendation 1)
APASL[11]	Liver resection is a first-line curative treatment of solitary or multifocal HCC confined to the liver, anatomically resectable, and with satisfactory liver function reserve (evidence 2B, recommendation B)	Local ablation is an acceptable alternative to resection for small HCC (< 3 cm) in Child-Pugh A cirrhosis (evidence 2B, recommendation B); Local ablation is a first-line treatment of unresectable, small HCC with 3 or fewer nodules in Child-Pugh A or B cirrhosis (evidence 2B, recommendation B)

Strength of evidence according to study design: Level 1, Randomized controlled clinical trials or meta-analyses of randomized studies; Level 2, Non-randomized controlled clinical trials; Level 3, Case series. Strength of evidence according to end-points: A, Total mortality; B, Cause-specific mortality; C, Carefully assessed quality of life; D, Indirect surrogates. Grading of recommendation: 1, Strong recommendation warranted; 2, Weaker recommendation. Grading of recommendation: A, Further research is very unlikely to change out confidence in the estimate of effect; B, Further research is likely to have an important impact on our confidence in the estimate of effect and may change the estimate; C, Further research is very likely to have an important impact on our confidence in the estimate of effect. BCLC: Barcelona Clinic Liver Cancer; HCC: Hepatocellular carcinoma; EASL: European Association for the Study of the Liver; EORTC: European Organisation For Research And Treatment Of Cancer; AASLD: American Association for the Study of Liver Diseases; APASL: Asian Pacific Association for the Study of the Liver.

10 mmHg to allow a safe resection[13], but the evidence for this recommendation is not very strong since it was based on data obtained in a very small cohort studied in the 1990s[30] and surgical techniques have substantially improved since then. Only one recent external validation was conducted on only 39 patients[31], whereas other studies could not confirm the influence of portal hypertension[32]. HVPG measurement can probably help to select surgical candidates, with a very low or null probability of post-operative liver failure, but it probably also excludes patients that can still benefit from surgery and that will be submitted to RFA with a lower chance of cure[32]. Thus, more restrictive criteria for resection result in a better outcome after surgery and a worse outcome after ablation that represents the alternative therapy to be adopted. It can be concluded that such discrepancies, evident even in international guidelines, are attributable to the relatively low level of evidence that can be obtained from the literature, as is pointed out in the following paragraphs.

COMPARATIVE STUDIES ON RESECTION *VS* ABLATION

The literature review retrieved 19 studies that directly compared resection and radiofrequency ablation; of them, three RCTs were available for comparison whereas the remaining studies were represented by retrospective observational studies. Randomized controlled studies were reviewed separately from observational studies. As can be noted from Table 2, the NOS scale of observational studies ranged from 5 to 8, none of them reached the maximum quality assessment of 9 and most of them had a quality scale below 8. In fact, the review of these studies showed that for the two treatment arms patients often have significant differences regarding most clinical and tumor variables, that are able to confound results. Thus, stratification for tumor size was attempted in order to reduce to a minimum potential biases resulting from covariate distribution. Differences observed between the two treatment arms were also highlighted. Characteristics of RCTs are reported in Table 3 and of observational studies in Table 4.

Randomized controlled studies

At December 2012, three RCTs were available for review and all were from Eastern countries[33-35] (Table 3). The first RCT was published by Chen *et al*[33]. Tumor recurrence rate at 2 years after treatment was used as the primary outcome measure to estimate the sample size of the study. After post-randomization exclusion, the study involved 71 patients submitted to ablation and 90 submitted to resection. The results showed that the 3-year overall survival was 71.4% after ablation and 73.4% after surgery. The corresponding disease-free survival rates were 64.1% and 69.0%, respectively. No statistical difference was observed and no differences were observed when patients were stratified by tumor size (*P*-values not provided). The authors concluded that the overall and

Table 2 Summary of published articles that directly compared hepatic resection and radio-frequency ablation identified through literature search

Ref.	Study period	Type of study	NOS
Feng *et al*[35]	2005-2008	RCT	-
Peng *et al*[36]	2003-2008	Retrospective	7
Wang *et al*[37]	2002-2009	Retrospective	6
Ruzzenente *et al*[47]	1995-2009	Retrospective	8
Nishikawa *et al*[42]	2004-2010	Retrospective	7
Hung *et al*[38]	2002-2007	Retrospective	7
Takayama *et al*[39]	2000-2003	Retrospective	5
Huang *et al*[34]	2003-2005	RCT	-
Ueno *et al*[41]	2000-2005	Retrospective	7
Abu-Hilal *et al*[48]	1991-2003	Retrospective	8
Guglielmi *et al*[43]	1996-2006	Retrospective	7
Hiraoka *et al*[40]	2000-2007	Retrospective	7
Hasegawa *et al*[46]	2000-2003	Survey	6
Lupo *et al*[45]	1999-2006	Retrospective	8
Chen *et al*[33]	1999-2004	RCT	-
Ogihara *et al*[49]	1995-2003	Retrospective	7
Montorsi *et al*[50]	1997-2003	Retrospective	6
Hong *et al*[51]	1999-2001	Retrospective	6
Vivarelli *et al*[44]	1998-2002	Retrospective	5

The Newcastle-Ottawa Score (NOS) scale can range from 5 to 9. RCT: Randomized controlled trials.

disease-free survivals were the same for patients with a single tumor ⩽ 5 cm treated with either ablation or resection; however, ablation showed an advantage over surgical resection in causing less post-treatment complications, less pain, and a shorter in-hospital stay[33].

The second RCT was published by Huang *et al*[34]. The 5-year overall survival rate after treatment was used as the primary outcome measure to estimate the sample size of the study. After post-randomization exclusion, the study involved 115 patients submitted to ablation and 115 submitted to resection. Results showed that the 5-year overall survival rates was 54.8% after ablation and 75.7% after surgery ($P = 0.001$). The corresponding recurrence-free survival rates were 28.7% and 51.3%, respectively ($P = 0.017$). The benefit of resection was maintained when patients were stratified by tumor size and number. The authors concluded that surgical resection may provide better survival and lower tumor recurrence rates than ablation for HCC within Milan criteria[34].

The third, and last, RCT was published by Feng *et al*[35]. The 3-year overall survival rate after treatment was used as the outcome measure to estimate the sample size of the study. After post-randomization exclusion, the study involved 84 patients submitted to ablation and 84 submitted to resection. Results showed that the 3-year overall survival rates was 67.2% after ablation and 74.8% after surgery ($P = 0.342$). The corresponding recurrence-free survival rates were 49.6% and 61.1%, respectively ($P = 0.122$). No stratification for tumor stage was provided. The authors concluded that percutaneous radiofrequency ablation may provide therapeutic effects similar to those of hepatic resection[35].

Thus, the available RCTs report different results and only Huang demonstrated a superiority of hepatic resection over ablation. Even if higher survival rates after resection were also observed in the analyses of Chen and Feng, they did not find a statistically significant superiority of surgery over ablation, leaving the question regarding the best therapeutic approach to be adopted unsolved. It should be noted, however, that the different proportions of HCC beyond the very early stage can, at least in part, explain the conflicting results, since it is known that ablation beyond this stage is less able to achieve complete tumor necrosis, thus biasing the final results[19,28,29]. Hence, a further review of the available observational studies is necessary to obtain more clinical, useful information.

Single tumors less or equal to 2 cm

Four observational retrospective studies analyzed outcomes of resection and ablation in single tumors ⩽ 2 cm[36-39] (Table 4) while none of the previous reported RCTs analyzed this specific tumor stage. None of the observational studies reported a convincing comparability between the two treatment arms, and the most frequent differences observed between ablated and surgical patients were that RFA patients were older than surgical patients, had a lower platelet count, belonged more frequently to Child-Pugh class B and were affected by smaller tumors ($P < 0.050$). Thus, results in terms of both patient survival and recurrence rate can be biased by covariate distribution. Three articles deserve some discussion for different reasons. The first article derives from a multi-institutional database of the Liver Cancer Study Group of Japan involving 2550 patients[39]. In this report, disease-free survival (DFS) was significantly better ($P = 0.001$) after resection ($n = 1235$) than after RFA ($n = 1315$), but patient survival was similar ($P = 0.280$). Ablated patients were more frequently in Child-Pugh class B, had higher ICG-R15 and smaller tumor size in comparison to resected patients ($P = 0.001$ in all cases). Therapy and Child-Pugh class were independent prognostic factors of DFS at Cox regression analysis but regression on patient survival was not performed. This report represents the largest series published in the literature that analyzed this specific tumor stage. It can be speculated that patient survival after RFA could be under-estimated, because of more advanced hepatic dysfunction, and, on the contrary, recurrence rate over-estimated because of smaller tumor size. These observations support the hypothesis that patient survival after ablation can be similar to that of surgery for tumors < 2 cm; unfortunately the choice of a composite end-point, as DFS is (in which the event is death or recurrence), does not allow a similar conclusion for just recurrence rate.

In a more recent report by Wang *et al*[37], the authors tried to handle the different covariate distribution by means of propensity score one-to-one match. In their sub-analysis of 104 matched patients with single tumor < 2 cm (52 patients for each arm), the authors reported that resection and RFA provide similar patient survival ($P = 0.296$), but that DFS of surgical patients was significantly better than that of RFA patients ($P = 0.031$). Unfortu-

Table 3 Characteristics of randomized controlled studies that compared hepatic resection *vs* radiofrequency ablation

Ref.	Liver function	Tumor features	Treatment	Study characteristics and main findings
Chen *et al*[33]	Child-Pugh class A ICG-R15 < 30% PLT > 40000/mm^3	Single < 5 cm	HR: 90 RFA: 71	21% of patients randomized to RFA withdrew their consent. The 1-, 3-, and 4-year overall survival rates after RFA and surgery were 95.8%, 71.4%, 67.9% and 93.3%, 73.4%, 64.0%, respectively. The corresponding DFS rates were 85.9%, 64.1%, 46.4% and 86.6%, 69%, 51.6%, respectively. Statistically, there was no difference. The 5-year rates were not reported
		Single tumor ⩽ 3 cm	HR: 42 RFA: 37	Authors stated that patient survival and DFS did not change in tumors < 3 cm but survival rates and *P*-values were not provided (only Kaplan-Meier curves were reported)
		Single 3.1-5.0 cm	HR: 48 RFA: 34	Authors stated that patient survival and DFS did not change in tumors between 3.1 and 5.0 cm but survival rates and *P*-values were not provided (only Kaplan-Meier curves were reported)
Huang *et al*[34]	Child-Pugh class A/B ICG-R15 < 20% PLT > 50000/mm^3	Single ⩽ 5 cm or up to 3 nodules < 3 cm	HR: 115 RFA: 115	Despite randomization, RFA patients had higher prevalence of nodules ⩽ 3 cm (P = 0.021). The 3- and 5-year survival rates for the RFA group and the HR group were 69.6%, 54.8% and 92.2%, 75.7%, respectively (P = 0.001). The corresponding RFS rates were 46.1%, 28.7% and 60.9%, 51.3%, respectively (P = 0.017)
		Single tumor ⩽ 3 cm	HR: 45 RFA: 57	The 3- and 5-year survival rates for the RFA group and the HR group were 77.2%, 61.4% and 95.6%, 82.2%, respectively (P = 0.030). Neither DFS nor RFS for this subgroup were provided
		Single 3.1-5.0 cm	HR: 44 RFA: 27	The 3- and 5-year survival rates for the RFA group and the HR group were 66.7%, 51.5% and 95.5%, 72.3%, respectively (P = 0.046). Neither DFS nor RFS for this subgroup were provided
		Multifocal < 3 cm	HR: 26 RFA: 31	The 3- and 5-year survival rates for the RFA group and the HR group were 58.1%, 45.2% and 80.8%, 69.2%, respectively (P = 0.042). Neither DFS nor RFS for this subgroup were provided
Feng *et al*[35]	Child-Pugh class A/B ICG-R15 < 30% PLT > 50000/mm^3	Up to 2 nodules < 4 cm	HR: 84 RFA: 84	The 1- and 3-year survival rates for HR and RFA groups were 96.0%, 74.8% and 93.1%, 67.2%, respectively (P = 0.342). The corresponding RFS rates were 90.6%, 61.1% and 86.2%, 49.6%, respectively (P = 0.122). Results at 5-year not reported (or not reached). On the basis of this lack of evidence, the authors did not include treatment as a variable in multivariate analysis

Other inclusion criteria common to all randomized controlled trials (RCTs): no radiological evidence of invasion into the portal/hepatic vein branches, no extra-hepatic metastases, no previous treatment of hepatocellular carcinoma (HCC), patient should be suitable to be treated by surgical resection and radiofrequency ablation. HR: Hepatic resection; RFA: Radiofrequency ablation; DFS: Disease-free survival; PLT: Platelet.

nately, the match was unconvincing and the inaccuracy of the match procedure is reinforced by the match provided in the same manuscript for patients with tumors < 3 cm, where covariates were still significantly different, after matching, among the two treatment arms (P < 0.001 in some cases)[37]. This work highlights the need for a rigorous statistical approach in the presence of significant covariate differences; without such an approach, the results can remain difficult to interpret with some degree of certainty.

The third report was published by Peng[36] in 2012, and involved 145 patients, submitted to resection, or ablation, for single tumor ⩽ 2 cm. The authors found that overall survival was better after RFA (P = 0.048) but that recurrence-free survival (RFS) was unaffected (P = 0.548). The results are intriguing since, when looking at the baseline characteristics, the two groups were quite similar as regards clinical and demographical covariates, except for lower prothrombin time and platelet count in the RFA arm. Thus, supposing an effect of worse liver function on survival, this would have to be shown in patients undergoing RFA, returning to an under-estimation of survival after ablation. Multivariate regression analyses showed that treatment allocation was the only significant prognostic factor for overall survival (P = 0.046). If a conclusion, regarding comparative analyses in this HCC stage, is to be drawn, it can be said that there is some evidence that for single nodules, not larger than 2 cm, RFA can provide survival similar to that of resection[24]. An increased recurrence rate, however, has to be expected after RFA even if the tumor is small but this could theoretically be the subject of re-treatment, justifying comparable survivals. For very early HCC, dedicated RCTs are warranted.

Single tumors less than or equal to 3 cm

There is greater experience published in the literature when this size threshold was selected as an inclusion criterion. Overall, seven studies were found to analyze ablation *vs* resection in single tumors ⩽ 3 cm, or that included a sub-analysis in this specific tumor stage (Tables 3, 4)[33,34,40-44]. Two of these studies were the previously cited RCTs by Chen *et al*[33] and Huang *et al*[34], which contained a sub-analysis for this specific tumor stage. In the RCT by Chen *et al*[33], the authors stated that both patient survival and DFS did not change in single tumors < 3 cm, but, unfortunately, both survival rates and *P*-values were not provided. The RCT by Huang *et al*[34] reported a survival advantage of surgery: in the subgroups of 45 resected patients *vs* 57 ablated patients with a solitary nod-

Table 4 Characteristics of observational studies that compared hepatic resection *vs* radiofrequency ablation

Ref.	Liver function	Tumor features	Treatment	Study characteristics and main findings
Peng *et al*[36]	Child-Pugh class A	Single tumor ≤ 2 cm	HR: 74 RFA: 71	RFA patients showed lower prothrombin activity (P = 0.001) and lower platelet count (P = 0.010). Other features were similar between the two groups The 3-, and 5-year survival rates were 87.7% and 71.9%, respectively, after RFA and 70.9% and 62.1% after HR (P = 0.048). The corresponding RFS rates were 65.2% and 59.8% with RFA and 56.1%, and 51.3% after HR (P = 0.548)
Wang *et al*[37]	Child-Pugh class A and B	BCLC early stage	HR: 208 RFA: 254	Patient characteristics were considerably different between the two treatments. RFA patients were significantly older, anti-HCV+, in Child-Pugh class B, with lower platelet count, with smaller and multifocal tumors than HR patients (P = 0.001 in all cases) The 3- and 5-year survival rates were 87.8% and 77.2% for HR, and 73.5% and 57.4% for RFA (P = 0.001). The 3- and 5-year DFS rates were 59.9% and 50.8% for HR and 28.3% and 14.1% for RFA, respectively (P < 0.001)
		BCLC early stage after PS match	HR: 208 RFA: 208	Patient characteristics were different between the two treatment arms. RFA patients were significantly older, anti-HCV+, in Child-Pugh class B, with lower platelet count, with smaller and multifocal tumors than HR patients (P = 0.001 in all cases). Patient and DFS rates not provided for this subgroup
		Single tumor < 2 cm	HR: 52 RFA: 91	Patient characteristics were different between the two treatment arms. RFA patients were significantly older, anti-HCV+, with lower platelet count than HR patients (P < 0.050). No Child-Pugh stratification was provided The 3- and 5-year survival rates were 98% and 91.5% for HR, and 80.3% and 72% for RFA (P = 0.073). The 3- and 5-year DFS rates were 62.1% and 40.7% for HR and 39.8% and 29.3% for RFA, respectively (P = 0.006)
		Single tumor < 2 cm after PS match	HR: 52 RFA: 52	Patient characteristics seem similar between the two treatments. The 3- and 5-year survival rates were 98% and 91.5% for HR, and 82.8% and 82.8% for RFA, respectively (P = 0.269). The 3- and 5-year DFS rates were 62.1% and 40.7% for HR and 46.8% and 38.0% for RFA (P = 0.031)
Ruzzenente *et al*[47]	Child-Pugh class A and B	Up to 3 tumors ≤ 6 cm after PS match	HR: 88 RFA: 88	Patient characteristics seem similar between the two treatments. The 3- and 5-year survival rates were 68.7% and 59.3% for HR, and 50.1% and 27.7% for RFA (P = 0.012). The 3- and 5-year DFS rates were 50.4% and 27.1% for HR and 30.2% and 18.6% for RFA, respectively (P = 0.001)
	Child-Pugh class A and B	Single tumor < 5 cm	HR: 45 RFA: 40	The 3- and 5-year survival rates were 66.1% and 54.5% for HR, and 63.7% and 43.8% for RFA (P = 0.633). The 3- and 5-year DFS rates were 42.4% and 22.6% for HR and 30.7% and 23.0% for RFA, respectively (P = 0.644). Patient and disease-free survival after HR were significantly superior to RFA, in patients with tumors ≥ 5 cm Further stratifications lead to very small groups (n < 10)
Nishikawa *et al*[42]	Child-Pugh class A and B	Single tumor ≤ 3 cm	HR: 78 RFA: 92	RFA patients had smaller tumors (P = 0.001) and lower platelet count (P = 0.004) in comparison to HR patients The 5-year overall survival rates after RFA and HR were 63.1% and 74.6%, respectively (P = 0.259). The corresponding RFS rates were 18.0% and 26.0%, respectively (P = 0.324). In the multivariate analysis treatment was not an independent risk factor for overall and RFS
Hung *et al*[38]	Child-Pugh class A and B	Up to 3 tumors ≤ 5 cm	HR: 229 RFA: 190	RFA patients were significantly older, anti-HCV+, with lower albumin and platelet count (P < 0.050) in comparison to HR patients The 3- and 5-year survival rates were 88.2% and 79.3% for HR, and 77.3% and 67.4% for RFA, respectively (P = 0.009). The 3- and 5-year RFS rates were 56.1% and 40.9% for HR and 29.0% and 20.5% for RFA (P = 0.001)
		Up to 3 tumors ≤ 5 cm after PS match	HR: 84 RFA: 84	Patient characteristics seem similar between the two treatments Patient and DFS rates not provided but only reported in Kaplan-Meier graphs. For patient survival no difference was found (P = 0.519); RFS was significantly worse after RFA (P < 0.001)
		Single tumor < 2 cm	HR: 50 RFA: 66	RFA patients were significantly older, anti-HCV+, with lower albumin and platelet count, higher bilirubin, AST and ICG-R15 and with smaller tumors (P = 0.001) in comparison to HR patients The 3- and 5-year survival rates were 91.1% and 84.6% for HR, and 86.5% and 77.8% for RFA, respectively (P = 0.358). The 3- and 5-year RFS rates were 42.6% and 21.8% for HR and 59.5% and 45.2% for RFA (P = 0.104)
Takayama *et al*[39]	Child-Pugh class A and B	Single tumor ≤ 2 cm	HR: 1235 RFA: 1315	Data from the Liver Cancer Study Group of Japan database. Results were reported in the form of brief communication. RFA patients were significantly more frequently in Child-Pugh class B, had higher ICG-R15 and smaller tumor size (P = 0.001 in all cases) in comparison to HR patients The 1- and 2-year survival rates were 98% and 94% for HR, and 99% and 95% for RFA, respectively (P = 0.280). The 1- and 2-year DFS rates were 91% and 70% for HR and 84% and 58% for RFA, respectively (P = 0.001) Multivariate analysis on DFS confirmed alpha-fetoprotein, therapy and Child-Pugh class as independent factors

Ueno *et al*[41]	Child-Pugh class A and B	BCLC early stage	HR: 123 RFA: 155	RFA patients were significantly more frequently in Liver Damage class B or C, had higher ICG-R15, MELD score and smaller tumor size (P = 0.001 in all cases) in comparison to HR patients The 3- and 5-year survival rates were 92% and 80% for HR, and 92% and 63% for RFA, respectively (P = 0.06). The 3- and 5-year DFS rates were 47% and 38% for HR and 36% and 20% for RFA (P = 0.02)
		Single tumor ⩽ 3 cm	HR: 78 RFA: 92	The 3- and 5-year survival rates were 95% and 95% for HR, and 90% and 60% for RFA, respectively (P = 0.01). The 3- and 5-year DFS rates were 56% and 44% for HR and 37% and 11% for RFA (P = 0.02)
		Single tumor 3.1-5.0 cm	HR: 32 RFA: 9	The 3- and 5-year survival rates were 92% and 72% for HR, and 73% and 73% for RFA, respectively (P = 0.15). The 3- and 5-year DFS rates were 33% and 25% for HR and 14% and 14% for RFA (P = 0.12)
		2 or 3 nodules ⩽ 3 cm	HR: 13 RFA: 54	The 3- and 5-year survival rates were 67% and not reached for HR, and 93% and 63% for RFA, respectively (P = 0.002). The 3- and 5-year DFS rates were 29% and not reached for HR and 35% and 22% for RFA (P = 0.59)
Abu-Hilal *et al*[48]	Child-Pugh class A and B	Single tumor ⩽ 5 cm	HR: 34 RFA: 34	This was a matched analysis for age, sex, tumor size, and Child-Pugh grade The 5-year survival was 56% for HR, and 57% for RFA (P = 0.302). The 5-year DFS was 28% for HR and 21% for RFA (P = 0.028)
Guglielmi *et al*[43]	Child-Pugh class A and B	Up to 3 tumors ⩽ 6 cm	HR: 91 RFA: 109	RFA patients were significantly older, belonged more frequently to Child-Pugh class B and more frequently had multinodular tumors (P = 0.010) in comparison to HR patients The 3- and 5-year survival rates were 64% and 48% for HR, and 42% and 20% for RFA, respectively (P = 0.010). The 3- and 5-year DFS rates were 56% and 27% for HR and 22% and 22% for RFA (P = 0.001) Superiority of HR was confined to patients in Child-Pugh class A. Further stratifications resulted in groups of patients not large enough (n < 10) to obtain realistic comparisons Type of treatment was significantly related to survival and DFS at multivariate analyses
	Child-Pugh class A	Single tumor ⩽ 3 cm	HR: 20 RFA: 11	The 3- and 5-year survival rates were 93% and 71% for HR, and 50% and not reached for RFA, respectively (P = 0.060)
	Child-Pugh class A	Single tumor > 3 cm	HR: 33 RFA: 23	The 3- and 5-year survival rates were 64% and 55% for HR, and 63% and 45% for RFA, respectively (P = 0.700)
Hiraoka *et al*[40]	Child-Pugh class A and B	Single tumor ⩽ 3 cm	HR: 59 RFA: 105	RFA patients belonged more frequently to Child-Pugh class B (P = 0.011), more frequently had tumors < 2 cm (P = 0.001), and had worse ICG-R15 (P = 0.026) in comparison to HR patients The 3- and 5-year survival rates were 91.4% and 59.4% for HR, and 87.8% and 59.3% for RFA, respectively (P = NS). The 3- and 5-year DFS rates were 64.3% and 22.4% for HR and 58.7% and 24.6% for RFA (P = NS) No multivariate analysis provided
Hasegawa *et al*[46]	Child-Pugh class A and B	Up to 3 tumors ⩽ 3 cm	HR: 2857 RFA: 3022	Data were analyzed together with a population of 1306 patients submitted to percutaneous ethanol injection. RFA patients were significantly older, belonged more frequently to Child-Pugh class B, had lower serum albumin, higher bilirubin, worse ICG-R15 and more frequently had multinodular and smaller tumors (P < 0.001 in all cases) in comparison to HR patients Results were limited to 24 mo. The 1- and 2-year survival rates were 98.3% and 94.5% for HR, and 98.5% and 93.0% for RFA, respectively (P = 0.640) The 1- and 2-year recurrence rates were 17.0% and 35.5% for HR and 26.0% and 55.4% for RFA (P < 0.001) At multivariate analysis, type of treatment did not affect overall survival but affected recurrence rate
Lupo *et al*[45]	Child-Pugh class A and B	Single tumor 3-5 cm	HR: 42 RFA: 60	The groups were similar in terms of median age, Child-Pugh score and tumor size The 3- and 5-year survival rates were 57% and 43% for HR, and 53% and 32% for RFA, respectively (P = 0.824). The 3- and 5-year DFS rates were 35% and 14% for HR and 18% and 0% for RFA (P = 0.283) No multivariate analyses were performed
Ogihara *et al*[49]	Child-Pugh class A and B	Single tumor without size limit	HR: 47 RFA: 40	RFA patients were significantly older, belonged more frequently to Child-Pugh class B and had smaller tumors (P < 0.001 in all cases) in comparison to HR patients The 3- and 5-year survival rates were 65% and 31% for HR, and 58% and 39% for RFA, respectively (P = NS). DFS not provided. No multivariate analysis was provided
	Child-Pugh class A and B	Single tumor ⩽ 5 cm	HR: 18 RFA: 26	In these subgroups, RFA patients were still significantly older and belonged more frequently to Child-Pugh class B (P < 0.050) in comparison to HR patients The 3- and 5-year survival rates were 64% and 21% for HR, and 53% and 32% for RFA, respectively (P = NS). The 3- and 5-year DFS rates were 37% and 37% for HR and 31% and 23% for RFA (P = NS) Results did not change in single tumors > 5 cm
Montorsi *et al*[50]	Child-Pugh class A and B	Single tumor ⩽ 5 cm	HR: 40 RFA: 58	All RFA were performed with laparoscopic approach. RFA patients had significantly worse INR and higher AST (P < 0.050). A trend toward higher bilirubin, lower platelet count and higher ALT was also reported (P < 0.10)

				The 3- and 4-year survival rates were 73% and 61% for HR, and 61% and 42% for RFA, respectively (P = 0.139). The RFS rates were not reported and only plotted in a Kaplan-Meier curve reporting a P = 0.024. Five-year rates not reported. Multivariate analysis on survival did not include the primary exposure variable (HR *vs* RFA)
Hong *et al*[51]	Child-Pugh class A	Single tumor ⩽ 4 cm	HR: 93 RFA: 55	RFA patients were significantly older (P < 0.001) but the other characteristics reported were not statistically different between the two groups The 1- and 3-year survival rates were 97.9% and 83.9% for HR, and 100% and 72.7% for RFA, respectively (P = 0.24). The 1- and 3-year RFS rates were 75.9% and 54.7% for HR and 74.1% and 40.2% for RFA (P = 0.54). Five-year rates not reported. Results did not change when patients were stratified by AJCC or CLIP stages No multivariate analyses were performed
Vivarelli *et al*[44]	Child-Pugh class A and B	No inclusion criteria specified	HR: 79 RFA: 79	RFA patients belonged more frequently to Child-Pugh class B and more frequently had multinodular tumors (P < 0.001 in both cases) The 1- and 3-year survival rates were 83% and 65% for HR, and 78% and 33% for RFA, respectively (P = 0.002). The 1- and 3-year DFS rates were 79% and 50% for HR and 60% and 20% for RFA (P = 0.001). Five-year rates not reported. No multivariate analyses were performed
	Child-Pugh class A and B	Single tumor ⩽ 3 cm	HR: 21 RFA: 22	The 1- and 3-year survival rates were 89% and 79% for HR, and 89% and 50% for RFA, respectively (P = NS). The 1- and 3-year DFS rates were 84% and 67% for HR and 70% and 34% for RFA (P = NS). Five-year rates not reported
	Child-Pugh class A and B	Single tumor > 3 cm	HR: 58 RFA: 57	The 1- and 3-year survival rates were 81% and 59% for HR, and 74% and 24% for RFA, respectively (P = 0.007). The 1- and 3-year DFS rates were 77% and 43% for HR and 56% and 12% for RFA (P = 0.003). Five-year rates not reported. These differences were confirmed when the analyses were confined to Child-Pugh class A patients

HR: Hepatic resection; RFA: Radiofrequency ablation; RFS: Recurrence-free survival; DFS: disease-free survival; PS: Propensity score; AST: Aspartate aminotransferase; NS: Not significant; BCLC: Barcelona Clinic Liver Cancer; HCV: Hepatitis C virus; AJCC: American Joint Committee on Cancer; CLIP: Cancer of the Liver Italian Program; MELD: Model for end-stage liver disease.

ule ⩽ 3 cm, the 5-year survival after surgery was 82.2%, significantly higher than the 61.4% after RFA of (P = 0.030). Disease-free or recurrence-free survivals were not analyzed. One limitation is represented by the fact that covariate distribution among the two treatment arms was not provided for these specific subgroups of patients; however, since in the whole study population tumor size was the only variable that proved to be slightly different among the two groups, this sub-analysis seems quite realistic and is, at present, the most robust evidence of the superiority of one treatment (surgery) over the competing one (ablation)[34].

Similar comments regarding covariate distribution, made for single tumors < 2 cm, can be repeated for analyses on single tumors < 3 cm. Of the five retrospective studies found, two series deserve particular discussion. In 2008, Hiraoka published results from a population of 59 surgical and 105 RFA patients: no significant differences were found in terms of both patient survival and DFS[40]. However, the magnitude of the differences observed between the two treatment arms, in terms of Child-Pugh class, ICG-R15, serum albumin, bilirubin, and tumor size that were all in favor of resection, was so large that the comparison was evidently unrealistic. Furthermore, the authors did not provide an inferential analysis, leaving the doubt unsolved[40]. In 2009, Ueno *et al*[41] published a report from the Kagoshima Liver Cancer Study Group reporting that patients with a single nodule ⩽ 3 cm achieved a 5-year survival of 95% after resection (n = 78), significantly higher than that of 60% after RFA (n = 92; P = 0.010), but 75.6% of the resected patients had a Liver damage A whereas 66.3% of ablated patients had a Liver damage B or C (P = 0.001). Stratification of survival for Liver damage returned to non-significant differences in terms of both patient and disease-free survivals and these results did not help clarify, with a convincing degree of evidence, the real superiority of resection over ablation[41]. The remaining studies report results on very small subgroups, often less than 10 patients[42-44], or suffered from wrongful comparison[37], making it hard to consider findings to provide enough degree of evidence.

Single tumors 3-5 cm

Four articles were identified that analyzed comparative results of surgery and ablation in single nodules between 3 and 5 cm or that included a sub-analysis in this specific tumor stage (Tables 3 and 4)[33,34,41,45]. Two of these studies were, again, the RCT by Chen *et al*[33] and the one by Huang *et al*[34], which contained a sub-analysis for this specific tumor stage. In the RCT by Chen *et al*[33], the authors stated that both patient survival and DFS did not change between treatment arms but survival rates and p-values were again not provided. Huang's results reported a 5-year survival after surgery of 72.3% *vs* 51.5% after ablation (P = 0.046); neither DFS nor RFS were provided (Table 3)[34]. Thus, with the limitations of subgroup analyses, the available RCTs reported a limited difference between surgery and ablation for single nodules between 3 and 5 cm. When observational studies were analyzed, the findings became very difficult to interpret. In a subgroup analysis by Ueno *et al*[41] (resection: 32 patients; RFA: 9 patients), no differences were found in terms of either patient survival (5-year rate after resection: 72%; ablation: 73%; P = 0.15) or DFS (5-year rate after resec-

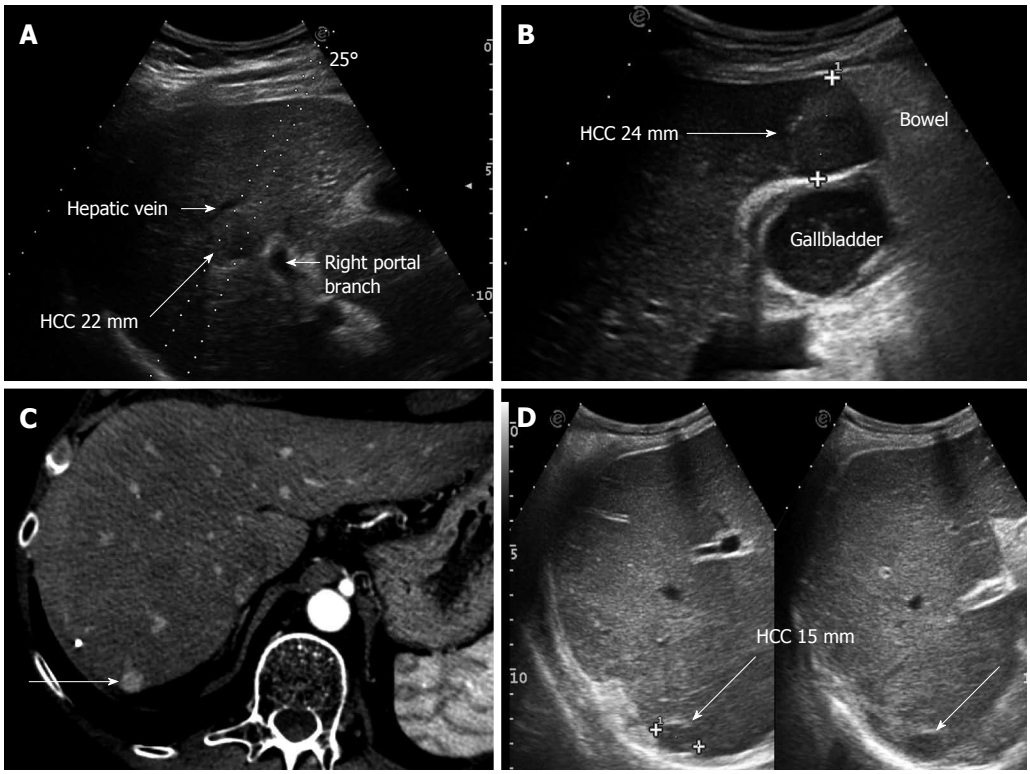

Figure 1 Clinical cases in which performing hepatic resection or radiofrequency ablation had to be decided. A: Small hepatocellular carcinoma (HCC), 22 mm in diameter, located centrally in the right liver lobe in a patient with MELD 10 and clinical signs of portal hypertension. Surgery would have required a right hepatectomy, thus, radiofrequency ablation was preferred even if a reduced rate of complete necrosis could be expected due to the possible heat sink effect of the nearby large vessels; B: The tumor is located sub-capsular, close to the bowel loops and in strict contact with the gallbladder, implying various technical contraindications to percutaneous ablation. Open surgery was the strategy adopted; C: The tumor (long arrow), shown in the arterial phase of contrast enhancement at computed tomography scan, is located sub-capsular at the liver dome; D: Ultrasonography confirms the tumor (long arrow) to lie very deep and without a safe needle track; in fact, these images are taken in deep inspiration, the lesion being hardly visible during normal breathing. The location was considered to contraindicate percutaneous ablation and surgery was performed.

tion: 25%; ablation: 14%; $P = 0.15$) but, as can be immediately noted, the sample size was very small. Another retrospective study published by Lupo *et al*[45] reported that resection and ablation provide very similar results. In particular, the 5-year survival was 43% after resection ($n = 42$) and 32% after ablation ($n = 60$; $P = 0.824$), and the corresponding DFS rates were 14% and 0% ($P = 0.283$). Thus, it must be noted that resection repeatedly leads to better patient survival and recurrence-rate, but the inability to detect a statistical difference between the two treatments leaves the question of the superiority of surgery unsolved. It could be speculated that it is paradoxical for ablation to be inferior to resection for nodules < 3 cm and equivalent for larger tumors, since the ability of RFA to achieve tumor necrosis decreases with the increase in tumor size[19,28,29,46]. Thus, for this single HCC 3-5 cm, it can be said that the literature consistently reports higher patient and disease-free survival rates that do not achieve statistical significance likely only for the small sample size of study populations. This specific tumor stage also probably deserves dedicated studies.

Multiple tumors

The presence of multiple tumors, at diagnostic evaluation prior to treatment, represents the most frequent indication for radiofrequency ablation. Except for the three RCTs and the studies conducted on solitary tumors, multifocal tumor prevalence was almost always higher in ablated patients in comparison to surgical ones[37,43,44,46]. Only two studies reported a subgroup analysis on two or three nodules less than 3 cm, thus within BCLC early stage, excluding single nodules[34,41]. The RCT by Huang reported a survival advantage of surgery ($P = 0.042$): in the subgroups of 26 resected patients *vs* 31 ablated patients with a solitary nodule ⩽ 3 cm, the 5-year survival after surgery was 69.2%, significantly higher than the 45.2% after RFA of[34]. Disease-free or recurrence-free survivals were not analyzed. In the report by Ueno *et al*[41], the 5-year survival was not reached for surgical patients ($n = 13$) and the 3-year survival was in favor of RFA ($n = 54$; $P = 0.002$), while DFS was similar ($P = 0.590$). The difficulty to obtain a comparison within this stage was highlighted by the sub-analysis by Guglielmi *et al*[43] who tried to stratify for Child-Pugh class 11 ablated patients (6 in Child-Pugh class A) *vs* 7 resected patients (all belonging to Child-Pugh class A) without obtaining any reliable result. For multiple tumors, the current comparative literature leaves the impression that the prognosis will be relatively lower despite the treatment adopted.

Other studies

Five studies remain to be briefly discussed[47-51]. The re-

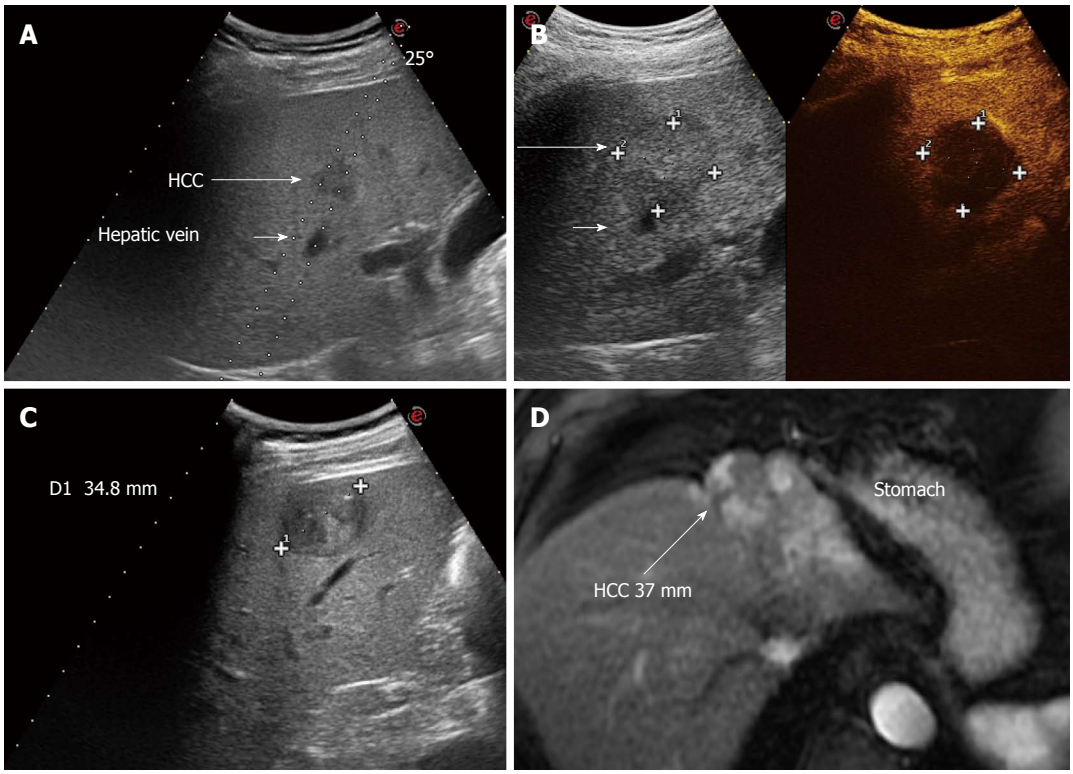

Figure 2 Clinical cases in which performing hepatic resection or radiofrequency ablation had to be decided. A: Ultrasonography through a right inter-costal scan shows a very early hepatocellular carcinoma (HCC) in segment 5 that can be reached with a safe needle track for thermal ablation. Given the small size and easy access, radiofrequency ablation was carried out; B: Post treatment assessment with contrast enhanced ultrasound shows a necrotic devascularized area (34 mm × 35 mm) that includes the tumor with a safety margin > 5 mm; C: Superficial HCC of 35 mm in hepatitis B virus related cirrhosis with preserved liver function. This lesion could be treated by either ablation or resection, but resection is preferable given the superficial location in segment 5 and the size > 3 cm; D: Tumor lesion partially treated by a previous trans-catheter arterial chemoembolization performed in another hospital, in a sub-capsular location close to the stomach. The theoretical path for radiofrequency ablation would lead the needle to puncture the tumor directly and thermal ablation would imply a risk of heat damage to the stomach wall. Laparoscopic resection was the strategy adopted. The long arrow indicates the HCC after treatment.

ports from Ruzzenente *et al*[47], in 2012, and from Abu-Hilal *et al*[48], in 2008, are examples of the attempt to account for confounding variables through matching. The first study used a propensity score match to select patients, submitted to surgery or RFA, having similar covariate distributions[47], and the second used an "a-priori" match based on age, sex, tumor size and Child-Pugh grade[48]. Both studies included tumors larger than 2 cm in both arms and reported an advantage of surgery in determining DFS over ablation but not in terms of patient survival that was similar for single tumors < 5 cm. Of note, the study by Abu-Hilal included only 34 patients for each arm and of the one by Ruzzenente included Child-Pugh B patients. The remaining articles reported results from comparative analyses without tumor size limit[49], or with large (up to 5 cm) size limit, but unfortunately they lack inference analyses[50,51].

DISCUSSION

The present review shows the lights and shadows of the comparative literature regarding hepatic resection and radiofrequency ablation for HCC. It is evident that most studies are affected by questionable methodological approaches since surgical patients and ablated patients represent patient populations that appear quite different as regards clinical and tumor features that are known to affect prognosis. Despite the inconclusive results and the interest in understanding which treatment strategy is best, it is worthwhile pointing out that the situations in which surgery and ablation would be both really equally feasible, and in which they could thus truly compete, occur in less than half of the cases seen in daily clinical practice. In fact, most studies did not report how many patients were excluded from either surgery or ablation, because of the presence of absolute or relative contraindications to one or the other treatment, which might differ in the case of one or the other therapy (thus these patients were most likely offered the alternative therapy). Patients might not be considered suitable for surgery because of liver dysfunction and/or portal hypertension, according to the individual center's strategy, as well as the presence of comorbidities or advanced age contraindicating general anesthesia. Some clinical examples can be found in Figures 1 and 2. In some cases, surgery might not be considered because of the hepatic location of the tumor, which would require very extensive parenchymal sacrifice. Conversely, a subcapsular anterior location exposes the patient to a higher risk of bleeding and/or peritoneal seeding[52], unless a direct puncture of the tumor could be

avoided[53], which is however not always possible. Moreover, complete necrosis of lesions close to the gallbladder is less often possible to achieved because of the potential risk of gallbladder wall damage[26]. Similarly, complete necrosis of lesions abutting the diaphragm may not be possible[27]. Finally, patients with compromised prothrombin time (prolonged International Normalized Radio) are invariably excluded from surgery because this alteration indicates liver dysfunction; similarly, a very low platelet count (< 50.000) is often also considered a contraindication to surgery since it indicates portal hypertension. Such patients should therefore be treated with ablation, as the first alternative therapy, but a clotting impairment might also contraindicate percutaneous ablation or at least increase the risk of adverse events, despite the possibility of preliminary transfusions. All these different variables affecting the choice between resection and ablation most likely justify the difference in the clinical covariates found in the various non randomized studies, as commented above, leading to rather heterogeneous patient populations. This is in keeping with the hypothesis that patients were not randomly allocated to one or the other treatment, but following clear preferences, so that in each case either surgery or ablation was specifically preferred on the basis of clinical variables. Only in rather a few remaining cases of early HCC within the Milan criteria might hepatic resection and radiofrequency ablation be considered truly competitive, and no definitive evidence exists strongly favoring one or the other technique.

However, based on the results reported and commented on above, we can conclude that, until further studies become available, it seems reasonable to offer radiofrequency ablation to very small HCC (< 2 cm) which present an easy access, with no technical contraindications, since in this instance complete necrosis, including the desired safety margin, is most likely to be achieved. At variance, in larger nodules, namely > 2 cm and especially if > 3 cm, and/or in tumor locations in which ablation is not expected to be effective or safe (which often correspond to subcapsular locations, which instead make atypical resections possible), surgical removal is to be preferred in our opinion. For future explorative research, it can be suggested that: (1) intention-to-treat analysis should be included in the studies; (2) further RCTs are warranted, especially for single tumors < 2 cm in which ablation can achieve a sustained pathological response; (3) retrospective observational studies should include in their analyses an inference approach that includes the primary exposure variable (that is resection *vs* ablation) regardless of its statistical difference at univariate analysis; and (4) retrospective observational studies should include stratification for tumor size and liver degree dysfunction together with an attempt at matching, as propensity score can provide.

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REVIEW

Epithelial toll-like receptor 9 signaling in colorectal inflammation and cancer: Clinico-pathogenic aspects

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Abstract

Toll-like receptors (TLRs) recognize specific motifs which are frequently present in bacteria, fungi, prokaryotes and viruses. Amongst TLRs, TLR9 can be activated by such bacterial or viral DNA fragments, immunoglobulin-DNA complexes or synthetic oligonucleotides, which all contain unmethylated cytosine-guanine nucleotide sequences (CpGs). Emerging data indicate that TLR9 signaling has a role in, and may influence, colorectal carcinogenesis and colonic inflammation. CpGs are classified into three groups according to their influence on both the antigen-specific humoral- and cellular immunity, and the production of type 1 interferons and proinflammatory cytokines. TLR9 activation *via* CpGs may serve as a new therapeutic target for several cancerous and various inflammatory conditions. Due to its probable anti-cancer effects, the application possibilities of TLR9-signaling modulation may be extremely diverse even in colorectal tumors. In this review we aimed to summarize the current knowledge about TLR-signaling in the pathogenesis and therapy of inflammatory bowel diseases and colorectal cancer. Due to the species-specific differences in TLR9 expression, however, one must be careful in translating the animal model data into the human system, because of the differences between CpG-oligodeoxynucleotide-responsive cells. TLR9 agonist DNA-based immunomodulatory sequences could also represent a promising therapeutic alternative in systemic inflammatory conditions and chronic colonic inflammations as their side effects are not significant.

Key words: Toll-like receptor 9; Synthetic oligodeoxynucleotide sequences; DNA-based immunomodulatory sequences; Colorectal cancer; Inflammatory bowel diseases

Core tip: Toll-like receptor 9 mediated signaling influences and regulates the severity of mucosal inflammation, and seems to have a protective role against malignant transformation. The modulation of toll-like receptor 9 signaling by synthetic oligodeoxynucleotide agonists or antagonists seems to have beneficial therapeutic effect in inflammatory and cancerous colonic disorders.

Fűri I, Sipos F, Germann TM, Kalmár A, Tulassay Z, Molnár B, Műzes G. Epithelial toll-like receptor 9 signaling in colorectal inflammation and cancer: Clinico-pathogenic aspects. *World J Gastroenterol* 2013; 19(26): 4119-4126 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i26/4119.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i26.4119

INTRODUCTION

The immunostimulatory effect of DNA was discovered by William Coley, a surgeon from New York[1]. He used living and heat-treated bacteria as a therapeutic option for different kinds of tumors. It has long been known that

microbes contain many immunostimulatory ingredients. In 1980, Tokunaga *et al*[2] identified the bacterial DNA as a main ingredient of the Coley-lysate. Later, they also showed that the same immunostimulatory effect could be achieved by using short synthetic oligodeoxynucleotide sequences (ODNs)[3,4]. In 2000, it was proven that DNA sequences are mainly recognized by the members of the Toll-like receptor (TLR) superfamily[5]. It has also been proven that in TLR9 knock out mice, microbial DNA fragments cannot result in an immune response[5]. It was also shown that the immunomodulating effect of natural and synthetic ODNs is mainly transmitted by TLR9[6].

Peyer's patches (PPs) and isolated lymphoid follicles (ILFs) are immunologic and regenerative organizers of the gut mucosa, and they also represent a unique switch point between colonic inflammation and cancer[7]. Microfold (M) cells are located in the follicle associated epithelium (FAE) of PPs and ILFs, where they mediate the uptake and transcytosis of luminal antigens to the underlying lymphoid tissue. TLR9 was found to be preferentially expressed in M cells[8]. Some TLR polymorphisms are known to be associated with the susceptibility of inflammatory bowel diseases (IBD)[9-11] and sporadic colorectal cancer (CRC) development[12,13], but the current and concrete pathogenic role of TLRs, including TLR9, remains uncertain in these conditions.

In this review we aimed to summarize the current knowledge about TLR-signaling in the pathogenesis of IBD and CRC, focusing especially on TLR9. Recent data indicate that targeting TLR9-signaling may yield new and promising therapeutic approaches to these conditions.

LIGANDS AND SIGNALING OF TOLL-LIKE RECEPTORS

TLRs belong to the type 1 transmembrane glycoproteins, which contain extracellular leucin-reach repeated sequences and Toll/interleukin-1 receptor signaling domains. TLR4 was the first to be identified, and currently 10 TLRs have been identified in humans, while 13 have been identified in mice[14]. TLRs are mainly expressed in the cells of the innate and adaptive immunity (*i.e.*, monocytes, macrophages, lymphocytes, mast cells, dendritic cells), however, some (TLR4, -5 and -9) may be expressed by modified epithelial cells as well[15]. Apical epithelial TLR9 activation by bacterial DNA fragments has been reported to maintain colonic homeostasis[16].

TLRs usually recognize microbial wall components, DNA and ribonucleic acid (RNA) fragments. TLR1, -2, -4, -5, and -6 are localized to the cell surface, while TLR3, -7, -8, and -9 are present in the intracellular compartment[17-20]. TLRs bind specific motifs, which frequently appear in bacteria, fungi, protozoa, and viruses[21,22]. These motifs can be lipids and lipopeptides (TLR1, -2, -4, -6), bacterial flagelline (TLR5), and fragments of nucleic acids (TLR3, - 7, -8, -9). TLR3 binds double stranded RNA from viruses, while TLR7 and -8 can recognize single stranded RNAs. Moreover, TLR7 recognizes immunoglobuline-self-RNA complexes in autoimmune disease conditions. Imiquimod is a specific ligand for TLR7. TLR9 could be activated by bacterial and viral DNA, immunoglobuline-DNA complexes, and synthetic ODNs, which contain unmethylated CpG sequences[21,22].

The signals transmitted by TLRs activate both innate and adaptive immunity. Due to the immune evasion nature of tumor cells, the dysregulated activation of adaptive and innate immune systems could result in cytotoxic effects. This could in turn eradicate the diseased cells or even control the tumorous progression. TLRs recognize pathogen-associated molecular patterns (PAMPs) originating from microbiota and could also bind endogenous ligands, such as danger-associated molecular patterns (DAMPs)[23]. Both bacterial DNA and synthetic ODNs activate the innate and adaptive immune system *via* plasmacytoid dendritic cells (pDCs) and macrophages[24].

TOLL-LIKE RECEPTOR 9 SIGNALING

Due to TLR9-associated activation, pDCs produce interferon-α which influences the cytokine production of B cells[24] resulting in pro- (*e.g.*, interleukin 6, tumor-necrosis factor-α) and anti-inflammatory (*e.g.*, interleukin 10) cytokine release and co-expression of MHC Ⅱ type surface antigens.

The activation of TLR9 is a complex pathway. The uptake of DNA sequences is the most unclear process, which is influenced by the structure of the DNA fragments. Many cell types can easily take up single stranded DNA, but the uptake of double stranded DNA may be more effective if a cationic lipid is used for packing it in, because TLR9 is located in the intracellular compartment of endosomes[24,25]. It was shown that fluorescein isothiocyanate labelled CpG DNA is transferred to the intracellular compartment by non-specific endocytosis[25]. This transport is non-specific, because DNA sequences lacking CpG dinucleotides may also be recognized by TLR9, and this way of immune activation can be competitively inhibited by non-CpG sequences[25]. After transportation to the intracellular compartment, endosomal acidic maturation occurs. This process may be inhibited by pH raising agents (*e.g.*, chloroquine, bafilomycin A)[25]. Finally pro- and anti-inflammatory cytokines may be released and B cell proliferation may be enhanced[25]. The main steps of TLR9-signaling are summarized in Figure 1.

The signal molecules of this pathway [*e.g.*, myeloid differentiation primary response gene 88 (Myd88), tumor necrosis factor receptor-associated factor 6 (TRAF6), interleukin receptor associated kinase (IRAK)-1, -4; p50/p65 heterodimer of nuclear factor (NF)-κB] are non-specific and are also involved in the signaling of other TLRs. Interferons may also be released by a mitogen activated protein kinase (MAPK)-associated pathway, which is also intensively being investigated[26].

CPG OLIGODEOXYNUCLEOTIDE CLASSES

The immunostimulatory effect of unmethylated CpG

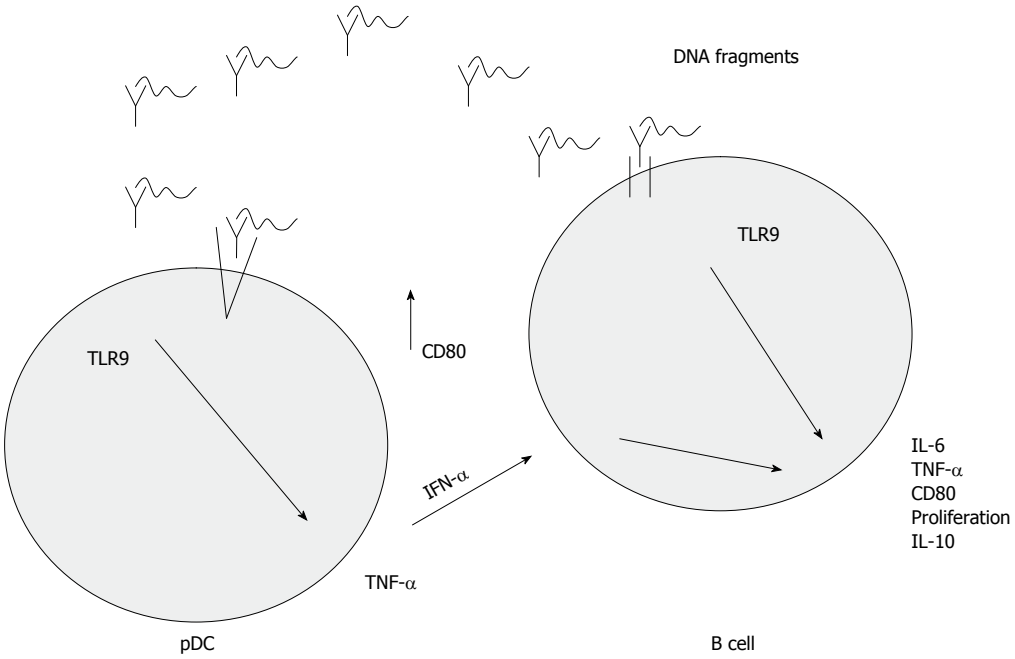

Figure 1 Toll-like receptors 9-mediated cytokine release in the colonic mucosa. Binding DNA fragments by toll-like receptors (TLRs) of plasmacytoid dendritic cells (pDCs) results in pro-inflammatory cytokine release and subsequent B cell activation together with both CD80 overexpression and B cell proliferation. CD80 provides a costimulatory signal necessary for T cell activation and survival. IFN: Interferon; TNF: Tumor necrosis factor; IL: Interleukin.

sequences has been proven in mice and in other species, as well as in *in vitro* human cell line experiments[27,28]. The CpG DNA sequences can be classified into three classes based on the different immune cell-mediated immune responses and their chemical structure. It has already been documented how these differences in the chemical structure may determine the immunostimulatory effect of these sequences on immune cells[29]. Liu *et al*[29] demonstrated in mice that three CpG-ODN classes can differentially affect antigen-specific humoral and cellular immune responses. Specifically, the B- and C-class CpG-ODNs induced a potent Th1-mediated immunity with comparable antibody levels as well as CD4+ and CD8+ T cell responses. In contrast, although the A-class CpG-ODNs weakly enhanced antibody titers and CD8+ T cell response regarding cytotoxic activity, they were not able to change the IgG1/IgG2a ratio or elicit antigen-specific, interferon γ-secreting CD4+ and CD8+ T cells. Consistent with this, three CpG-ODN classes provided differential antigen-specific protection against an intracellular bacterial infection (*i.e.*, Listeria monocytogenes). These three classes of CpG-ODNs did not show significant differences regarding the interleukin 12 producing effect[30,31]. These results may provide not only better understanding of the adjuvant activities of three CpG-ODN classes, but also of implications for the rational design of CpG-ODN adjuvants.

CONTRIBUTION OF TOLL-LIKE RECEPTOR 9 POLYMORPHISM TO DISEASE DEVELOPMENT

Components of Gram-negative bacterial cell walls alert the host to invading bacteria and activate innate immunity. These responses are usually effective in combating infection and restoring normal host function. However, in individuals susceptible to IBD, they may become excessive and lead to mucosal damage.

In genes for all the contributing proteins, single nucleotide polymorphisms (SNPs) have been identified that may increase IBD susceptibility[32]. There are several lines of sensing bacterial components (described earlier), all of which result in activation of NF-κB, and thereby stimulate the innate immune response. In genes of TLR9 signaling, SNPs have been found that may increase IBD susceptibility[9,33]. Török *et al*[9] reported that a SNP in the promoter region of the TLR9 gene was associated with increased risk of Crohn's disease in a German cohort. These genetic findings confirm an important role for innate immunity, pro- and anti-inflammatory immune responses for both gut homeostasis and the development of chronic inflammation in IBD.

Regarding the connection between polymorphisms in TLR9 genes and the risk of colorectal cancer no data are currently available.

ROLE OF TOLL-LIKE RECEPTOR 9 SIGNALING IN COLONIC INFLAMMATION AND CARCINOGENESIS

The commensal microbiota of the intestinal tract confer multiple health benefits to the host, including amelioration of IBD. It was recently identified that TLR9-induced type 1 interferons mediate the anti-inflammatory effects in experimental colitis[34]. The addition of neutralization antibodies to type 1 interferons abolished the anti-inflam-

matory effects, whereas the administration of recombinant interferon-β mimicked the anti-inflammatory effects induced by TLR9 agonists.

The relapse of IBD may occur following an infection with *Campylobacter jejuni* (*C. jejuni*). In a murine model of dextran sulfate sodium (DSS) induced colitis, the infection of the animals by *C. jejuni* disrupted TLR9-induced reinforcement of the intestinal epithelial barrier and colonization by *C. jejuni* increased the severity of DSS-induced colitis[35].

In humans, the gene expression and protein expression level of not just TLR2, -4, and -8, but also TLR9 increased in the biopsy samples of active ulcerative colitis patients. Furthermore, the levels of these TLRs positively correlated with the severity of intestinal inflammation as well as with inflammatory cytokine production[36]. Based on these results, it is plausible that TLR9 mediated signaling influences and regulates the severity of the mucosal inflammation.

In colonic carcinogenesis the role of TLR9 signaling is not well studied. It was recently published that ODNs targeting TLR9 oppositely modulate DNA repair genes in tumor versus immune cells and enhance the biologic effects of chemotherapy[37]. The first publication about the relation of TLR9 expression to colonic carcinogenesis was also published nowadays[38]. Eiró *et al*[38] found TLR9 expression to be higher in hyperplastic or adenomatous polyps compared to other polyp types. TLR9 expression was decreased in hyperplastic and villous polyps from patients who developed colorectal cancer. Their findings suggest a possible protective role of TLR9 expression against malignant transformation in the colorectal mucosa.

THERAPEUTIC POTENTIAL OF TOLL-LIKE RECEPTOR AGONISTS

The therapeutic targeting of TLRs may be useful in diseases such as tumors, allergies or viral infections. In these disorders, TLR agonists and antagonists result in a different immune response. In allergic diseases, like asthma or inflammatory conditions, such as IBD, these agents have an important effect on T cells. For a wider spectrum of anti-tumoral immune response TLR agonists in tumorous diseases require the involvement of the innate immunity, pDCs, monocytes and macrophages, as well as the activation of Th1-dependent immunity and induction of apoptosis[28].

Toll-like receptor agonists in colorectal cancer

In 2011, Rosa *et al*[39] demonstarted that an immunomodulatory oligonucleotide sequence (IMO) in combination with cetuximab has an antitumorous effect on a K-ras mutated colorectal carcinoma model. This is probably based on the alteration of MAPK phosphorylation and results in structural and functional changes in the relationship between epidermal growth factor receptor (EGFR) and TLR9[39]. They used a synthetic IMO having free 5' end. The CpG DNA sequence had dimer structure, where the 3-3' ends were connected by glycerin or 2'-deoxy-7-deazaguanosine modification. Mutation of the K-ras gene has a critical role in colon, lung and pancreatic cancers, and may cause a resistance to anti-EGFR therapy[40,41]. This is the reason why panitumumab and cetuximab therapy do not show a positive effect on the control of proliferation and metastasis of K-ras mutated colon cancer. This kind of biologic therapy could be only useful in the case of patients carrying the wild type K-ras gene[40].

It was shown in an *in vivo* murine xenograft model and *in vitro* human cancer cell lines (GEO, SW48 and LS174T) that IMOs can restore the therapy sensitivity for K-ras mutant colon and pancreatic cancers[40]. These cell lines, except GEO, were resistant for EGFR inhibition therapy, if they had a K-ras mutation. A small number of GEO cells carrying K-ras mutations showed sensitivity to anti-EGFR antibodies. This demonstrates that cells could carry a different K-ras mutation and could respond to EGFR inhibition therapy in a different way based on their K-ras status[39-41].

TLR9 agonists were also tested on a breast cancer cell line which was estrogen receptor positive[42]. After estrogen-TLR9 agonist combination the test showed significant reduction of transactivation *via* the estrogen receptor. Estrogen receptors may also take part in colorectal carcinogenesis[43,44], therefore, this interaction may have further therapeutic importance in colorectal cancer as well.

Currently, TLR9 agonist therapy has been tested clinically on colon, pancreatic and breast cancers[45-48], and experiments are running on oesophageal squamous cell cancer[49], melanomas[50], lymphomas[51,52], non-small cell lung carcinomas[53], renal tumors[47] and androgen resistant prostate cancers[54].

Toll-like receptor agonists in inflammatory bowel disease

It has long been known that in IBD patients, antibodies against own or microbial antigens can be detected. Antibodies against Saccharomyces cerevisiae, outer membrane porin, Pseudomonas fluorescens, pancreas, bacterial flagelline as well as anti-chitobioside-, anti-laminaribioside-, and anti-mannobioside antibodies[55] have all been identified. These antibodies are recognized by PAMP and DAMP receptors. The most important members of these receptor families are the nucleotide oligomerization domain (NOD) - caspase recruitment domain (CARD) system (mainly NOD2 receptor in Crohn's disease) and TLRs. These receptors are localized in the intestinal mucosa, and by increased activation and genetic polymorphisms these receptors create an excessive immune response. At the end of the pathway pro-inflammatory cytokines are released, regulatory T cells are thought to loose their control function and the Th1/Th17 cell subpopulation becomes over-expressed[55,56-58].

Rachmilewitz *et al*[56] used IMOs in a DSS-induced

colitis mouse model and found decreased IL-6, IL-12 and interferon mRNA levels. The levels of matrix metalloproteinases were found to be proportionally decreased. The immunological, clinical, biochemical and histological results showed decreased activity index of the inflammation. From these data one could suggest that the continuous presence of bacteria and bacterial DNA, which densely contain non-methylated CpG sequences, may act as a physiological factor. Furthermore, they could influence the release of inflammatory cytokines in IBD and thus may serve as a therapeutic tool[57].

A newly developed therapeutic agent is a synthetic DNA-based immunomodulatory sequence (DIMS0150), which acts through TLR9 signaling[57]. Based on the results of clinical trials, DIMS0150 seems to restore the steroid sensitivity of the mucosa in steroid-resistant ulcerative colitis patients. In the third phase of clinical trials, 71% of patients achieved remission after 12 weeks of administration of this drug[57]. Although it has no notable side effects, the mode of its administration, namely it has to be spread over the inflamed mucosa with the help of a spray catheter during colonoscopy, makes its use widely intolerable for patients. New ways of drug administration (*i.e.*, colon solvent capsules) must be developed in the near future.

Based on the results of clinical trials[45-54], TLR9 agonists are therapeutically safe *in vivo*. Only some minor side effects, mainly a dose-dependent local inflammation of the connective tissue were observed.

THERAPEUTIC POTENTIAL OF TOLL-LIKE RECEPTOR 9 ANTAGONISTS

Due to complex signaling of oligodeoxynucleotide binding TLRs (including TLR9) a dynamic regulation of pro- and anti-inflammatory cytokines is present[59]. Therefore, TLR9 antagonists and inhibitory oligodeoxynucleotides (inh-ODNs) also may represent new therapeutic options[60] in the treatment of autoimmune diseases. The mechanism of their action is by controlling and blocking the dangerous immune response activated by the self-antigen recognizing receptors. Interestingly, inh-ODNs have TLR9 (and TLR7) antagonist activity, but this effect is sequence dependent. These inhibitory oligonucleotides competitively inhibit TLR9 activation in a manner that competitively antagonizes the binding of ligands to the active, proteolytically cleaved TLR9 sequence. Their therapeutic use shows promise in systemic autoimmune diseases, DNA-mediated sepsis, and chronic inflammatory conditions (*e.g.*, IBD) in which TLR9 plays an important role[60].

SPECIES-SPECIFIC DIFFERENCE IN TOLL-LIKE RECEPTOR 9 EXPRESSION

In the majority of *in vivo* studies mice were used as animal models for showing that CpG-ODNs are effective both as adjuvants and for therapeutic intervention in infectious and tumour model systems. However, one must be careful in translating the murine data into the human system, because of the differences between CpG-ODN-responsive cells in mice and humans. One major and important difference between mice and humans refers to the expression pattern of TLR9. In humans, only pDC and B cells express TLR9 and respond directly to TLR9 activation. All other effects of TLR9 ligands on human immune cells seem to be indirect and depend on factors produced by pDCs and B cells[61].

The situation in mice is different because not only pDCs and B cells, but other dendritic cell subsets and macrophages express TLR9 and thus respond directly to TLR9 activation[61]. Given this important species-specific difference in TLR9 expression, mice are not ideal animal models for establishing TLR9-based therapeutic strategies. The natural ligands for TLR9 can be mimicked by special CpG-ODNs[62].

Besides rodent models, a few studies have analysed the immune response of CpG-ODNs in other animals[62]. Guzylack-Piriou *et al*[63] demonstrated that pig pDCs are the main producers of interferon-α in response to certain CpG-ODNs. Importantly, they additionally showed that myeloid DCs and monocytes/macrophages are refractory to CpG-ODNs. Thus, the CpG-ODN responsiveness in pigs seems to mimic the situation in humans, and therefore recommends the pig as an animal model for preclinical studies with CpG-ODN.

CONCLUSION

Since the immunomodulatory effects of TLRs are known, they are the center of biological, immunological and therapeutic research. Most of the research teams are dealing with the potential therapeutic use of TLR9 agonists and antagonists because their use is not restricted to a specific group of patients. They can be widely applied in almost all diseases where dysregulated immunity plays a central role *via* antibody production or phagocytosis by macrophages.

TLR9 plays a central role in both innate and adaptive immunity. The signalling cascade mediated by CpG ODNs is a complicated pathway and contains many steps, including the synthesis of proinflamatory cytokines and the production of interferons, and thus significant activation of pDCs and T-lymphocytes. The activation of TLR9 acts as a new therapeutic modality in bacterial, viral, inflammatory and neoplastic diseases. In inflammatory circumstances, TLR9 agonists act by both decreasing the enormous immune activation, especially in IBD, and setting the balance of the Th1/Th2 immune response. They may have an effect on the suppression of Th1/Th17 overexpression as well. In tumorous conditions, especially in colorectal cancer, these agents were able to restore anti-EGFR therapy sensitivity caused by a K-ras mutation. They also seem to be effective therapeutic agents in estrogen receptor positive breast

cancers, androgen-resistant prostate tumors, melanomas, lymphomas, large cell lung cancers and renal tumors. The side effects of TLR9 agonists are not significant. Further investigations of these new therapeutic modalities may have promising results in the near future.

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ORIGINAL ARTICLE

Hugl-1 induces apoptosis in esophageal carcinoma cells both *in vitro* and *in vivo*

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Abstract

AIM: To determine whether the human giant larvae homolog 1 gene (Hugl-1/Llg1/Lgl1) exerts tumor suppressor effects in esophageal cancer.

METHODS: We constructed a Hugl-1 expression plasmid, pEZ-M29-Hugl1, for gene transfection. We transfected the pEZ-M29-Hugl1 plasmid into Eca109 esophageal cancer cell lines with Lipofectamine 2000 to overexpress Hugl-1. Real-time reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting were performed to determine the effects of the plasmid on Hugl-1 expression. *In vitro* cell proliferation and apoptosis were examined separately by cell counting Kit-8 (CCK-8) assay, flow cytometry, and Western blotting before and after the transfection of the plasmid into Eca109 cells. Cell cycle distribution was assessed with flow cytometry. The effect of Hugl-1 overexpressing on tumor growth *in vivo* was performed with a xenograft tumor model in nude mice. Expression of Hugl-1 in xenograft tumor was analyzed by immunohistochemistry. The transferase-mediated dUTP nick end-labeling (TUNEL) technique was performed to detect and quantitate apoptotic cell.

RESULTS: The transfection efficiency was confirmed with real-time RT-PCR and Western blotting. Our results show that compared with control groups the mRNA levels and protein levels of Hugl-1 in pEZ-M29-Hugl1-treated group were remarkably increased ($P < 0.05$). The CCK-8 assay demonstrated that the growth of cells overexpressing Hugl-1 was significantly lower than control cells. Cell cycle distribution showed there was a G_0/G_1 cell cycle arrest in cells overexpressing Hugl-1 (64.09% ± 3.14% *vs* 50.32% ± 4.60%, 64.09% ± 3.14% *vs* 49.13% ± 2.24%). Annexin V-fluorescein isothiocyanate revealed that apoptosis was significantly increased in cells overexpressing Hugl-1 compared with control group (17.33% ± 4.76% *vs* 6.90% ± 1.61%, 17.33% ± 4.76% *vs* 6.27% ± 0.38%). Moreover, we found that Hugl-1 changes the level of the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax and the activation of both caspase-3 and caspase-9. With a TUNEL assay, we found that Hugl-1 markedly increased the apoptosis rate of Eca109 cells *in vivo* (60.50% ± 9.11% *vs* 25.00% ± 12.25%). It was shown that Hugl-1 represents a significantly more effective tumor suppressor gene alone in a xenograft tumor mouse model. This data suggest that Hugl-1 inhibited tumor growth and induced cell apoptosis *in vivo*.

CONCLUSION: These results suggest that Hugl-1 induces growth suppression and apoptosis in a human esophageal squamous cell carcinoma cell line both *in vitro* and *in vivo*.

Key words: Esophageal squamous cell carcinoma; Human giant larvae homolog 1; Proliferation; Apoptosis

Core tip: In this paper, we constructed a plasmid to express Hugl-1 which has significant homology to the Drosophila tumor suppressor gene lethal giant larvae. The human esophageal squamous cell carcinoma cell line Eca109 was used as the object of study. We found a positive correlation between Hugl-1 expression and cell apoptosis in Eca109 cells both *in vitro* and *in vivo*. These data suggest that Hugl-1 is a tumor suppressor gene in esophageal cancer and may provide a novel target for the treatment of esophageal cancer patients.

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INTRODUCTION

Esophageal cancer has two major histological types: squamous cell carcinoma (ESCC) and adenocarcinoma (EAC)[1,2]. ESCC is one of the most frequently diagnosed cancers in China[3]. It has been well established that surgical treatment can prolong the survival time of cancer patients, yet the 5-year survival rate for ESCC after surgery is still low (ranging 14%-22%)[4]. Most esophageal cancers are diagnosed in the advanced stages[5]. Thus, detecting gene alternations that promote the carcinogenesis process leading to esophageal cancer will have a profound impact on the diagnosis and treatment of the disease.

Lethal giant larvae (lgl), an evolutionarily conserved and widely expressed cytoskeletal protein, is indispensable for the establishment and maintenance of cell polarity and is a regulator of cell proliferation[6,7]. In Drosophila, mutations in three neoplastic tumor suppressor genes, discs large (dlg), scribble (scrib) and lgl, have revealed a link between the regulation of cell polarity and cell proliferation[8-13]. The human homologs of lgl are Hugl-1 (Llgl1) and Llgl2. The Hugl-1 protein shares 62.5% similarity with Lgl[14]. Several studies have shown that Hugl-1 transcripts are reduced or absent in a high proportion of breast cancers, lung cancers, prostate cancers, ovarian cancers, colorectal cancers, melanomas, endometrial cancers and hepatocellular carcinomas[15-19]. These studies have also shown that Hugl-1 may function as a tumor suppressor gene in various cancer types. In ESCC tissue samples, Hugl-1 is notably lower than in normal tissues[20]; however, the effect of Hugl-1 on tumor progression and prognosis in ESCC is not clear.

In the present study, we analyzed Hugl-1 expression in the esophageal carcinoma cell line Eca109 as well as in tissue samples. By using a forced overexpression technique, we explored the biological activity of Hugl-1 and the underlying mechanism *in vitro* and *in vivo*. We demonstrated that Hugl-1 inhibits proliferation in the esophageal carcinoma cell line as well as in ESCC tissue samples and that it promotes apoptosis in esophageal carcinoma cells and xenograft tumors through a mitochondria-related pathway.

MATERIALS AND METHODS

Cells, cell culture

The human ESCC cell line, Eca109, purchased from the China Center for Type Culture Collection (Wuhan Province, China) and cultured in RPMI-1640 medium (Gibco, United States) containing 10% fetal bovine serum (Gibco, United States), in a humidified atmosphere of 5% CO_2 at 37 ℃.

Plasmid construction and purification of cultured Eca109 cells

Hugl-1 expression plasmids were constructed with pEZ-M29 as the vector, Hugl-1 as the expression gene and ampicillin resistance for antibiotic selection (GeneCopoeia, United States). An empty expression plasmid of the same type was used as a control. Eca109 cells were seeded into a 6 cm dish at a density of 5×10^5 cells per well and incubated overnight with 5% CO_2 at 37 ℃. For each transfection, 9 μL of lipofectamine 2000 (Invitrogen, United States) and 3 μg of the Hugl-1 expression plasmid were added to 1 mL of Opti-MEM (Invitrogen, United States) and incubated for 5 min at room temperature. The diluted plasmid and lipofectamine were mixed together and incubated for 30 min before adding them directly to the cells. Eca109 cells overexpressing Hugl-1 were grown in RPMI-1640 medium with 200 μg/mL of G418 for stable clone selection.

Real-time RT-PCR

Total RNA was prepared from the Eca109 cells with TRIzol reagent (Invitrogen, United States) according to the manufacturer's protocol. First-strand cDNA was synthesized using the PrimeScript™ RT reagent kit (Takara, Japan). The isolated RNA (1 μg) was used as template to perform one-step RT-PCR according to the protocol, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. All reactions were conducted in a 25 μL volume.

Real-time RT-PCR was conducted on the resulting cDNA with the SYBR Green method and the AB7500 Real-time RT-PCR system. The sequences of the primer sets used were as follows: forward 5'-AGAAGGCTGGGGCTCATTTG-3' and reverse 5'-AGGGGCCATCCACAGTCTTC-3' for GAPDH (258 bp); forward 5'-GCTGCTTCGATCCCTACAGTGAC-3' and reverse 5'-CGGCACATCCTAAGCTCCAG-3' for Hugl-1 (131 bp). PCR was performed by initial denaturation at 95 ℃ for 30 s followed by 40 cycles of 5 s at 95 ℃, 30 s at 60 ℃ and 1 min at 72 ℃. The threshold cycle (Ct) values of each sample were used in the $2^{-\Delta\Delta CT}$ data analysis method.

Western blotting

Cells were harvested from 6-well culture plates, and

aliquots of cell extracts were separated on an 8%-12% SDS-polyacrylamide gel. The proteins were then transferred to a polyvinylidene difluoride membrane (Millipore, United States) and incubated overnight at 4 ℃ with the following rabbit polyclonal antibodies: anti-Hugl1 (ab39292, Abcam), anti-Bcl2 (SC-492, Santa Cruzc), anti-Bax (5023, Cell Signaling), anti-p21 (2947, Cell Signaling), anti-cyclin D1 (2978, Cell Signaling), anti-survivin (2808, Cell Signaling), anti-caspase9 (9502, Cell Signaling), anti-caspase3 (9662, Cell Signaling), anti-p65 (3037, Cell Signaling), anti-p-p65 (3033, Cell Signaling) or anti-GAPDH (2118, Cell Signaling).

The blots were rinsed three times in TBST and incubated with a 1:10000 diluted goat-anti-rabbit secondary antibody (LICOR, United States) conjugated to horseradish peroxidase for 1 h at room temperature before they were washed extensively with TBST. Finally, the membranes were scanned with a two-color infrared imaging system (Odyssey, LICOR, United States). Membranes were also probed for GAPDH as an additional loading control.

Cell proliferation analysis

Cells were seeded into 96-well plates at a density of 3000 cells per well 48 h after transfection. The effects of let-7a on cell proliferation were examined with CCK-8 (Dojindo, Japan) according to the manufacturer's instruction 0, 24, 48, 72 and 96 h after seeding.

Cell cycle analysis

Cell cycle analysis was performed with flow cytometry (BD FACS Aria Ⅲ, United States). Cultured cells were harvested 48 h after transfection with pEZ-M29-eGFP and pEZ-M29-Hugl1, respectively, washed with ice-cold phosphate buffered solution (PBS), and fixed in 70% ethanol overnight at 4 ℃. After centrifugation at 500 × *g* for 5 min at 4 ℃, the cell pellets were stained with 10 μg/mL propidium iodide (PI) and 10 μg/mL RNase A in phosphate buffered saline (PBS) buffer for 20 min at room temperature in the dark. Cell cycle analysis was performed with three independent experiments.

Flow cytometric analysis of apoptotic cells using Annexin V-fluorescein isothiocyanate kit

The cultured cells were harvested after treatment with pEZ-M29-eGFP and pEZ-M29-Hugl1, respectively, washed with ice-cold PBS and centrifuged for 5 min at 500 × *g* at 4 ℃. The supernatants were discarded, and the cell pellets were resuspended in ice-cold binding buffer. Double staining with Annexin V-fluorescein isothiocyanate (FITC) and PI was performed using the Annexin V-FITC kit (Beyotime, China) according to the manufacturer's recommendations, and the cells were then analyzed by FACS (BD FACS Aria Ⅲ, United States).

Nude mice xenograft experiments

BALB/c nude mice (5-6 week old) were obtained from the Beijing HFK Experimental Animal Center and were quarantined for one week before tumor implantation. Animal welfare and experimental procedures were performed in strict accordance with guidelines. Mice were randomly divided into two groups (six mice per group). A xenograft tumor model was established by subcutaneously injecting either Hugl1-overexpressing cells or PBS-treated cells (2×10^6) suspended in 0.1 mL of PBS into the right flank of mice, and the tumor volume was measured every week until the mice were sacrificed. At the end of the experiment (day 21), tumors were harvested for additional analyses. Differences in tumor growth were tested for statistical significance.

Immunohistochemistry analysis

The xenograft tumors were embedded in paraffin, cut into 4 μm sections, and either stained with hematoxylin and eosin or treated with Hugl-1 antibody for immunohistochemical evaluation. The results were captured by microscopy (Olympus, Japan).

Transferase-mediated dUTP nick end-labeling assay

The transferase-mediated dUTP nick end-labeling (TUNEL) technique was performed to detect and quantitate apoptotic cell death using the in situ Cell Death Detection Kit (Roche, United States) according to the manufacturer's instructions. Chamber slides were fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton X-100. The slides were then incubated with the TUNEL reaction mixture for 1 h at 37 ℃. After the slides were washed with PBS, they were incubated with peroxidase-conjugated antibody for 30 min at 37 ℃ and were developed with the DAB system. A minimum of 3 fields were randomly selected, and the total cells were counted in each field to achieve a minimum number of 100 total cells. Apoptotic rates (the number of apoptotic cells/total cells) were expressed as mean ± SD from different fields.

Statistical analysis

The statistical analysis was performed using SPSS software (version 17.0 for Windows). Data were presented as means ± SD and comparisons were made using Student's *t* test. A probability of 0.05 or less was considered statistically significant.

RESULTS

Overexpression of Hugl-1 in vitro

The eGFP was used as an marker to detect whether the pEZ-M29-Hugl1 plasmid vectors were successfully transduced *in vitro*. The transfection efficiency is shown in Figure 1A, *in vitro* approach to 60%.

To analyze the effect of pEZ-M29-Hugl1 on the expression of cancer genes, we assessed the mRNA levels of Hugl-1 in the treated cells by Real-time RT-PCR. Our results demonstrated that, compared with group 1 (PBS-

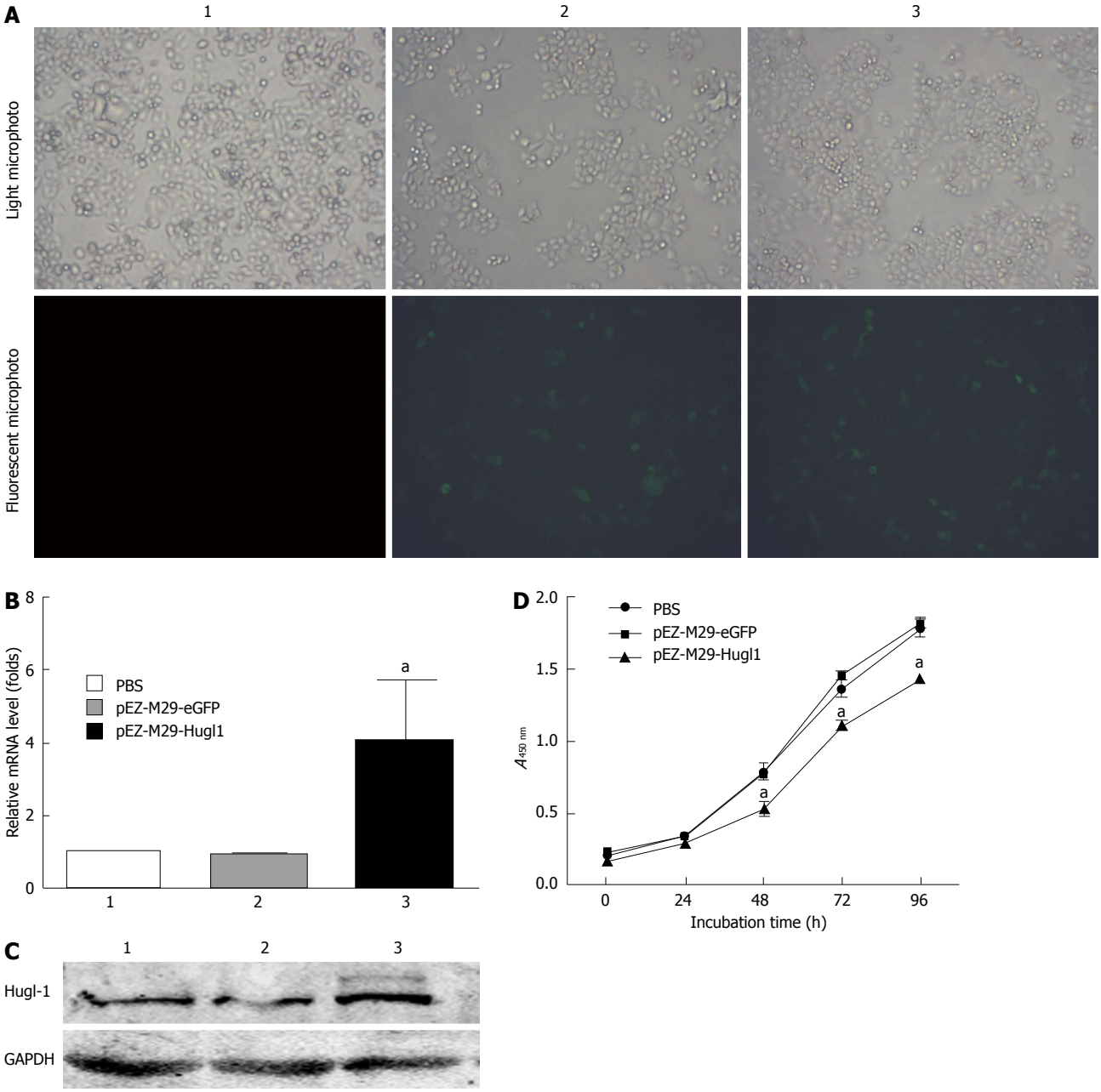

Figure 1 Transfection with pEZ-M29-Hugl1 increased Hugl-1 expression and inhibited the proliferation in Eca109 cells. A: Fluorescent expression in Eca109 cells (× 200); B: Real-time reverse transcription-polymerase chain reaction data of Hugl-1 mRNA levels following transfection with pEZ-M29-Hugl1 plasmids (group 3), pEZ-M29-eGFP (group 2), or treatment with phosphate buffered saline (PBS) (group 1); C: Western blotting data showing Hugl-1 protein expression levels following pEZ-M29-Hugl1 transfection compared with control groups; D: The effect of Hugl-1 on cell proliferation was assessed by cell counting Kit-8. Results represent mean values of three experiments and are indicated as mean ± SD. $^{a}P < 0.05$ *vs* the pEZ-M29-eGFP-treated and PBS-treated groups. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

treated), the mRNA levels of Hugl-1 in group 3 (pEZ-M29-Hugl1-treated) were remarkably increased ($P < 0.05$), but the mRNA levels in group 2 (pEZ-M29-eGFP-treated) were not noticeably different (Figure 1B).

We next assessed the expression of Hugl-1 protein by Western blotting. The expression of Hugl-1 was consistent with results from real-time RT-PCR, and compared with group 1, the protein level of group 3 was increased (Figure 1C).

Effect of Hugl-1expression on the proliferation of Eca109 cells

Cell proliferation assays were performed with the cell counting Kit 8 assay 48 h after transfection. The proliferation of Eca109 cells 48 h after being transfected with pEZ-M29-Hugl1 was slower than that of the other two control groups ($P < 0.05$). Therefore, Hugl-1 inhibited the proliferation of Eca109 cells (Figure 1D).

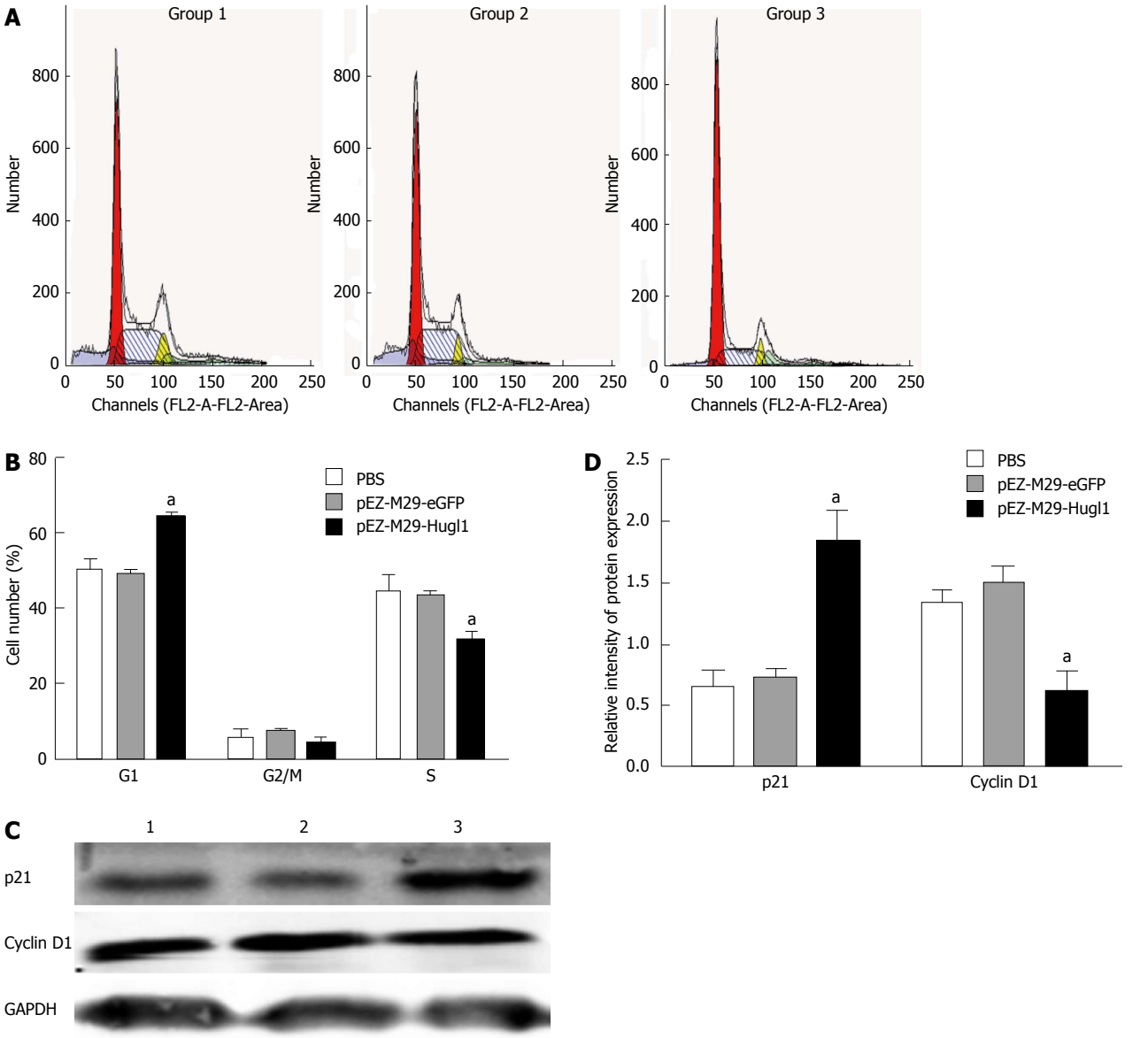

Figure 2 Effect of Hugl-1 on cell cycle distribution of Eca109 cells *in vitro*. A: Cells were treated with pEZ-M29-Hugl1, pEZ-M29-eGFP or phosphate buffered saline (PBS) for 48 h and were then prepared for fluorescence-activated cell sorting analysis; B: Data are presented as mean ± SD of three independent experiments; C: Western blotting data of p21 and cyclin D1 protein expression levels following transfection with pEZ-M29-Hugl1 or controls; D: Analysis of the expression of proteins. $^{a}P < 0.05$ *vs* the pEZ-M29-eGFP-treated and PBS-treated groups. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

Effect of Hugl-1 protein on the cell cycle of Eca109 cells

The mechanism underlying the inhibition of cell proliferation in Eca109 cells was investigated by analyzing the cell cycle with FACS following pEZ-M29-Hugl1 transfection. It was observed that Hugl-1 overexpression arrested the cell cycle in the G_1 phase (Figure 2A). The pEZ-M29-Hugl1 transfected cells were found to contain 64.09% ± 3.14% of cells in the G_1 phase and 31.47% ± 4.90% of cells in the S phase, whereas in the PBS-treated group, 50.32% ± 4.60% of cells were in the G_1 phase and 49.30% ± 4.98% of cells were in the S phase (Figure 2B). There was no difference between the group 3 and the group 2 (49.13% ± 2.24% in the G_1 phase and 43.47% ± 2.09% in the S phase).

Figure 2C shows that cells overexpressing Hugl-1 exhibited down-regulation of cyclin D1 and up-regulation of p21; these results suggest that the G_0/G_1 cell cycle arrest induced by Hugl-1 involved a reduced level of cyclin D1 and an increased level of p21. The protein levels of p21 was upregulated (1.83 ± 0.25 *vs* 0.64 ± 0.14, 1.83 ± 0.25 *vs* 0.72 ± 0.08, $P < 0.05$) and cyclin D1 was down-regulated (0.61 ± 0.18 *vs* 1.33 ± 0.12, 0.61 ± 0.18 *vs* 1.48 ± 0.15, $P < 0.05$) in the pEZ-M29-Hugl1 transfected cells (Figure 2D).

Effect of Hugl-1 protein on Eca109 cell apoptosis

After transfection, cells were incubated with Annexin V-FITC in a buffer containing PI and were then analyzed by flow cytometry (Figure 3A). The results show that group 3 had a higher apoptosis rate (17.33% ± 4.76%)

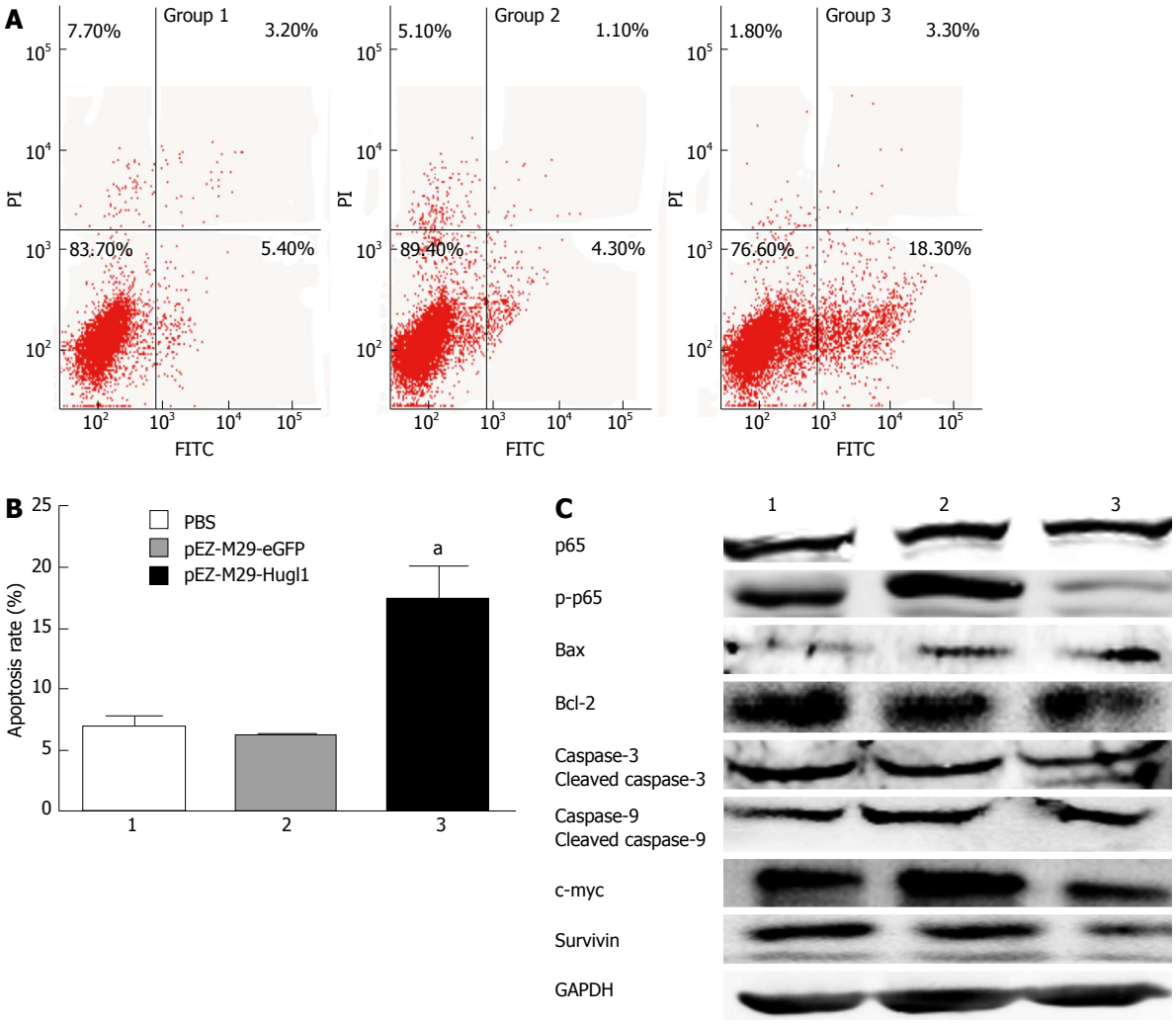

Figure 3 Effect of Hugl-1 on apoptosis of Eca109 cells *in vitro*. A: Cells were treated for 48 h and were then processed for FACS by staining with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI); B: After transfection with pEZ-M29-Hugl1, a significant number of cells were in an early state of apoptosis, and a population of cells had progressed to a later stage of apoptosis; C: Up-regulation of Hugl-1 led to a change of the protein levels of p65, p-p65, Bax, Bcl-2, caspase-3 and -9, survivin and c-myc among the three cell lines. All experiments were performed three times independently. $^{a}P < 0.05$ *vs* the pEZ-M29-eGFP-treated and phosphate buffered solution (PBS)-treated groups. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

compared with group 1 (6.90% ± 1.61%) ($P < 0.05$), and as we expected, no difference was observed between the apoptosis rates of group 1 and group 2 (6.27% ± 0.38%) ($P > 0.05$) (Figure 3B).

To identify the mechanisms that were affected by Hugl-1 expression, Eca109 cells expressing Hugl-1 were analyzed by Western blotting for changes in the levels of various cell-signaling proteins. Figure 3C shows that phospho-p65 was essentially absent in Hugl-1-overexpressing cells, but the total p65 level decreased only slightly. These results suggest that Hugl-1 down-regulated the nuclear factor kappa B (NF-κB) signaling pathway through inhibition of IKKα/β and p65 phosphorylation.

To establish that Hugl-1 induced apoptosis, we examined the activation of the classical caspases and the Bcl-2 family of proteins by Western blotting. Figure 3C shows that Hugl-1 up-regulated the expression of Bax, of cleaved caspase-3, and of cleaved caspase-9, and it down-regulated Bcl-2, survivin, and c-myc expression. These results suggest that Hugl-1 induced apoptosis in Eca109 cells through activation of the mitochondrial apoptotic pathway.

Effect of Hugl-1 expression on xenograft tumor growth

To study the effect of Hugl-1 on tumor growth, nude mice were inoculated with Hugl-1-overexpressing cells, and the resulting tumor growth was compared to that in a control group of mice injected with PBS-treated cells. The difference in tumor growth between the two groups of mice was statistically significant at $P < 0.05$. In the control group, tumors displayed rapid and continued outgrowth during the course of the experiment, and the mean tumor volume was 1126.56 ± 141.70 mm^3. In contrast, the mean tumor size for the experimental group was 606.03 ± 22.49 mm^3 (Figure 4A).

Hematoxylin and eosin staining revealed a significant level of cell death in tumor tissues treated with the pEZ-M29-Hugl1 plasmid compared to the control group (Fig-

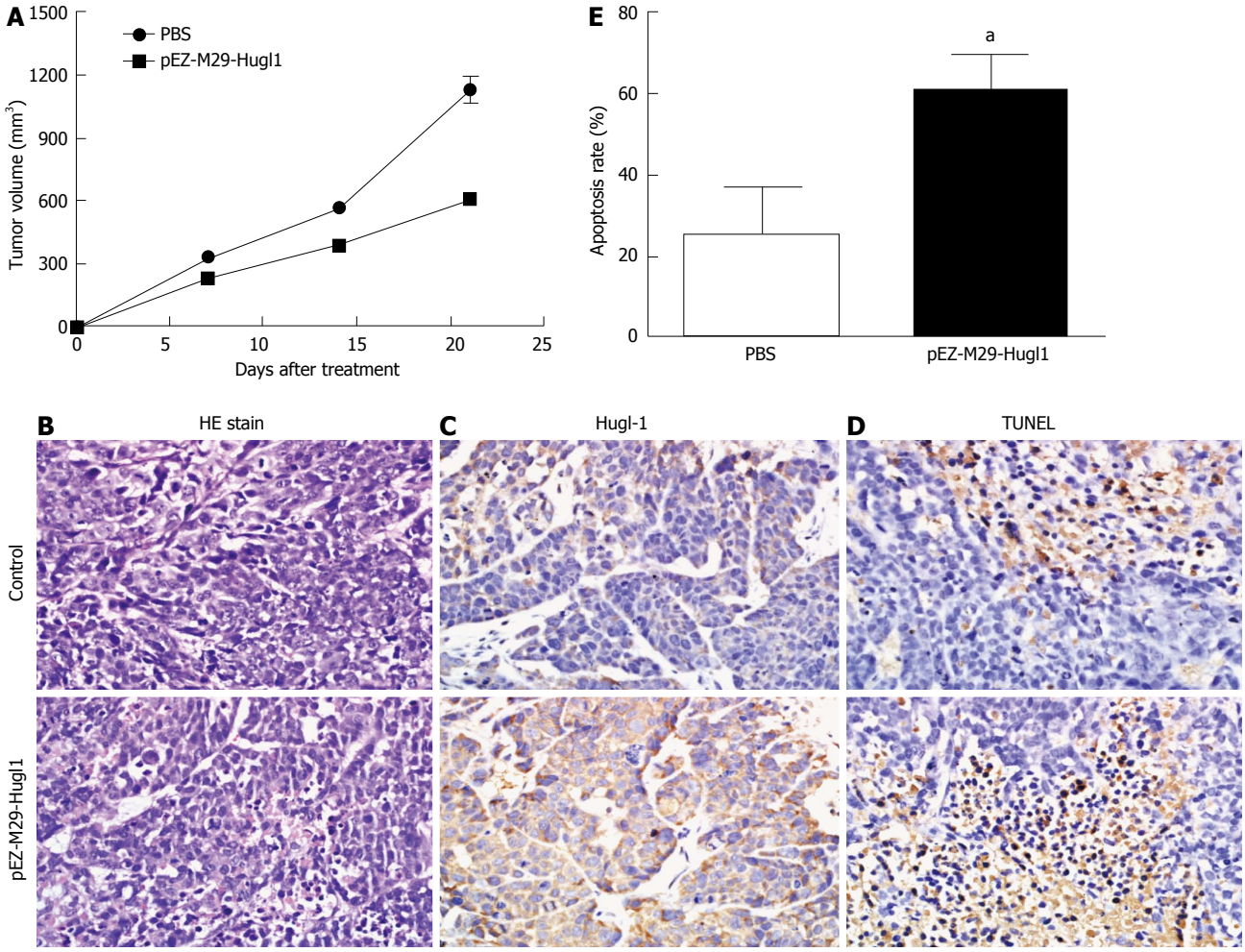

Figure 4 Effect of Hugl-1 on xenograft tumor *in vivo*. A: Cells were injected subcutaneously into nude mice, and with one group of mice receiving pEZ-M29-Hugl1-treated cells and another receiving phosphate buffered saline (PBS). Tumor volume was measured at 7-d intervals for 21 d; B: Tumor sections were observed by hematoxylin and eosin (HE) staining (× 400); C: Expression of Hugl-1 in tumor tissues was analyzed by immunohistochemistry. Many cells were strongly positive for Hugl-1 in the pEZ-M29-Hugl1-treated tumor sections (× 400); D: Representative photomicrographs showing transferase-mediated dUTP nick end-labeling (TUNEL) staining for evidence of apoptosis in transplantation tumors undergoing various treatments (× 400); E: Quantitative analysis of apoptotic cells in tumors treated with PBS or pEZ-M29-Hugl1. Apoptotic cells, shown by TUNEL, were significantly increased in tumors treated with pEZ-M29-Hugl1. Data are presented as mean ± SD (× 400). $^{a}P < 0.05$ *vs* the pEZ-M29-eGFP-treated and PBS-treated groups.

ure 4B). As shown in Figure 4C, many cells were strongly positive for Hugl-1 in the pEZ-M29-Hugl1-treated tumor sections. Apoptotic cells in the tumor sections were analyzed by TUNEL staining (Figure 4D), which showed markedly more positive cells in the pEZ-M29-Hugl1-treated group (60.50% ± 9.11%) than in the PBS-treated group (25.00% ± 12.25%) (Figure 4E).

DISCUSSION

The oncogenesis of esophageal cancer involves accumulated alternations of oncogenes, tumor suppresser genes and other epigenetic regulations[21,22]. Hugl-1 gene is one of the tumor suppresser genes involved in tumor cell proliferation. In this study, we reported that Hugl-1 was a potent anticancer gene for Eca109 cells through inducing G_0/G_1 cell cycle arrest and apoptosis. We concluded that up-regulation of Hugl-1 suppressed esophageal cancer cell proliferation by CCK-8 assay. Flow cytometry showed that overexpression of Hugl-1 reduced the number of cells in S-phase while increasing the number of cells in G_0/G_1-phase, indicating a G_0/G_1 arrest. To understand the mechanism by which Hugl-1 induces esophageal cancer apoptosis, we analyzed the expression of p65, p-p65, Bcl-2, Bax, survivin, c-myc, cyclin D1, p21, and caspase-3 and -9 between cells transfected with the Hugl-1-expressing plasmid and those transfected with the control plasmid. Immunoblotting analysis revealed that Hugl-1 overexpression significantly decreased the expression of p-p65, cyclin D1, Bcl-2, survivin, and c-myc and that it increased the expression of p21 and Bax. In addition, Hugl-1 significantly increased the expression and activity of caspase-3 and caspase-9. More importantly, Hugl-1 potently suppressed the growth of Eca109 cells xenografted in nude mice by inducing cell apoptosis.

Cell cycle arrest and apoptosis are two main ways by which cell growth can be inhibited. In higher eukaryotes, multiple cyclin-dependent kinases associate with multiple cyclins to regulate cell cycle progression[23,24]. Cyclin D1, a member of the G_1 cyclins, controls the cell cycle tran-

sit from G_1 to S phase[25]. The activities of CDKs and CDK/cyclin complexes are known to be regulated by the CIP/KIP family member p21[26]. P21 is, in turn, under transcriptional control of the tumor suppressor p53 and is required for p53-dependent cell cycle arrest[27]. In this study, we found that up-regulation of Hugl-1 in Eca109 cells resulted in a G_0/G_1 cell cycle arrest that was accompanied by down-regulation of cyclin D1 and up-regulation of p21. These data suggest that the mechanism of Hugl-1-induced cell cycle arrest involves down-regulation of cyclin D1 and up-regulation of the CDK inhibitor p21, causing inhibition of CDK activity.

Furthermore, the increase in Hugl-1 expression induced down-regulation of the anti-apoptotic gene Bcl-2 and up-regulation of the pro-apoptotic gene Bax. The central cast of players in the mitochondrial pathway of programmed cell death is the extended Bcl-2 family of proteins[28,29]. Therefore, the balance between the levels of Bcl-2 and Bax is critical in determining the fate of cells in terms of survival or death[30]. Homo-oligomerization of Bax leads to permeabilization of the mitochondrial membrane and subsequent release of cytochrome C to activate apoptosis[31]. Bcl-2 interacts with Bax, preventing its homo-oligomerization and, ultimately, apoptosis[31]. In this case, we found that Hugl-1 induced apoptosis in Eca109 cells, as evidenced by the increase in Annexin V-positive cells, the reduced cell proliferation, and the changes in caspase activation. Currently, there are two known pathways that activate the apoptotic caspase cascade, the intrinsic (mitochondrial) and extrinsic pathways[32]. Our results disclosed that the caspase-9-regulated intrinsic pathway was involved in Hugl-1-induced cell apoptosis. In Hugl-1-treated cells, we observed an increase in the cleaved caspases-9 and caspases-3. These results suggest that Hugl-1 induces apoptosis in Eca109 cells through activation of the mitochondrial pathway.

Considerable evidence indicates that NF-κB is constitutively active in esophageal cancer and that its activation is correlated with tumor progression[33]. The relationship between the NF-κB signaling pathway and tumor cell apoptosis has been extensively studied[34]. It has been presumed that the NF-κB pathway was involved in suppressing apoptosis[35]. The NF-κB family is composed of homodimers and heterodimers of the Rel family of proteins, including p65 (RelA), c-Rel, RelB, p52 and p50[36]. The most abundant form of NF-κB is a heterodimer with two subunits: p50 and p65. Our study showed that phospho-p65 was essentially absent in Hugl-1-overexpressing cells, but the total level of p65 was only slightly reduced. It has been suggested that Hugl-1 down-regulates the NF-κB signaling pathway through inhibition of the IKKα/β and p65 phosphorylation.

In addition, in the nude mice xenografted with Eca109 cells, we found that up-regulation of Hugl-1 reduced tumor growth (606.03 ± 22.49 mm^3 *vs* 1126.56 ± 141.70 mm^3). With a TUNEL assay, we found that Hugl-1 markedly increased the apoptosis rate of Eca109 cells *in vivo* (60.50% ± 9.11% *vs* 25.00% ± 12.25%). Thus, our results indicate that Hugl-1 may be a tumor suppressor of esophageal cancer.

In summary, our study has demonstrated that Hugl-1 exerts tumor suppressor effects by inducing growth suppression and apoptosis both *in vitro* and *in vivo*. G_0/G_1 cell cycle arrest induced by Hugl-1 occurs through a pathway that is mediated by p53-dependent p21 and cyclin D1 and that apoptosis induced by Hugl-1 occurs through the mitochondria pathway. The data presented here also indicate that Hugl-1 interferes with cell proliferation by affecting the NF-κB signaling pathway. The observations that Hugl-1 expression led to the loss of activated IKKα/β and p65 suggest that Hugl-1 is a negative regulator of NF-κB signaling. More importantly, Hugl-1 induced growth suppression and apoptosis in a human esophageal carcinoma cell line *in vivo*. Taken together, we show that Hugl-1 induces growth suppression and apoptosis in a human esophageal squamous cell carcinoma cell line both *in vitro* and *in vivo*. These data suggest that Hugl-1 may provide a novel target for treatment of esophageal cancer patients.

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COMMENTS

Background

Esophageal squamous cell carcinoma (ESCC) is one of the most frequently diagnosed cancers in China. Most esophageal cancers are diagnosed in the advanced stages. Thus, detecting gene alternations that promote the carcinogenesis process leading to esophageal cancer will have a profound impact on the diagnosis and treatment of the disease. The human homologs of lethal giant larvae (lgl) are Hugl-1 (Llgl1) and Llgl2. The Hugl-1 protein shares 62.5% similarity with lgl. Several studies have shown that Hugl-1 transcripts are reduced or absent in a high proportion of breast cancers, lung cancers, prostate cancers, ovarian cancers, colorectal cancers and hepatocellular carcinomas. However, the effect of Hugl-1 on tumor progression and prognosis in ESCC is not clear. The authors aimed to determine whether the Hugl-1 exerts tumor suppressor effects in esophageal cancer.

Research frontiers

Targeted molecular therapy is a new effective treatment for cancer including esophageal cancer. Hugl-1 is a potential tumor suppressor in several cancers, but the role of Hugl-1 remains controversial and its exact role in ESCC remains unknown.

Innovations and breakthroughs

Authors constructed a Hugl-1 expression plasmid, pEZ-M29-Hugl1, for gene transfection. Authors transfected the pEZ-M29-Hugl1 plasmid into Eca109 esophageal cancer cell lines to overexpress Hugl-1. The results showed that overexpression of Hugl-1 could inhibit the growth of Eca109 cells and promote cell apoptosis, and modulate the expression of Bcl-2, Bax, caspase-3, caspase-9, *etc.* Hugl-1 may serve as a potential therapeutic target in ESCC. It suggested that Hugl-1 is a tumor suppressor and interact with the mitochondrial pathway in ESCC.

Applications

The results showed that Hugl-1 is a tumor suppressor and interact with the mitochondrial pathway in ESCC. It may contribute to the future research of ESCC and be a promising target for therapeutic intervention in ESCC.

Terminology

Lethal giant larvae homolog 1 (Human): This gene encodes a protein that is similar to a tumor suppressor in Drosophila.

Peer review

The function of Hugl-1 as a tumor suppressor has been studied in other cancers, but not in ESCC. Thus authors examined the effects of Hugl-1 up-regulation on cell growth and apoptosis in the ESCC cell line Eca109 and determined the conclusion. This conclusion is meaningful in the sense characterized the function of Hugl-1 in ESCC cells.

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ORIGINAL ARTICLE

Effects of rhein on intestinal epithelial tight junction in IgA nephropathy

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Abstract

AIM: To investigate the effects of rhein on intestinal epithelial tight junction proteins in rats with IgA nephropathy (IgAN).

METHODS: Twenty-eight female Sprague-Dawley rats were randomly divided into four groups (7 per group): Control, IgAN, Rhein-treated, and Rhein-prevented. Bovine serum albumin, lipopolysaccharide and CCl_4 were used to establish the rat model of IgA nephropathy. The Rhein-treated group was given rhein from week 7 until the rats were sacrificed. The Rhein-prevented group was given rhein from week 1. Animals were sacrificed at the end of week 10. We observed the changes in the intestinal epithelial tight junctions using transmission electron microscopy, and expression of intestinal epithelial tight junction proteins zona occludens protein (ZO)-1 and occludin by immunofluorescence using laser confocal microscopy. Changes in mRNA and protein expression of ZO-1 and occludin were measured by reverse transcriptase polymerase chain reaction and Western blotting. The ratio of urinary lactulose/mannitol was measured by high performance liquid chromatography (*HPLC*) for assessing the intestinal permeability.

RESULTS: In the control group, the tight junctions lied between epithelial cells on the top of the outer side of the cell membrane, and appeared in dense dotted crystal structures, the neighboring cells were binded tightly with no significant gap, and the tight junction protein ZO-1 and occludin were evenly distributed in the intestinal epithelial cells at the top of the junction. Compared with the control group, in the IgAN group, the structure of the tight junction became obscured and the dotted crystal structures had disappeared; the fluorescence of ZO-1 and occludin was uneven and weaker (5.37 ± 1.27 *vs* 10.03 ± 1.96, $P < 0.01$; 4.23 ± 0.85 *vs* 12.35 ± 4.17, $P < 0.01$); the mRNA expression of ZO-1 and occludin decreased (0.42 ± 0.19 *vs* 0.92 ± 0.24, $P < 0.01$; 0.40 ± 0.15 *vs* 0.97 ± 0.25, $P < 0.01$); protein expression of ZO-1 and occludin was decreased (0.85 ± 0.12 *vs* 1.98 ± 0.43, $P < 0.01$; 0.72 ± 0.15 *vs* 1.38 ± 0.31, $P < 0.01$); and the ratio of urinary lactulose/mannitol increased (3.55 ± 0.68 *vs* 2.72 ± 0.21, $P < 0.01$). In the Rhein-prevented and Rhein-treated groups, compared with the IgAN group, the intestinal epithelial tight junctions were repaired; fluorescence of ZO-1 and occludin was stronger (11.16 ± 3.52 and 8.81 ± 2.30 *vs* 5.37 ± 1.27, $P < 0.01$; 10.97 ± 3.40 and 9.46 ± 2.40 *vs* 4.23 ± 0.85, $P < 0.01$); mRNA of ZO-1 and occludin increased (0.81 ± 0.17 and 0.64 ± 0.16 *vs* 0.42 ± 0.19, $P < 0.01$; 0.82

± 0.22 and 0.76 ± 0.31 *vs* 0.40 ± 0.15, $P < 0.01$); protein expression of ZO-1 and occludin was increased (2.07 ± 0.41 and 1.57 ± 0.23 *vs* 0.85 ± 0.12, $P < 0.01$; 1.34 ± 0.21 and 1.15 ± 0.17 *vs* 0.72 ± 0.15, $P < 0.01$); and the ratio of urinary lactulose/mannitol decreased (2.83 ± 0.43 and 2.87 ± 0.18 *vs* 3.55 ± 0.68, $P < 0.01$).

CONCLUSION: Rhein can enhance the expression of ZO-1 and occludin, repair damaged tight junctions, and protect the intestinal barrier.

Key words: Intestine; Tight junction; Rhein; IgA nephropathy; Rat

Core tip: It has been reported that the incidence and aggravation of IgA nephropathy (IgAN) are often accompanied with intestinal mucosal damage. We speculate that various factors cause the destruction of the intestinal mucosal barrier, food proteins activate the mucosal immune system, and a large amount of secretory IgA is deposited in kidney and causes IgAN. Rhubarb has a protective effect on the intestine. Rhein is isolated from rhubarb and we speculate that it also has a protective effect, although this has not been reported to date. We used various biochemical approaches to confirm this.

Peng SN, Zeng HH, Fu AX, Chen XW, Zhu QX. Effects of rhein on intestinal epithelial tight junction in IgA nephropathy. *World J Gastroenterol* 2013; 19(26): 4137-4145 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i26/4137.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i26.4137

INTRODUCTION

IgA nephropathy (IgAN) is the most common primary glomerular disease worldwide[1]. Although the etiology and pathogenesis of IgAN are still not clear, and it lacks effective treatment, the incidence and aggravation of IgAN are often accompanied with intestinal mucosal damage[2]. We speculate that various virulence factors cause destruction of the intestinal mucosal barrier, the permeability of the intestinal mucosa increases, food proteins activate the intestinal mucosal immune system, and a large amount of secretory IgA is produced and deposited in the kidney, which causes renal damage and IgAN. We suggest that the protection of the intestinal mucosal barrier can decrease the permeability of intestinal mucosa and prevent or reduce the occurrence of IgAN.

It has been reported that rhubarb has a protective effect on the intestinal mucosal barrier[3]. Rhein (1.8-dihydroxy-3-carboxy-anthraquinone, CAS number: 478-43-3) is an anthraquinone monomer isolated from rhubarb, and we speculate that it may also have a protective effect on the intestinal mucosal barrier and delay or prevent the course of IgAN. The function of the intestinal mucosal barrier mainly depends on the integrity of the tight junction proteins in the intestinal epithelial cells. A decrease in tight junction proteins increases intestinal permeability and leads to dysfunction of the intestinal mucosal barrier[4]. As far as we are aware, a protective effect of rhein on the intestinal epithelial tight junction proteins in rats with IgAN has not yet been reported. Therefore, we used various biochemical approaches to determine how rhein regulates the expression of intestinal epithelial tight junction proteins in IgAN.

MATERIALS AND METHODS

Materials

Rhein (> 95% purity) was extracted and identified by Chengdu Mansite Pharmaceutical Co. Ltd. (batch number: MUST-11032801; China). Antibodies against occludin were purchased from Abcam (Cambridge, United Kingdom). Antibodies against zonula occludens protein (ZO)-1 were purchased from Invitrogen (Carlsbad, CA). Bovine serum albumin (BSA) was purchased from Roche (Mannheim, Germany). Lipopolysaccharide (LPS), lactulose and mannitol were purchased from Sigma (St Louis, MO). Carbon tetrachloride and castor oil were purchased from Shanghai Reagents (China). Antibodies against β-actin, horseradish-peroxidase-conjugated secondary antibodies and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were purchased from Beijing Zhongshan (China). Trizol and reverse transcriptase polymerase chain reaction (RT-PCR) kit were purchased from Transgen (Beijing, China).

Animal model

Twenty-eight female Sprague-Dawley rats weighing 180-220 g were obtained from the Animal Center of Nanchang University. They were housed in the animal facilities of the Nanchang University, with free access to food and water. Animals were treated humanely by use of protocols that were approved by the Institutional Animal Use and Care Committee of Nanchang University. Rats were divided randomly into the control group, IgAN group, Rhein-prevented group, and Rhein-treated group ($n = 7$ each). The IgAN experimental animal model was established by treatment with BSA, LPS and CCl_4[5], and specific implementation was as follows: BSA (400 mg/kg, oral every other day) for 6 wk plus LPS (0.05 mg, intravenous injection at wk 6 and 8) and CCl_4 (0.1 mL dissolved in 0.5 mL castor oil, subcutaneous injection weekly for 9 wk). The Rhein-treated group was given rhein (100 mg/kg per day)[6] from week 7 until sacrifice. The Rhein-prevented group was given rhein (100 mg/kg per day) from week 1. The control and IgAN groups were given the same volume of normal saline. All the rats were sacrificed at week 10.

Transmission electron microscopy

Seven rats per group were analyzed by transmission elec-

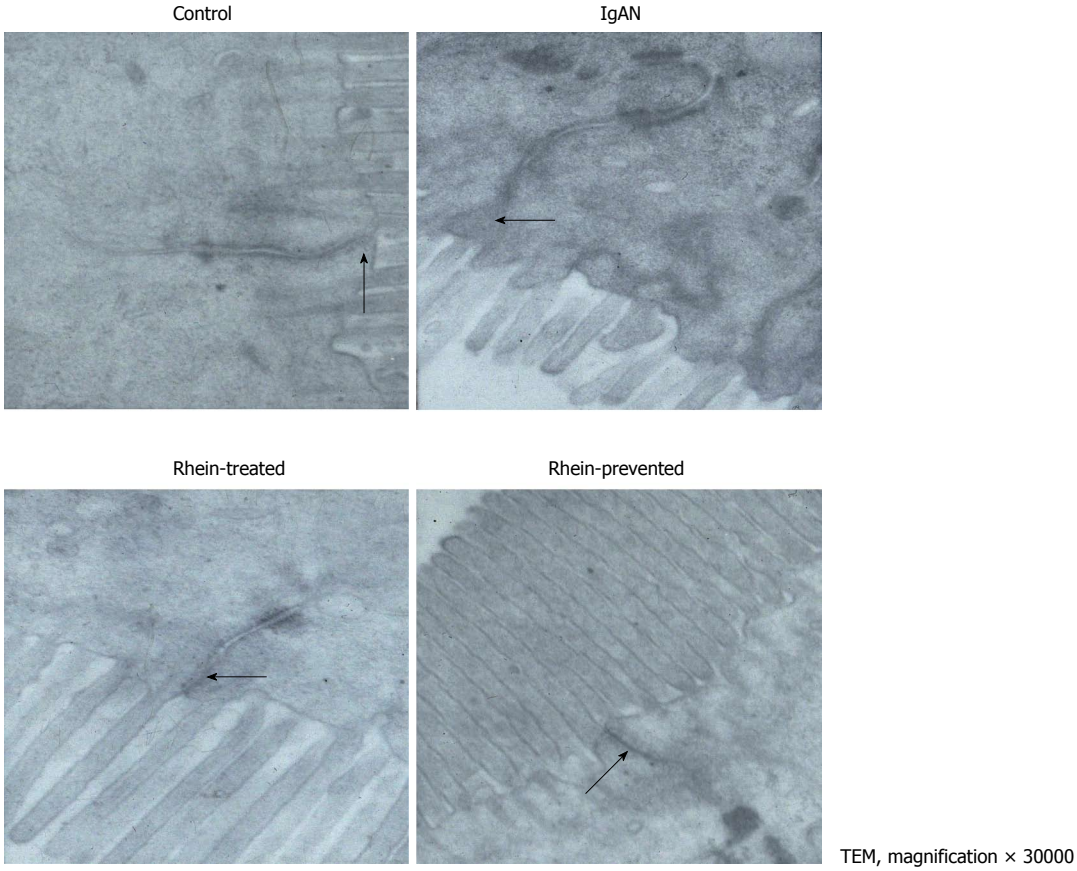

Figure 1 Electron micrograph of intestinal epithelial cells showing tight junction. A: In the control group, the tight junction appeared as an electron-dense belt at the apex of the intestinal epithelial cells (arrow), indicating an intact intestinal mucosal barrie; B: In the IgA nephropathy (IgAN) group group, the intercellular space was widened, the tight junction was indistinct, and the density was reduced (arrow); C and D: In the Rhein-treated and Rhein-prevented groups, the density of the tight junctions was increased compared with that in the IgAN group (arrows). TEM: Transmission electron microscopy.

tron microscopy (TEM) (H-600). Pieces of ileum, 2 mm × 2 mm, were fixed in 2.5% glutaraldehyde overnight at 4° C. The fixed tissues were then post-fixed in 1% osmium tetroxide for 2 h and then rinsed and stored in 0.1 mol/L sodium cacodylate buffer containing 6% sucrose for 12 h. The pieces of ileum were dehydrated through a graded acetone series and embedded in epoxy resin. Semi-thin sections (1.5 μm) were cut and stained with toluidine blue. Ultra-thin sections were stained with 4% uranyl acetate solution in 50% ethanol and lead citrate and then the intestinal epithelial tight junctions were examined by TEM.

Immunofluorescence analysis of occludin and ZO-1

Seven rats per group were analyzed by immunofluorescence. Pieces of ileum, 5 mm × 5 mm, were frozen in liquid nitrogen and 10-μm frozen sections were cut. The frozen sections were fixed with cold acetone for 10 min at 4 ℃. After extensive washing three times (5 min per wash) with cold PBS, the frozen sections were blocked with 10% normal sheep serum in PBS and then incubated with the antibodies against occludin (1:200, Abcam) and ZO-1 (1:100, Invitrogen) at 4 ℃ overnight, followed by staining with FITC-conjugated secondary antibodies. Stained frozen sections were examined with a laser confocal microscope equipped with a digital camera, identifying occludin and ZO-1 by light green color (excitation light wave length of 490 nm). Stained frozen sections were analyzed by a morphological analysis system to determine semi-quantitatively the expression of occludin and ZO-1. Five visual fields were randomly observed under high magnification, with two sections selected from each specimen. The integrated optical density of the positive material in each visual field and its area were measured by morphological analysis system; the ratio of which showed the relative content of occludin and ZO-1.

RT-PCR

Five rats per group were analyzed by RT-PCR. RT-PCR was used for mRNA detection and semi-quantitative assessment. Total RNA was extracted from the small intestine using Trizol reagent (Transgen), measured and verified with a UV spectrophotometer. cDNA was synthesized using an One-Step RT-PCR kit (Transgen) from 1 μg total RNA. Primers were designed by the Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA) according to mRNA sequences (by GenBank) of occludin, ZO-1 and β-actin (as control). The sequences of primers were as follows: forward primer of β-actin gene was 5'-TCAGGTCATCACTATCGGCAAT-3'

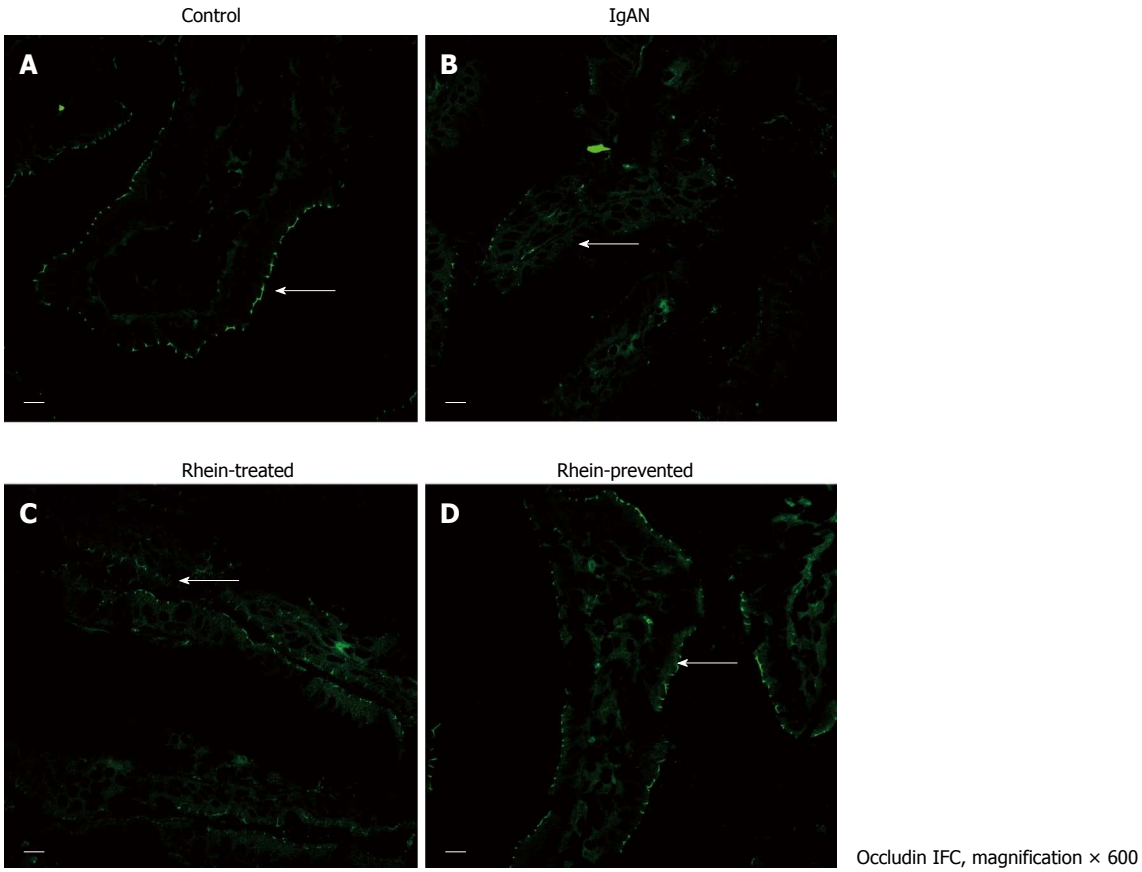

Figure 2 Location of tight junction protein occludin in rat ileum. Laser confocal microscope immunofluorescence staining of ileum from all four groups of rats. A: Cross-section of a normal intestinal villus. Immunoreactive occludin was localized at the apex of intestinal epithelial cells, consistent with the site of the intestinal mucosal barrier (arrow); B: Cross-section of an intestinal villus in the IgA nephropathy (IgAN) group. Occludin immunofluorescence staining became weak and discontinuous (arrow); C and D: Cross-section of an intestinal villus in the Rhein-treated group and cross-section of an intestinal villus in the Rhein-prevented group. Compared with the IgAN group, occludin immunofluorescence staining became stronger and continuous (arrows). Scale bars = 10 μm. IFC: Integrated fluidic circuit.

and its reverse primer was 5'-AAAGAAAGGGTGT AAAACGCA-3'. The forward primer of the occludin gene was 5'-TGCGTGGCTTCCACACTT GCT-3' and its reverse primer was 5'-TTTGCCGCTCTGGGGTCT-GT-3'. The forward primer of the ZO-1 gene was 5'-TGCCCGGCCATTTGAACGCA-3' and its reverse primer was 5'-TCAGG CGGCTGTGTGGAACT-3'. The PCR products were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide to confirm that products of the expected size were detected. The electrophoretic bands were analyzed using a gel image analysis system. The results were normalized to the respective β-actin expression. RT-PCR experiments were repeated twice.

Western blotting

Five rats per group were analyzed by Western blotting. For Western blotting, the small intestines were frozen in liquid nitrogen until further use. Protein extraction was carried out using the RIPA lysate (Solarbio, Beijing, China). Protein (20-50 μg per lane) was separated by SDS-PAGE. Occludin was separated on 10% gel and ZO-1 on 8% gel. Proteins were transblotted to polyvinylidene difluoride membranes (Solarbio) in standard Tris-glycine transfer buffer, pH 8.3, containing 0.5% SDS. After transfer, membranes were blocked for 1 h at room temperature in TBST (10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 0.2% Tween-20) containing 5% non-fat milk powder, and incubated overnight at 4 ℃ with either anti-occludin (Abcam) or anti-ZO-1 (Invitrogen) diluted 1:200 in TBST containing 1% non-fat milk powder. Membranes were then washed in TBST for 30 min, incubated with horseradish-peroxidase-conjugated goat anti-rabbit IgG, diluted 1:5000 (Beijing Zhongshan, China) in TBST, washed in TBST for 30 min, and resolved by chemiluminescence (Thermo, Waltham, MA). All membranes were stripped and reprobed with anti-β-actin antibodies (Beijing Zhongshan) as loading controls. Intensities of immunoreactive bands were quantified by densitometry, and normalized to the respective β-actin content. Western blotting experiments were repeated twice.

Measurement of intestinal permeability

Intestinal permeability was determined using two non-metabolized sugars. Three grams lactulose and 1.5 g mannitol were dissolved in 60 mL distilled water. After a fasting period of 12 h, all animals received 2 mL lactulose/mannitol solution by orogastric tube. One hour

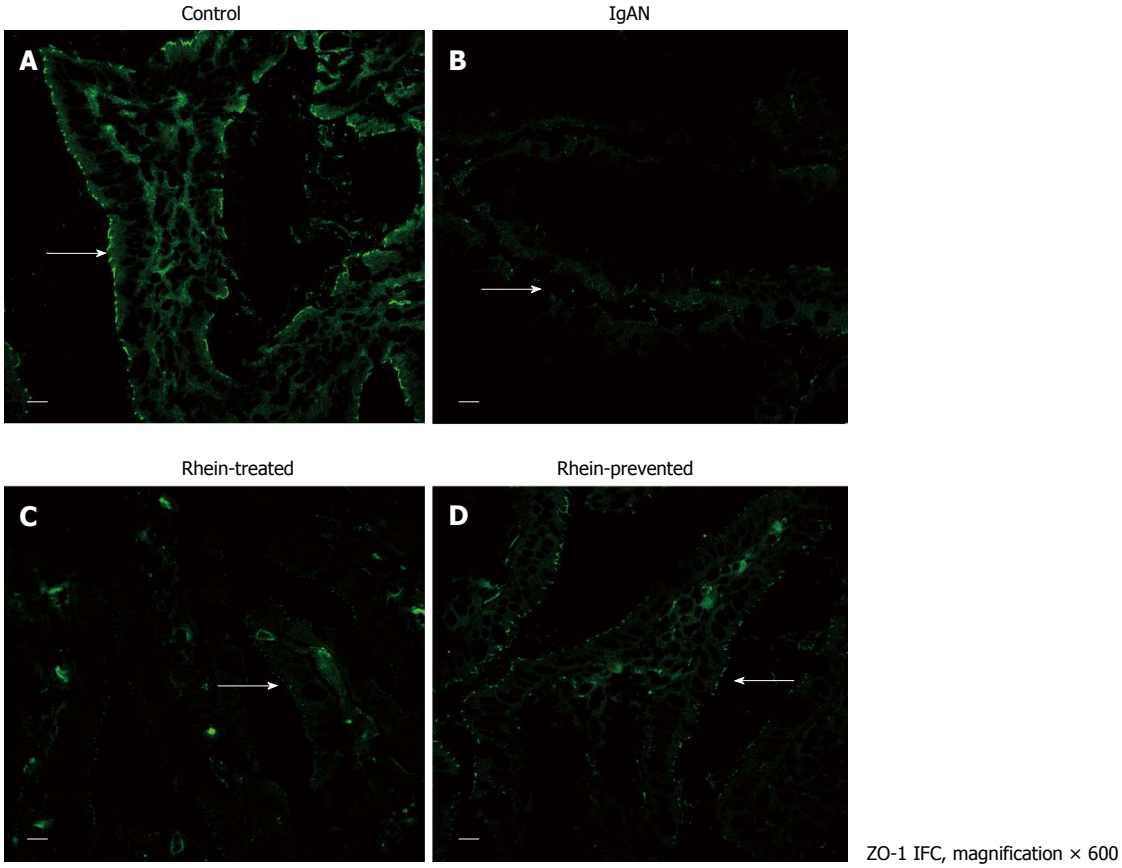

Figure 3 Location of tight junction protein zona occludens protein-1 in rat ileum. Laser confocal microscope immunofluorescence staining of ileum from all four groups of rats. A: Cross-section of a normal intestinal villus. Immunoreactive zona occludens protein (ZO)-1 was localized at the apex of intestinal epithelial cells, consistent with the site of the intestinal mucosal barrier (arrow); B: Cross-section of an intestinal villus in the IgA nephropathy (IgAN) group. ZO-1 immunofluorescence staining became weak and discontinuous (arrow); C and D: Cross-section of an intestinal villus in the Rhein-treated group and cross-section of an intestinal villus in the Rhein-prevented group. Compared with the IgAN group, ZO-1 immunofluorescence staining became stronger and continuous (arrows). Scale bars = 10 μm. IFC: Integrated fluidic circuit.

Table 1 Average optical density value of occludin and zona occludens protein-1

	Control group	IgAN group	Rhein-treated group	Rhein-prevented group
In immunofluorescence ($\times 10^{-2}$)				
Occludin	12.35 ± 4.17	4.23 ± 0.85[b]	9.46 ± 2.40[b,d]	10.97 ± 3.40[d]
ZO-1	10.03 ± 1.96	5.37 ± 1.27[b]	8.81 ± 2.30[d]	11.16 ± 3.52[d,f]
In reverse transcriptase polymerase chain reaction				
Occludin	0.97 ± 0.25	0.40 ± 0.15[b]	0.76 ± 0.31[a,d]	0.82 ± 0.22[d]
ZO-1	0.92 ± 0.24	0.42 ± 0.19[b]	0.64 ± 0.16[b,d]	0.81 ± 0.17[d,e]

The results were normalized to the respective β-actin expression. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group; [d]$P < 0.01$ *vs* IgA nephropathy (IgAN) group; [e]$P < 0.05$ *vs* Rhein-treated group; [f]$P < 0.01$ *vs* Rhein-prevented group.

after feeding, the 6-h urine was collected using metabolic cages before sacrifice. The ratio of urine concentrations of lactulose and mannitol was measured to assess the intestinal permeability.

Statistical analysis

All measurement data were expressed as mean ± SE. Statistical analysis was performed using SPSS 17.0 software. Comparison between groups was made using one-way analysis of variance followed by Student-Newman-Keuls test. $P < 0.05$ was considered to be statistically significant.

RESULTS

TEM results

ZO-1 and occludin are important components in tight junctions, so we performed a morphological analysis of the junctions (Figure 1). Tight junctions are belt-shaped and expand around the apex of epithelial cells. TEM indicated that the cell membrane was intact, and distinct junction complexes were observed in the Control Group. However, in the IgAN Group, the structure of the tight junctions became obscured and the dotted crystal structures disappeared. The microvilli were sparse with irregular length and arrangement. The situations in rhein-treated group and rhein-prevented group were improved compared with IgAN group. The intestinal epithelial tight junctions were repaired with respect to structural integration, with close intercellular connection and high electron density.

Laser confocal microscopy

Indirect immunofluorescence staining for occludin (Figure 2; intestinal epithelium by occludin immunofluorescence staining) and ZO-1 (Figure 3; intestinal epithelium

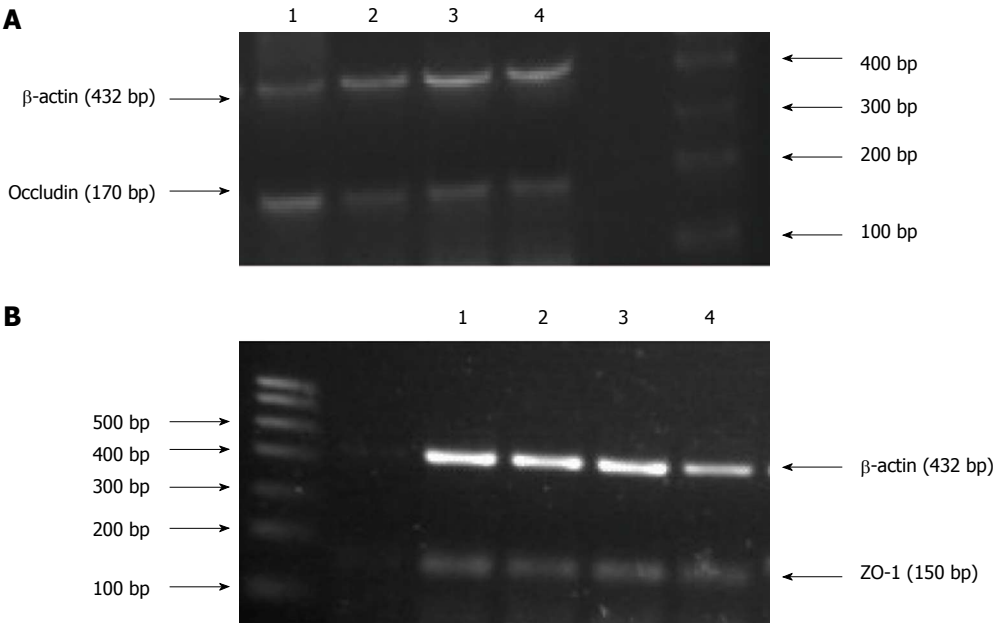

Figure 4 Reverse transcriptase polymerase chain reaction analysis for zona occludens protein-1 and occludin mRNA in rat ileum. By reverse transcriptase polymerase chain reaction, amplification products of expected size [150 bp for zona occludens protein (ZO)-1 and 170 bp for occludin] were obtained in the ileum in all four groups of rats. β-actin was the housekeeping protein. The levels of occludin and ZO-1 expression in the IgA nephropathy (IgAN) group were lower than in the control group. In the Rhein-treated and Rhein-prevented groups, the levels were higher than in the IgAN group. 1: Control; 2: IgAN; 3: Rhein-treated; 4: Rhein-prevented.

by ZO-1 immunofluorescence staining) was performed. In the control group, occludin and ZO-1 staining was found at the apical part of the lateral membranes of the polar epithelial cells and distributed continuously, similar to an intestinal mechanical barrier. In the IgAN Group, the green signals were intermittent and markedly weaker than those in the Control group ($P < 0.01$), and the integrity of the barrier was damaged. The condition of the Rhein-treated and Rhein-prevented groups was ameliorated compared with the IgAN group ($P < 0.01$). The green signals of ZO-1 in the Rhein-prevented group were stronger than in the Rhein-treated group ($P < 0.01$). In contrast, no change in the green signals of occludin was observed between these two groups (Table 1).

Rhein upregulated expression of occludin and ZO-1 mRNA in the small intestine

Occludin, ZO-1 and β-actin RNAs were 170, 150 and 432 bp long, respectively. RT-PCR semi-quantitative analyses showed that the levels of occludin and ZO-1 expression in the IgAN group were significantly lower than in the control group ($P < 0.01$). In the Rhein-treated and Rhein-prevented group, the levels were markedly higher than in the IgAN Group ($P < 0.01$). The level of ZO-1 expression in the Rhein-prevented group was higher than in the Rhein-treated group ($P < 0.05$). In contrast, no change in occludin expression was observed between these groups (Figure 4 and Table 1).

Rhein upregulated expression of occludin and ZO-1 protein in the small intestine

Western blotting analysis showed that occludin and ZO-1 protein expression decreased significantly in the IgAN group compared with the control group ($P < 0.01$). In the Rhein-treated and Rhein-prevented groups, occludin and ZO-1 protein expression was higher than in the IgAN group ($P < 0.01$). ZO-1 protein expression in the Rhein-prevented group was higher than in the Rhein-treated group ($P < 0.01$). However, no change in occludin protein expression was observed between the groups (Figure 5 and Table 2). These findings are consistent with the immunofluorescence results.

Rhein decreased intestinal permeability

The intestinal permeability was assessed by differential uptake of lactulose and mannitol in all four groups. Measurement of mannitol and lactulose by HPLC showed that the ratio of urinary lactulose/mannitol increased in the IgAN group compared with the control group ($P < 0.01$), indicating an increase of intestinal permeability. In the Rhein-treated and Rhein-prevented groups, the ratio of urinary lactulose/mannitol decreased compared with the IgAN group ($P < 0.05$), indicating decreased intestinal permeability. The decrease in intestinal permeability in the Rhein-prevented group was more obvious than that in the Rhein-treated group ($P < 0.05$) (Table 3).

DISCUSSION

IgAN is defined as the predominant deposition of IgA in the glomerular mesangium[7]. The etiology of IgAN has not been completely clarified, but one hypothesis involves the stimulation of antigen by an intestinal route causing an increase in IgA production in the intestinal

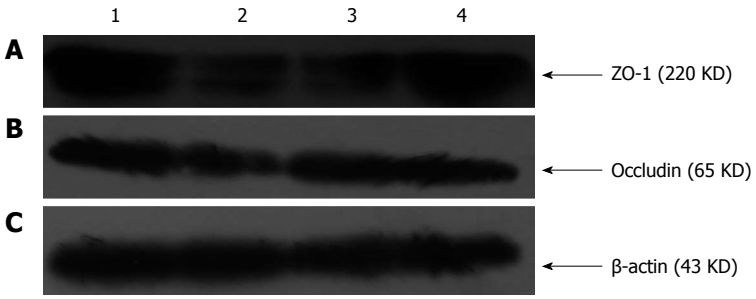

Figure 5 Western blotting analysis for zona occludens protein-1 and occludin of rat ileum. Western blotting analysis revealed zona occludens protein (ZO)-1 immunoreactivity by a band of 220 kDa in the control group and by a weaker band at the same level in the IgA nephropathy (IgAN) group. Compared with the IgAN group, there were stronger bands at the same level in the Rhein-treated and Rhein-prevented groups. Occludin immunoreactivity was revealed by a band of 65 kDa in the control group and by a weaker band at the same level in the IgAN group. Compared with the IgAN group, there were stronger bands at the same level in the Rhein-treated and Rhein-prevented groups. β-Actin was the housekeeping protein. 1: Control; 2: IgAN; 3: Rhein-treated; 4: Rhein-prevented.

Table 2 Grey level of occludin and zona occludens protein-1 in Western blotting

	Control group	IgAN group	Rhein-treated group	Rhein-prevented group
Occludin	1.38 ± 0.31	0.72 ± 0.15[b]	1.15 ± 0.17[d]	1.34 ± 0.21[d]
ZO-1	1.98 ± 0.43	0.85 ± 0.12[b]	1.57 ± 0.23[a,d]	2.07 ± 0.41[d,f]

The results were normalized to the respective β-actin expression. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group; [d]$P < 0.01$ *vs* IgA nephropathy (IgAN) group; [f]$P < 0.01$ *vs* Rhein-treated group.

Table 3 Ratio of urinary lactulose/mannitol

	Concentration of lactulose	Concentration of mannitol	Ratio of them
Control	5.73 ± 0.37	2.10 ± 0.05	2.72 ± 0.21
IgAN group	7.38 ± 1.42[b]	2.08 ± 0.11	3.55 ± 0.68[b]
Rhein-treated group	6.02 ± 0.31[d]	2.10 ± 0.05	2.87 ± 0.18[d]
Rhein-prevented group	6.06 ± 0.97[c]	2.15 ± 0.07	2.83 ± 0.43[d]

[b]$P < 0.01$ *vs* control group; [d]$P < 0.01$ *vs* IgA nephropathy (IgAN) group; [c]$P < 0.05$ *vs* Rhein-treated group.

mucosa, such as ulcerative colitis or Crohn's disease[8,9]. In recent years, an IgAN animal model induced by oral immunization has supported this hypothesis[10].

It has been reported that the permeability of the intestinal mucosa of IgAN patients is significantly higher than normal[11]. This is consistent with the experimental results, but its mechanism is not yet clear. Intestinal mucosal permeability is closely related to the integrity of the intestinal barrier. Mucosal barrier plays an important role in protecting the body from food antigens, microorganisms and their harmful metabolites[12]. The mucosal barrier includes mechanical, immune, chemical and biological barriers, among which, the mechanical barrier is essential for maintaining the integrity of the intestinal barrier. It is mainly composed of the intestinal epithelial cells and cellular junctions among them. The function of the intestinal barrier is affected by the morphology and number of epithelial cells and cellular junctions[13]. The cellular junctions include tight junctions, intermediate junctions, desmosomes, and gap junctions, and tight junctions are closely related to the mechanical barrier[14]. Tight junctions, or ZO, expand around the apex of epithelial cells and form a semipermeable barrier in the paracellular pathway in most vertebrate epithelia[15]. Disruption of the tight junctions can cause increased permeability and leakiness[16,17]. Three groups of macromolecules are considered as integral components of the tight junctions: occludins, claudins and junction adhesion molecules[18]. ZO-1 is the major tight junction protein that binds to the intracellular domain of occludins[19]. The interaction between occludin and ZO-1 plays a crucial role in maintaining the structure of tight junctions and epithelial barrier function[20,21]. Therefore, detection of occludin and ZO-1 reflects the condition of the tight junctions and intestinal mucosa barrier.

Our results showed that expression of intestinal epithelial tight junction proteins occludin and ZO-1 was significantly reduced in the IgAN Group. Therefore, we hypothesized that increased intestinal permeability in that group might be related to the decrease in expression of intestinal epithelial tight junction proteins. This decrease may be due to excess secretion of inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, NO, and oxygen free radicals, which is caused by intestinal antigen stimulation in the modeling process[22,23]. It has been reported that TNF-α affects the interaction of occludin and ZO-1 with components of the actin cytoskeleton[24]. Moreover, a series of recent reports has indicated that TNF-α disrupts tight junction assembly and decreases expression of ZO-1[25,26]. The synergy between TNF-α and IFN-γ can downregulate occludin expression of the occludin promoter[27].

Increased expression of tight junction proteins improves intestinal mucosal barrier function[28,29]. It has been reported that rhubarb can promote intestinal mucosal barrier function recovery and alleviate intestinal bacterial translocation in animal models of burns[30]. Therefore, we speculated that rhein, as the main pharmacological component of rhubarb, would also have a protective effect on the intestinal mucosal barrier and delay or prevent progression of IgAN. Western blotting showed that occludin and ZO-1 protein expression in the Rhein-treated

and Rhein-prevented Groups was higher than that in the IgAN Group, which was consistent with the immunofluorescence and RT-PCR results. The protective effect of rhein on tight junction proteins may be associated with the following aspects. First, rhein lowers the activity of macrophages and inhibits the secretion of TNF-α and other inflammatory cytokines that damage the structure and function of tight junctions[31]. Second, rhein has a positive effect on the peristaltic reflex of the small intestine[32], speeds up the excretion of intestinal bacteria and LPS, and reduces LPS-induced secretion of inflammatory cytokines. Third, reactive oxygen species destroy tight junction proteins by affecting the signal transduction pathway[33]. Rhein can remove reactive oxygen species and alleviate oxidative damage[34]. Lastly, dysfunction of the intestinal microcirculation results in structural damage of tight junctions[35]. Rhein inhibits intestinal microvascular endothelial cell secretion of NO, endothelin-1 and other vasoconstrictor substances, which improves the intestinal microcirculation[36].

In summary, rhein reduces intestinal permeability by protecting intestinal epithelial tight junction proteins ZO-1 and occludin, which alleviates the damage to the intestinal mucosa in IgAN. In this regard, rhein may be a potential therapeutic agent for protecting the intestinal mucosa in IgAN.

COMMENTS

Background

IgA nephropathy (IgAN) is the most common primary glomerular disease worldwide. The etiology and pathogenesis of IgAN are still not clear, and it lacks effective treatment. The incidence and aggravation of IgAN are often accompanied with damage to the intestinal mucosa. Rhein is an anthraquinone monomer isolated from rhubarb. It has been reported that rhubarb has a protective effect on the intestinal mucosal barrier in burns and pancreatitis, but a protective effect on the intestinal mucosal barrier in IgAN has not yet been reported.

Research frontiers

The intestinal mucosal barrier is an important area in research related to the etiology and pathogenesis of IgAN. The research hotspot is how to protect the intestinal mucosal barrier to prevent the occurrence of IgAN.

Innovations and breakthroughs

It has been reported previously that rhubarb has a protective effect on the intestinal mucosal barrier in burns and pancreatitis. Rhein is one of the anthraquinone monomers isolated from rhubarb. The protective effect of rhein on the intestinal mucosal barrier in IgAN has not yet been reported. The authors observed the protective effect of rhein on the intestinal mucosa in a rat model of IgAN; further demonstrated the pathogenesis of intestinal mucosal barrier injury in IgAN; and showed that intestinal protection and repair play an important role in the prevention and treatment of IgAN.

Applications

The results suggest that rhein is a potential therapeutic material that could be used in protecting intestinal mucosa barrier and preventing and treating IgAN.

Terminology

Rhein (1.8-dihydroxy-3-carboxy-anthraquinone, CAS number: 478-43-3) is a substance in the anthraquinone group obtained from rhubarb. Originally the rhubarb plant was used as a laxative, and it was believed that rhein along with other anthraquinone glycosides imparted this activity.

Peer review

This was a good descriptive study in which the authors analyzed the preventive effect of rhein on the intestinal mucosa barrier in rats with IgAN. The results are interesting and suggest that rhein is a potential therapeutic substance that could be used in protecting the intestinal mucosa and preventing IgAN.

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ORIGINAL ARTICLE

Restoring the Treg cell to Th17 cell ratio may alleviate HBV-related acute-on-chronic liver failure

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Abstract

AIM: To investigate the role of T helper 17 cells (Th17) and regulatory T cells (Treg) in hepatitis B virus (HBV)-related acute-on-chronic liver failure (ACLF).

METHODS: We enrolled 79 patients with HBV infection into the study, 50 patients with HBV-related ACLF and 29 patients with chronic hepatitis B (CHB), from the First Affiliated Hospital of Medical College from January 2009 to June 2012. The ACLF patients were diagnosed according to the criteria recommended by The 19th Conference of the Asian Pacific Association for the Study of the Liver in 2009. Twenty healthy individuals with a similar gender and age structures to the two patient groups were also included as the normal controls (NC). Of the 50 ACLF patients, 28 were subsequently classified as non-survivors: 19 patients died from multi-organ failure, 3 underwent liver transplantation, and 6 discontinued therapy during follow-up because of financial reasons. The remaining 22 ACLF patients whose liver and anticoagulation function recovered to nearly normal levels within the next 6 mo were classified as survivors. The number of circulating Treg and Th17 cells was determined upon diagnosis and during the 8th week of follow-up through flow cytometry.

RESULTS: The percentage of circulating Treg cells in the ACLF group was significantly higher than that in the CHB group (5.50% ± 1.15% *vs* 3.30% ± 1.13%, $P < 0.01$). The percentages of circulating Th17 cells in the ACLF and the CHB groups were significantly higher than that in the NC group (6.32% ± 2.22% *vs* 1.56% ± 0.44%, $P < 0.01$; 3.53% ± 1.65% *vs* 1.56% ± 0.44%, $P < 0.01$). No significant difference in Treg cell to Th17 cell ratio was observed between the ACLF group and the CHB group (0.98 ± 0.44 *vs* 1.12 ± 0.64, $P = 0.991$), whereas those in the two HBV infection groups were significantly lower than that in the NC group (1.85 ± 1.22; both $P < 0.01$). The percentage of Treg cells in the survivors during the 8th week of follow-up was significantly lower than that during peak ACLF severity [total bilirubin (TBIL) peak] (3.45% ± 0.97% *vs* 5.18% ± 1.02%, $P < 0.01$). The percentage of Th17 cells in survivors during the 8th week of follow-up was significantly lower than that during the peak TBIL (2.89% ±

0.60% *vs* 5.24% ± 1.46%; $P < 0.01$). The Treg cell to Th17 cell ratio during the 8th week of follow-up was significantly higher than that during the TBIL peak (1.22 ± 0.36 *vs* 1.10 ± 0.54; $P < 0.05$).

CONCLUSION: Restoring the Treg cell to Th17 cell ratio during the follow-up phase of ACLF could maintain the immune system at a steady state, which favours good prognosis.

Key words: Hepatitis B virus; Acute-on-chronic liver failure; Regulatory T cells; T helper 17 cells; Treg cell to Th17 cell ratio

Core tip: In this study, the expression of circulating Treg and Th17 cells in hepatitis B virus-related acute-on-chronic liver failure (ACLF), chronic hepatitis B (CHB), and normal controls (NC) was measured using flow cytometric analysis. The percentages of circulating Treg and Th17 cells in ACLF group increased significantly compared with that in CHB group and NC group. Furthermore, the ratio of Treg to Th17 cells increased significantly upon recovery. Our study suggests that the reverting ratio of Treg to Th17 cells at the follow-up phase of ACLF could maintain the immune system at a steady state in favour of good prognosis.

Niu YH, Yin DL, Liu HL, Yi RT, Yang YC, Xue HA, Chen TY, Zhang SL, Lin SM, Zhao YR. Restoring the Treg cell to Th17 cell ratio may alleviate HBV-related acute-on-chronic liver failure. *World J Gastroenterol* 2013; 19(26): 4146-4154 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i26/4146.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i26.4146

INTRODUCTION

Acute-on-chronic liver failure (ACLF) is defined as an acute hepatic insult that manifests as jaundice and coagulopathy complicated by ascites and/or encephalopathy within four weeks among patients with chronic liver disease. The major aetiologic agents of ACLF are alcohol and drugs in the West and infectious agents in the East[1]. A characteristic feature of ACLF is its rapid progression and high incidence of short- and medium-term mortality, ranging from 50% to 90%[2]. Recent advances in medical therapy have only slightly decreased the mortality rate of HBV-related ACLF. Antiviral treatment improves the survival of patients with HBV-related ACLF[3,4]. Administering granulocyte-colony stimulating factor improves survival of patients with ACLF[5]. Liver transplantation can also improve outcomes, even in critically ill patients with multi-organ failure[6]. However, these advances have not significantly decreased the mortality associated with HBV-related ACLF.

In China, hepatitis B virus (HBV) infections account for 82% of all ACLF[7]. The exact mechanism of HBV-related ACLF is currently unclear. HBV is not directly cytopathic[8] and the hepatocellular injury caused by HBV infection is predominantly immune-mediated[9,10]. Wasmuth *et al*[11] demonstrated that the immunopathology of ACLF is similar to "sepsis-like" immune paralysis. Cytokines also play an important role in ACLF[12,13]. Evidence shows that circulating IL-17$^+$ T cells accumulate in large numbers in the liver of CHB patients, increasing with progression from CHB to ACLF[14,15]. By contrast, Treg cells suppress immune responses and inflammatory diseases[16,17], and they regulate chronic inflammatory responses that contribute to the pathologic events in the liver during HBV infection[18]. Several research groups have demonstrated increased Th17 cells in the peripheral blood and liver tissues, as well as changes in the balance between Th17 and Treg cells in ACLF patients[19,20].

In this study, we focus on the balance between CD4$^+$CD25$^+$FoxP3$^+$ Treg cells and CD4$^+$IL17$^+$ Th17 cells in HBV-related ACLF and examine the effects of this balance on patient responses to therapy and outcomes.

Table 1 Clinical characteristics of subjects in hepatitis B virus-related acute-on-chronic liver failure, chronic hepatitis B, and normal control groups *n* (%)

	ACLF (n = 50)	CHB (n = 29)	NC (n = 20)
Male/female	42/8	24/5	16/4
Age (yr)	40.2 ± 12.0	34.4 ± 12.0	34.5 ± 9.2
ALT (IU/L)	176.6 ± 430.0	328.4 ± 386.8	22.3 ± 6.7
TBIL (μmol/L)	566.3 ± 133.5	68.8 ± 90.9	12.2 ± 2.3
INR	1.83 ± 0.44	1.09 ± 0.10	0.99 ± 0.03
HBeAg	17 (34)	18 (62.1)	NA
Anti-HBe	30 (60)	8 (27.6)	NA
HBVDNA (Log10 IU/mL)	5.25 ± 1.30	6.11 ± 1.28	NA
MELD score	22.5 ± 4.7	4.6 ± 6.5	-2.7 ± 1.9

This table shows the mean ± SD of age, alanine aminotransferase (ALT), total bilirubin (TBIL), international normalized ratio (INR), and hepatitis B virus (HBV) DNA of all patients. Model for End-stage Liver Disease (MELD) scores are shown for the HBV-related acute-on-chronic liver failure (ACLF) group, chronic hepatitis B (CHB) group, and normal control (NC) group. The proportion of patients positive for hepatitis B e antigen (HBeAg) or hepatitis B e antibody (anti-HBe) in the ACLF and CHB groups is also displayed. NA: Not available.

MATERIALS AND METHODS

Subjects

We enrolled 79 patients with HBV infection into the study, 50 patients with HBV-related ACLF and 29 patients with CHB, from the First Affiliated Hospital of Medical College, Xi'an Jiaotong University (Xi'an, China) from January 2009 to June 2012. The ACLF patients were diagnosed according to the criteria recommended by The 19th Conference of the Asian Pacific Association for the Study of the Liver in 2009[1]. Patients were excluded if their liver disease was caused by conditions other than HBV infection. No patient received steroids or other immunosuppressive drugs within 6 mo before sampling.

Table 2 Predisposing factors of hepatitis B virus-related acute-on-chronic liver failure non-survivors and survivors *n* (%)

	Variceal bleeding	Infection			Drug	Alcohol	Indeterminant reasons
		Gastrointestinal tract	Upper respiratory tract	Ascites			
Non-survivors (*n* = 28)	2 (7.1)	5 (17. 8)	4 (14.3)	2 (7.1)	7 (25.0)	2 (7.1)	8 (28.6)
Survivors (*n* = 22)	0	7 (31.8)	5 (22.7)	0	5 (22.7)	1 (4.5)	4 (18.2)

Table 3 Clinical characteristics of non-survivors and survivors in hepatitis B virus-related acute-on-chronic liver failure group

	Non-survivors (*n* = 28)	Survivors (*n* = 22)	*P* value
Male/female	24/4	18/4	ND
Age (yr)	40.8 ± 11.0	39.5 ± 13.3	0.570
TBIL (μmol/L)	607.6 ± 117.5	513.7 ± 136.7	0.006
INR	2.00 ± 0.44	1.62 ± 0.33	0.088
MELD scores	23.7 ± 4.6	20.9 ± 4.4	0.021

This table shows the mean ± SD of age, total bilirubin (TBIL), international normalized ratio (INR) and Model for End-stage Liver Disease (MELD) scores for non-survivors and survivors in the hepatitis B virus (HBV)-related acute-on-chronic liver failure (ACLF) group. The *P* values of each parameter between groups are also displayed. ND: Not done.

The normal controls (NC) consisted of 20 healthy individuals with similar gender and age structures to the two patient groups.

Blood samples were collected from the ACLF patients 1 wk after diagnosis and again on the 2nd, 4th, 6th, 8th, and 12th week. The blood samples of the CHB patients were collected upon diagnosis and before receiving antiviral therapy. The clinical and biochemical details of ACLF patients at the time of their highest total bilirubin (TBIL), as well as those of the CHB patients are listed in Table 1. The Model for End-stage Liver Disease (MELD) score was calculated using the following formula: 3.8 × $\log_e$[bilirubin (mg/dL)] + 11.2 × $\log_e$(INR) + 9.6 × $\log_e$[creatinine (mg/dL)] + 6.4 × (aetiology: 0 if cholestatic or alcoholic, 1 otherwise)[21].

We classified 28 patients as non-survivors: 19 patients died from multi-organ failure, 3 underwent liver transplantation, and 6 discontinued therapy during follow-up because of financial reasons. The remaining 22 patients whose liver function and coagulation recovered to nearly normal within the next 6 mo were classified as survivors.

The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Medical College, Xi'an Jiaotong University, and written informed consent was obtained from each subject prior to enrolment in the study.

Cell stimulation and culture

Heparinized whole blood (200 μL) from study subjects was incubated for 4 h in phorbol 12-myristate 13-acetate (PMA) (final concentration 25 ng/mL) and ionomycin (final concentration 1 μg/mL) with monensin (end concentration 1.4 μg/mL) at 37 ℃ under a 5% CO_2 atmosphere.

Then, the whole blood was separated and incubated with anti-CD4-fluorescein isothiocyanate (FITC) and anti-CD25-phycoerythrin conjugate (PE) or anti-CD4-FITC. After simultaneous fixation and permeabilization, the cells were incubated for 30 min with anti-Foxp3-phycoerythrin-cyanine 5 conjugate (PE-Cy5) or anti-IL17A-PE. Then, the cells were washed again and were resuspended in PBS for flow cytometric analysis.

Flow cytometric analysis

To detect the expression of circulating Th17 and Treg cells, the whole blood was subjected to flow cytometry on a CyFlow® SL machine (PARTEC Company, Germany) using FloMax software.

Virologic and immunologic assessment

The levels of HBsAg, anti-HBs, HBeAg, anti-HBe, anti-HCV, anti-HDV, anti-HEV, and anti-HIV antibodies were detected *via* qualitative enzyme immunoassays. The serum HBV DNA levels were measured using a real-time polymerase chain reaction (PCR) assay with a detection limit of 1 × 10^3 IU/mL. All tests were performed in a clinical laboratory according to standardized methods.

Statistical analysis

Results are expressed as mean ± SD. Statistical comparisons between groups were performed using a Mann-Whitney non-parametric *U* test. A Spearman's correlation analysis was performed to evaluate the relationship between variables. The data were analyzed using SPSS version 16.0 for Windows (SPSS Inc., Chicago, IL). Differences with *P* values < 0.05 were considered statistically significant in all analyses.

RESULTS

Clinical characteristics of different short-term outcomes of ACLF patients

The characteristics of survivors and non-survivors in the ACLF group are listed in Table 2. The biochemical parameters and MELD scores at the time of peak TBIL level of the survivors and non-survivors are listed in Table 3.

Peripheral Th17 and Treg cells expression between groups

The percentages of $CD4^+CD25^+FoxP3^+$ (Treg) cells and $CD4^+IL\text{-}17^+$ (Th17) cells and the ratio of Treg to Th17 cells between groups are relative to $CD4^+$ T cells (Figure 1A). The percentages of circulating Treg cells in the two HBV infection groups were significantly higher than that in the NC group [2.46% ± 0.78%, *P* = 0.000 (*vs* ACLF),

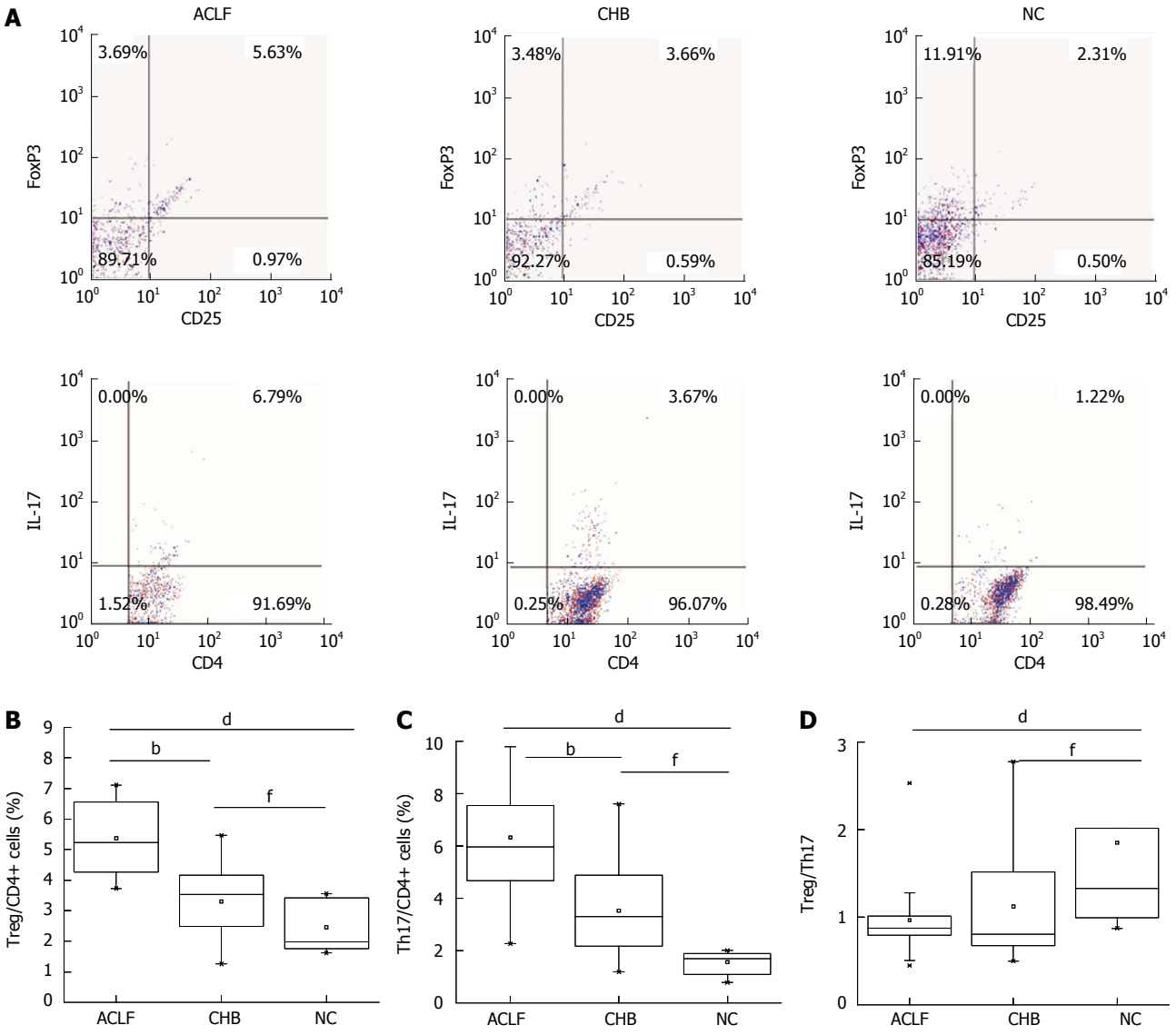

Figure 1 Percentages of T helper 17 cells and regulatory T cells, and the ratio of regulatory T cells to T helper 17 cells in hepatitis B virus-related acute-on-chronic liver failure, chronic hepatitis B, and normal control groups. Typical four-quadrant graphs of $CD25^+FoxP3^+$ cells [regulatory T (Treg)] and $IL17^+$ cells [T helper 17 (Th17)] in $CD4^+$ cells were divided into three groups (A). The proportion of Treg cells (B) and Th17 cells (C) among $CD4^+$ cells, and the Treg cell to Th17 cell ratios (D) were shown as box plot graphs. Acute-on-chronic liver failure (ACLF) *vs* chronic hepatitis B group (CHB) $^bP < 0.01$; ACLF *vs* normal control group (NC) $^dP < 0.01$; CHB *vs* NC $^fP < 0.01$.

$P = 0.008$ (*vs* CHB)]. The percentage of Treg cells in the ACLF group was also significantly higher than that in the CHB group (5.50% ± 1.15% *vs* 3.30% ± 1.13%, $P = 0.001$) (Figure 1B). Strikingly, the percentages of circulating Th17 cells in the ACLF and CHB groups were significantly higher than those in the NC group (6.32% ± 2.22% *vs* 1.56% ± 0.44%, $P = 0.000$; 3.53% ± 1.65% *vs* 1.56% ± 0.44%, $P = 0.000$) (Figure 1C). No significant difference in the Treg cell to Th17 cell ratio was observed between the ACLF group and the CHB group (0.98 ± 0.44 *vs* 1.12 ± 0.64, $P = 0.991$). However, the Treg cell to Th17 cell ratios in the two HBV infection groups were significantly lower than that in the NC group [1.85 ± 1.22; $P = 0.000$ (*vs* ACLF), $P = 0.004$ (*vs* CHB)] (Figure 1D). In summary, the percentages of Th17 and Treg cells in the peripheral blood of ACLF patients increased, whereas the ratio of Treg to Th17 cells was lower in the ACLF and CHB groups compared with that in the NC group.

Clinical correlation of Th17 and Treg expression in HBV-related ACLF

The percentage of Treg cells in the ACLF group was correlated with ALT ($r = 0.381$ $P = 0.006$), TBIL ($r = 0.378$, $P = 0.007$), and INR ($r = 0.381$, $P = 0.006$) (Figure 2A-C). The percentage of Th17 cells was positively correlated with ALT ($r = 0.360$, $P = 0.012$), TBIL($r = 0.323$, $P = 0.025$), and MELD scores (r = 0.293, $P = 0.043$) (Figure 2D-F). The percentage of Treg cells was significantly correlated with the percentage of Th17 cells ($r = 0.425$, $P = 0.003$; Figure 2G). The ratio of Treg to Th17 cells was not significantly correlated with any of the biochemical parameters. Neither the percentages of Treg

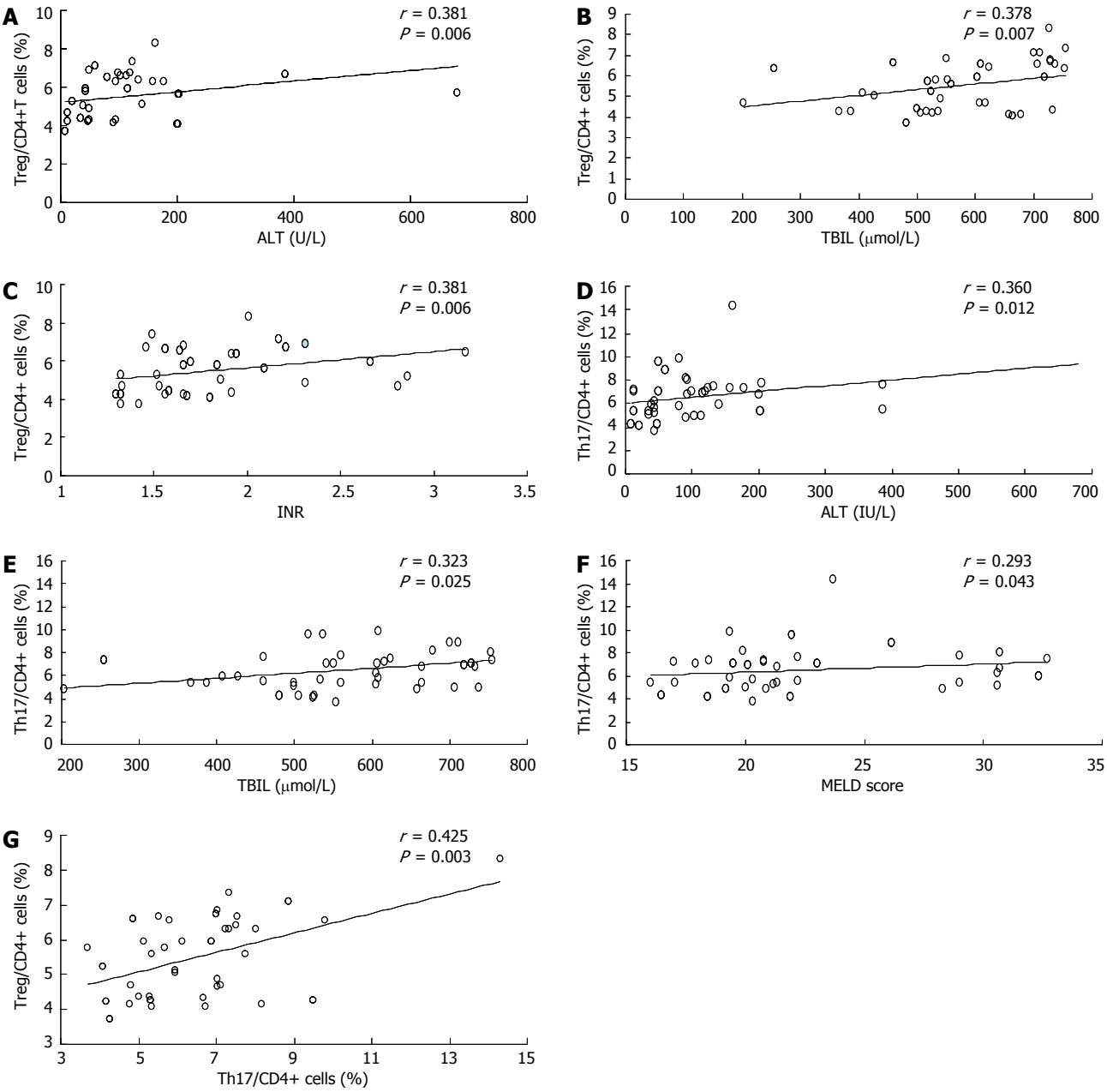

Figure 2 Correlation between T helper 17 cells and regulatory T cells counts and clinical parameters in hepatitis B virus-related acute-on-chronic liver failure group. The percentage of regulatory T (Treg) cells was significantly correlated with alanine aminotransferase (ALT) (A), total bilirubin (TBIL) (B), and international normalized ratio (INR) (C). The percentage of T helper 17 (Th17) cells was significantly correlated with ALT (D), TBIL (E), and Model for End-stage Liver Disease (MELD) scores (F).The percentage of Treg cells was significantly correlated with that of Th17 cells (G).

and Th17 cells, nor the ratio of Treg to Th17 cells, was correlated with serum HBV DNA levels.

In CHB group, the percentage of Treg cells was positively correlated with TBIL ($r = 0.431$, $P = 0.02$), whereas the percentage of Th17 cells was significantly correlated with ALT ($r = 0.367$, $P = 0.05$). Neither the percentages of Treg and Th17 cells, nor the ratio of Treg to Th17 cells, showed a relationship with serum HBV DNA levels.

Percentages of Treg and Th17 cells and the ratio of Treg to Th17 cells during follow-up phase in ACLF patients

The percentages of Treg and Th17 cells were measured serially in the ACLF group during a follow-up phase, which lasted for 8 wk. During TBIL peak, the percentage of Treg cells in non-survivors increased slightly compared with that in survivors (5.76% ± 1.21% *vs* 5.18% ± 1.02%), but the difference was not statistically significant ($P = 0.103$; Figure 3A). The percentage of Th17 cells in non-survivors was significantly higher than that in survivors (7.17% ± 2.37% *vs* 5.24% ± 1.46%; $P = 0.002$) (Figure 3B). No significant difference in Treg cell to Th17 cell ratio was observed between non-survivors and survivors (0.88 ± 0.32 *vs* 1.10 ± 0.54; $P = 0.233$; Figure 3C).

During 8th week follow-up, the percentages of Treg and Th17 cells and the Treg cell to Th17 cell ratio of the survivors were compared with those at the time of TBIL

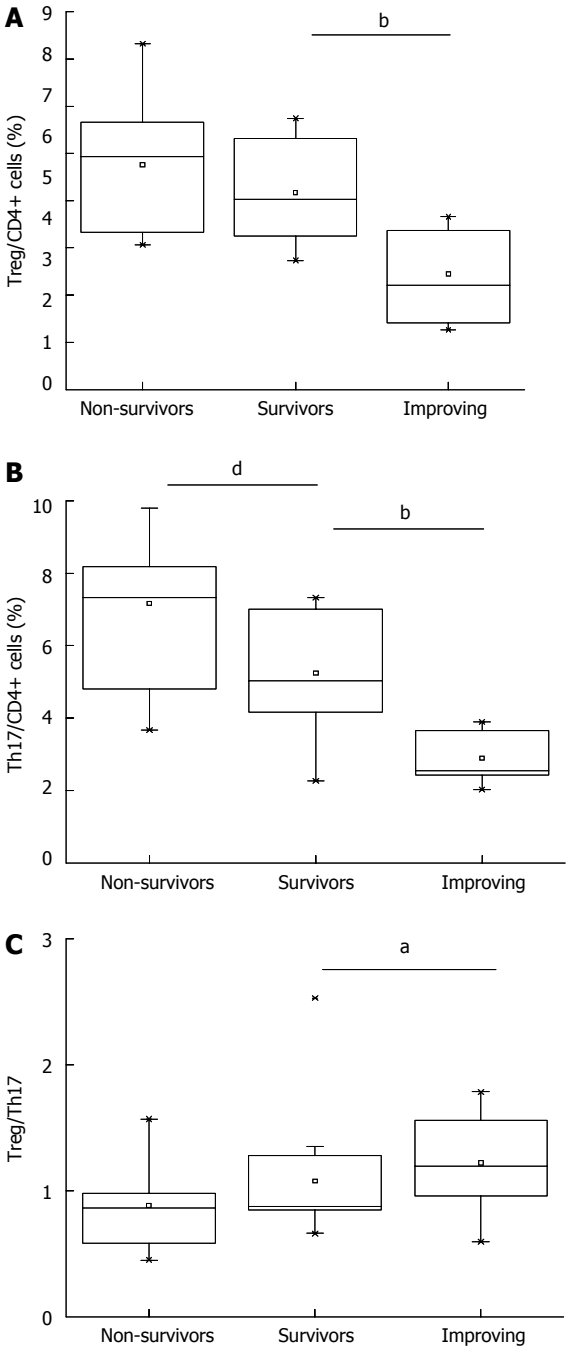

Figure 3 Comparison of regulatory T cells and T helper 17 cell counts, and the regulatory T cell to T helper 17 cell ratio between acute-on-chronic liver failure survivors and non-survivors, as well as those during the peak of acute-on-chronic liver failure severity (total bilirubin peak) and those during the 8th week of follow-up. Regulatory T (Treg) cell counts (A), T helper 17 (Th17) cell counts (B), and the Treg cell to Th17 cell ratios (C) of acute-on-chronic liver failure (ACLF) non-survivors, as well as those of ACLF survivors during the total bilirubin (TBIL) peak and during the 8th week of follow-up. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* the 8th week of follow-up in ACLF survivors, $^{d}P < 0.01$ *vs* non-survivors. Non-survivors: The non-survivors in ACLF group; Survivors: The survivors in ACLF group during the TBIL peak; Improving: The survivors in ACLF group during the 8th week of follow-up.

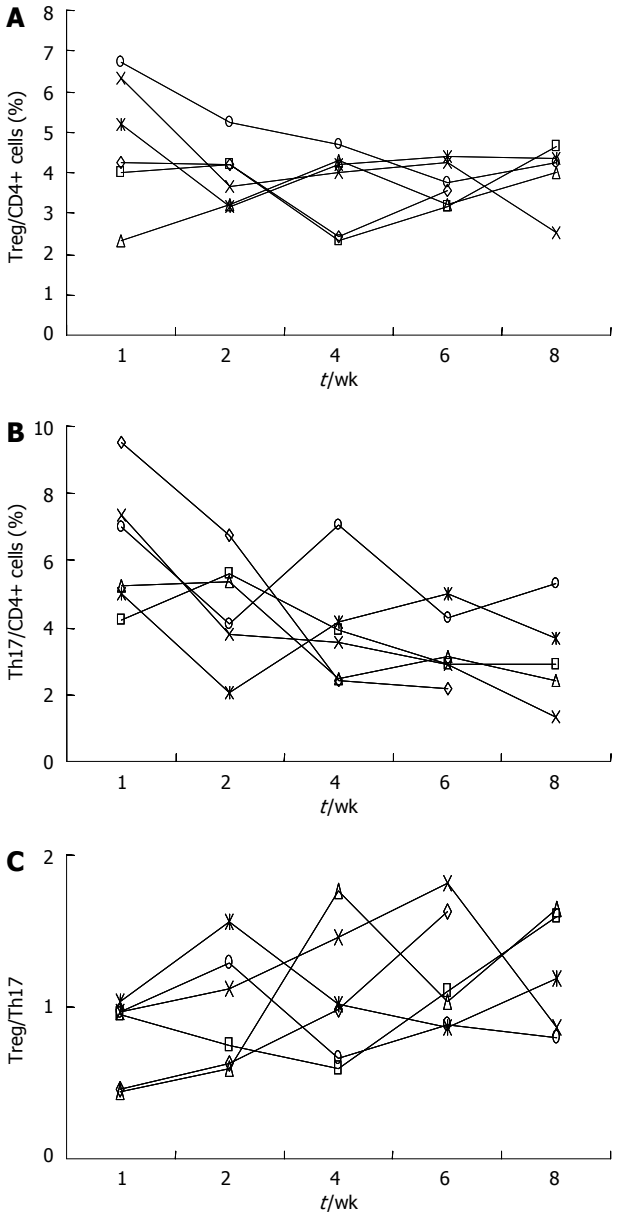

Figure 4 Changes in regulatory T cells and T helper 17 cell counts, and regulatory T cells and T helper 17 cell ratios of acute-on-chronic liver failure survivors. Changes in regulatory T (Treg) cell counts (A), T helper 17 (Th17) cell counts (B), and the Treg cell to Th17 cell ratio (C) of six acute-on-chronic liver failure survivors (5 males and 1 female).

peak. The percentage of Treg cells during the 8th week was significantly lower than that during the TBIL peak (3.45% ± 0.97% *vs* 5.18% ± 1.02%, $P = 0.000$) (Figure 3A), whereas the percentage of Th17 cells during the 8th week was significantly lower than that during the TBIL peak (2.89% ± 0.60% *vs* 5.24% ± 1.46%; $P = 0.000$) (Figure 3B). The Treg cell to Th17 cell ratio was significantly higher during the 8th week than that during the TBIL peak (1.22 ± 0.36 *vs* 1.10 ± 0.54; $P = 0.039$) (Figure 3C).

We examined changes in the percentage of Treg cells and Th17 cells, as well as the Treg cell to Th17 cell ratios, of 6 ACLF survivors (5 males and 1 female) who exhibited decreased TBIL with obvious clinical improvement. The percentage of Treg cells (Figure 4A) decreased together with the percentage of Th17 cells (Figure 4B) during the follow-up period, whereas the Treg cell to Th17

cell ratio increased gradually (Figure 4C).

DISCUSSION

Th17 and Treg cells are subsets of $CD4^+$ T helper cells with developmental pathways that contribute significantly to immune responses[22-27]. Th17 cells are implicated in host defence against a number of microorganisms[28,29]and they participate in autoimmune and chronic inflammatory diseases[30,31]. By contrast, Treg cells display suppressive and surveillance functions in immune responses and inflammatory diseases[16,17]. A study suggested that Th17 cells mediate airway inflammatory responses whereas antigen-specific Treg cells suppress Th17-mediated lung inflammation[32]. Yang *et al*[33] reported that an imbalance between Th17 and Treg cells contributes to the pathogenesis of systemic lupus erythematosus (SLE) and that regulating the balance between Treg and Th17 cells may be a promising strategy for SLE treatment. However, our aim is to determine whether an imbalance in Treg and Th17 cells contributes to the pathogenesis of acute hepatocellular injury in HBV-related ACLF and its mechanism.

Treg and Th17 cells were significantly higher in ACLF patients than in CHB patients and the normal controls. Furthermore, in ACLF patients, the percentage of Treg cells was significantly correlated with the percentage of Th17 cells, as well as with ALT and TBIL. Several recent studies demonstrated that the percentages of circulating Th17 and Treg cells increase with disease progression and are parallel to the severity of liver inflammation as CHB progresses to ACLF[14,18,19]. These results suggest that Th17 cells are a potential marker for the degree of liver injury in ACLF, whereas Treg cells may contribute to the suppression of the immune system[11].

We then analyzed the Treg and Th17 cell counts of ACLF survivors and non-survivors to determine their correlation with their clinical outcomes. The percentage of Th17 cells in ACLF non-survivors was significantly higher than that in survivors, but the percentage of Treg cells and the Treg cell to Th17 cell ratio were not significantly different between the two groups. Our findings are consistent with those of Zhai *et al*[20] who also found increased Th17 cells and Treg cells in ACLF patients. However, they found no significant difference in Th17 and Treg cell counts between ACLF survivors and non-survivors and they observed significantly lower Th17 cell to Treg cell ratios in the survivors than in non-survivors. These differences between the two studies may be attributed to differences in the severity of hepatic injury, as well as differences in the timing of sample acquisition. However, both studies found that patients with ACLF have higher Treg and Th17 cell counts and that higher Th17 cell to Treg cell ratios may predict poorer prognosis.

In China, ACLF occurs mainly in patients with CHB or HBV-related cirrhosis. Spontaneous or treatment-induced inflammatory flare ups are frequently observed in chronic hepatitis B[34]. Several researchers in Asia have demonstrated that early intervention using antiviral therapy improves the short- and long-term outcomes of HBV-related ACLF by aggressively targeting the precipitating events to prevent multi-organ failure[3,4,35]. Entecavir-induced suppression of HBV replication in nine CHB patients showed rapid increases in Th17 cells and decreases in Treg cells, which significantly reduced the Treg cell to Th17 cell ratio[36]. Thus, the effects of antiviral therapies with ACLF will affect Treg and Th17 cells.

We measured the Treg and Th17 cell counts in ACLF survivors during follow-up to determine whether antiviral therapy affects the balance between Treg and Th17 cells. The percentages of Treg and Th17 cells decreased significantly during follow-up, whereas the Treg cell to Th17 cell ratio increased significantly compared with that during the peak of illness, which coincides with the peak serum total bilirubin. Restoring the Treg cell to Th17 cell ratio could help maintain the immune system in a steady state, favouring good outcomes among patients with HBV-related ACLF.

In conclusion, Th17 cell counts may reflect the degree of liver injury, where Treg cells may regulate the protective suppression of the immune system of patients with ACLF. Treg cells and Th17 cells increased in patients with ACLF, but higher Th17 cell to Treg cell ratios may be correlated with poorer prognosis. Restoring the Treg cell to Th17 cell ratio during ACLF could help maintain the immune system in a steady state, which may improve patient prognosis.

COMMENTS

Background

Acute-on-chronic liver failure (ACLF) is an acute hepatic insult with rapid progression and high short- and medium-term mortality. In China, hepatitis B virus (HBV) infections account for 82% of all ACLF cases. Studies have demonstrated that the hepatocellular injury caused by HBV infection is predominantly immune-mediated.However, the mechanism of HBV-related ACLF is currently unclear.

Research frontiers

Several studies showed that Treg and Th17 cells may contribute to the pathologic events in the liver during HBV infection and the balance between Treg cells and Th17 cells may be disrupted during ACLF progression.

Innovations and breakthroughs

The authors found that ACLF patients have significantly increased Treg cell to Th17 cell ratios. The Treg cells and Th17 cells decreased significantly with improvement of the ACLF, but the Treg cell to Th17 cell ratio significantly increased. Thus, Th17 cells may be a marker for the degree of liver injury and poor prognosis in ACLF. Restoring the Treg cell to Th17 cell ratio during the aggravating phase of ACLF could maintain the immune system at a steady state, which favours good prognosis.

Applications

Th17 cells and Treg cells are subsets of $CD4^+$ T helper cells with related developmental pathways. The balance between Th17 cells and Treg cells is important for maintaining human immune homeostasis. The balance between Th17 cells and Treg cells should be maintained before chronic hepatitis B progresses to ACLF. Once CHB has progressed to ACLF, restoring the Treg cell to Th17 cell ratio becomes even more significant to the outcome.

Terminology

ACLF is defined as an acute hepatic insult that manifests as jaundice and coagulopathy in a patient with chronic liver disease, complicated within 4 wk by ascites and/or encephalopathy. The major aetiologic agents of ACLF are alcohol

and drugs in the West and infectious agents in the East. A characteristic feature of ACLF is its rapid progression and high short- and medium-term mortality (50% to 90%).

Peer review

The manuscript focuses on the balance between $CD4^+CD25^+FoxP3^+$ Treg cells and $CD4^+IL17^+$ Th17 cells in HBV-related ACLF. The authors examined the effects of this balance on patient responses to therapy and outcomes. This manuscript is interesting. The references used in the study are updated.

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ORIGINAL ARTICLE

Reversal of multidrug resistance in gastric cancer cells by CDX2 downregulation

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Abstract

AIM: To explore the role of CDX2 in the multi-drug resistance (MDR) process of gastric cancer *in vitro* and *in vivo*.

METHODS: A cisplatin-resistant gastric cancer cell line with stable downregulation of CDX2 was established. mRNA and protein expression levels of CDX2, survivin, cyclin D1, and c-Myc were detected by western blotting and semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR). The influence of downregulation of CDX2 on MDR was assessed by measuring IC50 of SGC7901/DDP cells to cisplatin, doxorubicin, and 5-fluorouracil, rate of doxorubicin efflux, apoptosis, and cell cycle progression detected by flow cytometry. In addition, we determined the *in vivo* effects of CDX2 small interfering RNA (siRNA) on tumor size, and apoptotic cells in tumor tissues were detected by deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling and hematoxylin and eosin staining.

RESULTS: CDX2 siRNA led to downregulation of endogenous CDX2 mRNA (0.31 ± 0.05 *vs* 1.10 ± 0.51, 0.31 ± 0.05 *vs* 1.05 ± 0.21, $P = 0.003$) and protein (0.12 ± 0.08 *vs* 0.51 ± 0.07, 0.12 ± 0.08 *vs* 0.55 ± 0.16, $P = 2.57 \times 10^{-4}$) expression. It significantly promoted the sensitivity of SGC7901/DDP cells to cisplatin (0.12 ± 0.05 *vs* 0.33 ± 0.08, 0.12 ± 0.05 *vs* 0.39 ± 0.15, $P = 0.001$), doxorubicin (0.52 ± 0.13 *vs* 4.11 ± 1.25, 0.52 ± 0.13 *vs* 4.05 ± 1.44, $P = 2.81 \times 10^{-4}$), and 5-fluorouracil (0.82 ± 0.13 *vs* 2.81 ± 0.51, 0.82 ± 0.13 *vs* 3.28 ± 1.03, $P = 1.71 \times 10^{-4}$). Flow cytometry confirmed that the percentage of apoptotic cells increased after CDX2 downregulation (32.15% ± 2.15% *vs* 17.63% ± 3.16%, 32.15% ± 2.15% *vs* 19.3% ± 2.25%, $P = 1.73 \times 10^{-6}$). This notion was further supported by the observation that downregulation of CDX2 blocked entry into the S-phase of the cell cycle (31.53% ± 3.78% *vs* 65.05% ± 7.25%, 31.53% ± 3.78% *vs* 62.27% ± 5.02%, $P = 7.55 \times 10^{-7}$). Furthermore, downregulation of CDX2 significantly increased intracellular accumulation of doxorubicin (0.21 ± 0.06 *vs* 0.41 ± 0.11, 0.21 ± 0.06 *vs* 0.40 ± 0.08, $P = 0.003$). In molecular studies, semiquantitative RT-PCR and western blotting revealed that CDX2 downregulation could inhibit expression of c-Myc, survivin and cyclin D1.

CONCLUSION: CDX2 may be involved in regulating multiple signaling pathways in reversing MDR, suggesting that CDX2 may represent a novel target for gastric cancer therapy.

Key words: Homeobox gene CDX2; RNA interference; Gastric cancer; Drug resistance; Murine model

Core tip: Modulator of multidrug resistance (*MDR*) gene is a direct transcriptional target of CDX2. However, we still speculate whether CDX2 affects MDR through other ways. Our results showed that downregulation of CDX2 significantly promoted sensitivity of SGC7901/DDP cells to anticancer drugs, and increased the percentage of apoptotic cells. Downregulation of CDX2 potentiated G1 phase arrest of the cell cycle. Furthermore, it significantly increased intracellular accumulation of doxorubicin. We conclude that downregulation of CDX2 can efficiently reverse MDR *via* inhibition of apoptosis/cell-cycle-related gene expression (c-Myc, survivin and cyclin D1).

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INTRODUCTION

The transcription factor, CDX2, is a member of the caudal-related homeobox gene family. It is expressed exclusively in the small and large intestine, playing important roles in proliferation and differentiation of intestinal epithelial cells[1]. Several investigators have reported that low levels of CDX2 are a characteristic feature of human colon and squamous esophageal cancer[2,3], but others have shown that strong and robust expression of CDX2 is found in > 80% of colorectal cancer and non-small cell lung cancer[4,5]. In addition, CDX2 enhances proliferation and has tumorigenic potential in human colon cancer cell lines LoVo and SW48[6]. These studies have suggested that CDX2 also has oncogenic activity. Together, these conflicting findings point to a complex role for CDX2 in cell regulation. In adult humans, CDX2 is associated with intestinal metaplasia in the stomach in which ectopic expression of CDX2 is speculated to cause the gastric epithelial cells to trans-differentiate and assume the intestinal phenotype[7]. In addition, CDX2 transgenic mice have been shown to have intestinal metaplasia and a high incidence of gastric carcinoma[8,9].

In a previous study[10], it has been reported that RNA interference (RNAi)-mediated inhibition of CDX2 decreases endogenous MDR1 expression. MDR1 was originally identified as an overexpressed and amplified gene in multidrug-resistant cells. Its product, P-glycoprotein (P-gp), appears to play a critical role in drug resistance, which suggests that CDX2 is associated with multidrug resistance (MDR) of gastric cancer. Previously, we have reported that CDX2 affects the cell cycle and apoptosis of gastric cancer[11], Furthermore, apoptosis is just one of the important mechanisms of reversal MDR[12]. CDX2 may play a crucial role in the control of reversal MDR.

In the present study, we constructed small interfering RNA (siRNA) sequences that targeted CDX2, transfected them into a cisplatin-resistant gastric cancer cell line SGC7901/DDP, selected stable transfectants, and explored changes in IC50, rate of doxorubicin efflux, cell cycle, and apoptosis. We also observed the effect of CDX2 siRNA on the expression of genes associated with apoptosis, including c-Myc and survivin. Moreover, we investigated the effects of CDX2 downregulation on the growth and apoptosis of SGC7901/DDP cells in nude mice.

MATERIALS AND METHODS

Reagents

5-fluorouracil, cisplatin and doxorubicin were purchased from Sigma-Aldrich (St Louis, MO, United States). Cell culture medium RPMI-1640 was purchased from Invitrogen-Gibco (Carlsbad, CA, United States). Fetal bovine serum (FBS) was from Invitrogen-Gibco. Trypsin, streptomycin and penicillin were obtained from Sunshine Biotechnology (Nanjing, China). CDX2, c-Myc, survivin, cyclin D1, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and β-actin antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, United States). All other chemicals were of the highest commercial grade available.

Cell culture

The cells were cultured in RPMI 1640 supplemented with 10% FBS (Sijiqing Biotec, Co. Ltd., Hangzhou, China), antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) in a humidified 5% CO_2 atmosphere at 37.8 ℃. For SGC7901/DDP cells, 0.6 μg/mL cisplatin was supplemented in the medium to maintain the drug-resistance phenotype.

Gene transfection

Recombinant lentiviral vector for *CDX2* gene (siRNA-CDX2) and null vector (siRNA-NC) were stored in our laboratory[13]. SGC7901/DDP cells were seeded in six-well plates with antibiotic-free medium. After 24 h incubation, cells were infected with viral supernatant at a multiplicity of infection of 150 PFU per cell (MOI = 150), and the stable-transfected cell lines were obtained by culturing transfected cells in the presence of 700 mg/mL G418 (Invitrogen, Carlsbad, CA, United States) for 3-4 wk. The cells were divided into three groups: SGC7901/DDP + siRNA-CDX2, SGC7901/DDP + siRNA-NC, and SGC7901/DDP.

Measurement of cell drug sensitivity by MTT analysis

The IC50 was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazoliumbromide] assay. Cells were plated in 96-well plates (5000 cells/well), and after

adherence, the cells were exposed to cisplatin, doxorubicin, and 5-fluorouracil. After incubation for 48 h, the cells were incubated with 20 μL MTT (at a final concentration of 0.5 mg/mL) at 37 ℃ for 4 h. The medium was removed and the precipitated formazan was dissolved in 100 μL DMSO. The absorbance at 490 nm was detected using a microplate reader (Bio-Rad, Hercules, CA, United States). The IC50 was estimated by the relative survival curve. Each assay was performed in triplicate.

Measurement of pump rate of doxorubicin by flow cytometry

The cells were inoculated into six-well plates and 4 mg/mL doxorubicin was added, and all wells were placed at 37 ℃ for 30 min. Flow cytometry was used to measure the fluorescent intensity of doxorubicin in cells with an excitation wavelength of 488 nm and emission wavelength of 575 nm. The cells were then washed twice with fresh culture medium and incubated with the new medium at 37 ℃ for 1 h to detect the retained doxorubicin. Subtraction of the fluorescence retained from the total fluorescence was the fluorescent index of doxorubicin. The procedure was repeated three times and an average value was obtained to calculate the pump rate of doxorubicin. The pump rate of the drug from the cells = (accumulated quantity of doxorubicin-retained quantity of doxorubicin)/accumulated quantity of doxorubicin.

Cell cycle analysis by flow cytometry

SGC7901/DDP cells (1×10^6) were washed twice with ice-cold PBS, treated with trypsin, and fixed in cold 70% ethanol at 4 ℃ for 30 min. The cell pellet was incubated in a solution containing 50 ng/mL propidium iodide, 0.2 mg/mL RNase, and 0.1% Triton X-100 at room temperature for 30 min. The cells were analyzed by flow cytometry using an EPICS XL-MCL FACScan (Becton-Dickinson, Mountain View, CA, United States). The data was analyzed with the MultiCycle Software for Windows (Phoenix Flow Systems, San Diego, CA, United States).

Semiquantitative reverse-transcriptase polymerase chain reaction

Total RNA was extracted from SGC7901/DDP + siRNAi-CDX2 cells, SGC7901/DDP + siRNAi-NC cells, and SGC7901/DDP cells using TRIzol Reagent (Invitrogen). All gene segments were amplified and verified by semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR). cDNAs were reverse-transcribed from 2 μg total RNA. The PCR primer sequences (CDX2 primers were sense: 5'-CGG CAG CCA AGT GAA AAC-3' and antisense: 5'-GAT GGT GAT GTA GCG ACT GTA-3'. Survivin primers were sense: 5'-AAA TGC ACT CCA GCC TCT GT-3' and antisense: 5'-TGT CGA GGA AGC TTT CAGGT-3'. Cyclin D1 primers were sense: 5'-CCC TCG GTG TCC TAC TTC AA-3' and antisense: 5'-GGG GAT GGT CTC CTT CAT CT-3. c-Myc primers were sense: 5'-TTC TCT CCG TCC TCG GAT TC-3' and antisense: 5'-GTA GTT GTG CTG ATG TGT GG-3'. GAPDH primers were sense: 5'-ACC ACA GTC CAT GCC ATC AC-3' and antisense: 5'-TCA CCA CCC TGT TGC TGT A-3'). The products of PCR were checked by agarose gel electrophoresis, and the abundance of each mRNA was detected and normalized to that of GAPDH mRNA.

Western blotting

Cell lysates were prepared in a buffer containing 100 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 7.6), 1 mmol/L EDTA (pH 8.0), 1 μg/mL aprotinin, 100 μg/mL phenylmethylsulfonyl fluoride, and 1% (v/v) NP-40. After protein quantitation using the Lowery protein assay, equal amounts of proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes by the semi-dry blotting method using a three-buffer system. The membranes were incubated with a dilution of primary antibody (anti-CDX2: 1:500, anti-c-Myc: 1:1000, anti-survivin: 1:1500, anti-cyclin D1:1:3000), overnight at 4 ℃. The membrane was washed with TBST and incubated with a peroxidase-conjugated secondary antibody (1:1000) (Santa Cruz Biotechnology) for 1 h. Specific antibody binding was detected using a chemiluminescence detection system (Pierce, Rockford, IL, United States), according to the manufacturer's recommendations. Western blot film was scanned, and the net intensities of the bands were quantified using Image-QuanT software (Molecular Dynamics, Sunnyvale, CA, United States). After development, the membrane was stripped and reprobed with antibody against GAPDH (1:1000) or β-actin (1:1500) to confirm equal sample loading.

Effect of CDX2 siRNA on reversing MDR of human gastric cancer in vivo

BALB/c 5-wk-old male nude mice (Guangxi Animal Center, Nanning, China) were kept under specific pathogen-free conditions and tended to in accordance with institutional guidelines. All experimental studies were approved by the Guangxi Medical University Animal Care and Use Committee. SGC7901/DDP cells were used for tumor implantation. Approximately 2×10^6 tumor cells were harvested, resuspended in 100 μL PBS, implanted subcutaneously into the flanks of the BALB/c nude mice, and resulting tumor was named as SGC7901/DDP tumor. After 7 d, when the SGC7901/DDP tumor measured 3-5 mm in diameter, these nude mice were randomly divided into the following three groups (6 mice/per group): SGC7901/DDP + siRNA-CDX2, SGC7901/DDP + siRNA-NC, and SGC7901/DDP. The animals were administered an intratumoral injection of LV-siRNA- CDX2 or LV-siRNA-NC at a titer of 5×10^6 TU in 100 μL PBS, and injection of an equal volume of PBS was used as a blank control. After the first injection, the animals were administered a similar injection every 2 d. DDP was administered by intraperitoneal injection at a dose of 25 mg/kg. After the first injection, the animals were administered a similar injection every 2 d. The tumors were monitored every day and measured every 2 d with a caliper, and the diameters were recorded. The tumor volume (TV) was calculated by the formula: $TV =$

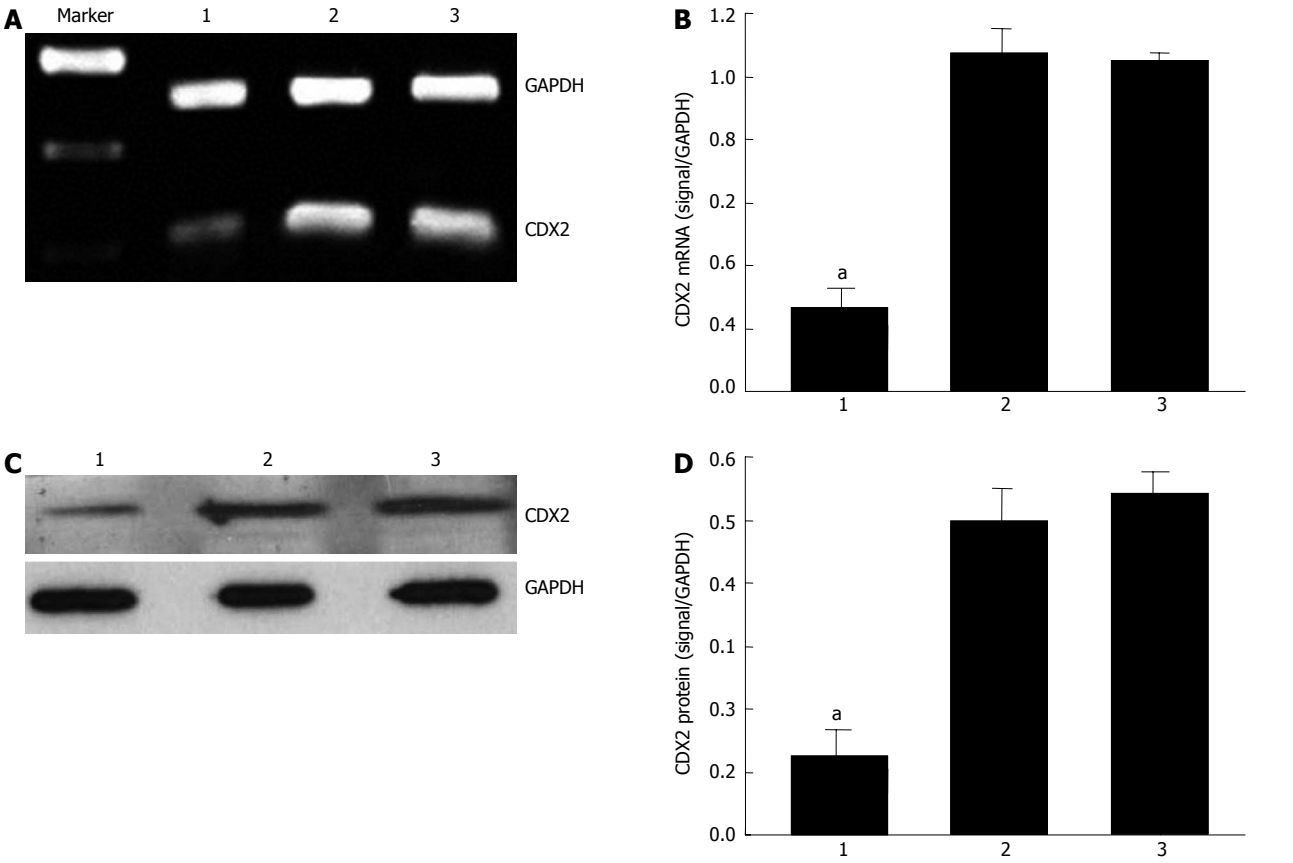


Figure 1 mRNA and protein expressions of CDX2 after RNA interference. A, B: Expression level of CDX2 mRNA was determined by semiquantitative reverse-transcriptase polymerase chain reaction; C, D: Expression level of CDX2 protein was determined by Western blotting. mRNA results were expressed as the ratio of CDX2 to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Western blotting results were expressed as the ratio of optical density of CDX2 bands to GAPDH bands. All values are mean ± SE. $^{a}P < 0.05$ for SGC7901/DDP + small interfering RNA (siRNA)-CDX2 cells *vs* SGC7901/DDP + siRNA-NC cells and SGC7901/DDP cells. Lane 1: SGC7901/DDP + siRNA-CDX2 cells; Lane 2: SGC7901/DDP + siRNA-NC cells; Lane 3: SGC7901/DDP cells.

$W^2 \times L/2$, where L is the length and W is the width of the tumor. The relative tumor volume (RTV) was calculated by the formula: $RTV = V_t/V_0$ (V_0 is the TV at the day when the chemicals were given, and V_t is the TV of subsequent measurement). The animals were sacrificed at 12 d after tumor injection and the tumors were analyzed.

Hematoxylin and eosin staining and deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay

For hematoxylin and eosin (HE) staining tumor tissues were fixed in 4% formaldehyde, dehydrated with gradient ethanol, and embedded in paraffin wax. Tissue sections were dewaxed and rehydrated according to a standard protocol. Sections were stained with HE. For the deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay, apoptotic cells in sections of mouse tumor tissue were detected using an *in situ* apoptosis detection kit (KEYGEN, Nanjing, China) as instructed by the manufacturer. Cells were visualized with a light microscope (Olympus IX70, Tokyo, Japan). The apoptotic index was calculated as follows: the apoptotic index = number of apoptotic cells/total number of cells. The *in vivo* experiments strictly obeyed the ethical principles and guidelines for scientific experiments on animals.

Statistical analysis

Data are expressed as mean ± SE. Statistical significance was determined using χ^2 test, Student's t test, or one-way analysis of variance (ANOVA). Statistical analysis were carried out using SPSS version 13.0 (Chicago, IL, United States) or Origin 7.5 software programs (OriginLab, Northampton, MA, United States). A value of $P < 0.05$ was considered as statistically significant.

RESULTS

CDX2 siRNA inhibits CDX2 mRNA and protein expression

Our previous study suggested that recombinant lentiviral vector for CDX2 gene (siRNA-CDX2) successfully inhibited CDX2 mRNA and protein expression in MGC-803 cells[13]. In the present study, we further tested the hypothesis that CDX2 siRNA downregulates CDX2 mRNA and protein expression in SGC7901/DDP cells. We treated SGC7901/DDP cells with siRNA-CDX2 and siRNA-NC (negative control). Transfection of siRNA-CDX2 into SGC7901/DDP cells led to marked inhibition of CDX2 mRNA (Figure 1A) and protein expression (Figure 1C). Densitometry analysis showed that CDX2 mRNA (Figure 1B) and protein (Figure 1D) in SGC7901/DDP

Table 1 IC50 values for anticancer drugs in SGC7901/DDP cells

	Doxorubicin (μg/mL)	5-fluorouracil (μg/mL)	Cisplatin (μg/mL)
SGC7901/DDP + siRNA-CDX2	0.12 ± 0.05[a]	0.52 ± 0.13[a]	0.82 ± 0.13[a]
SGC7901/DDP + siRNA-NC	0.33 ± 0.08	4.10 ± 1.25	2.81 ± 0.50
SGC7901/DDP	0.39 ± 0.15	4.05 ± 1.44	3.28 ± 1.03

IC50 values were evaluated by MTT assay. Each experiment was conducted in triplicate. Data are expressed as means ± SD. One-way analysis of variance followed by Dunnett's multiple comparison tests revealed statistical differences. [a]$P < 0.05$ for SGC7901/DDP + small interfering RNA (siRNA)-CDX2 cells *vs* SGC7901/DDP + siRNA-NC cells and SGC7901/DDP cells.

+ siRNA-CDX2 cells were about 3.5- and 4-fold lower, respectively, than those in SGC7901/DDP + siRNA-NC cells and SGC7901/DDP cells ($P < 0.05$). There were no differences between SGC7901/DDP + siRNA-NC cells and SGC7901/DDP cells. These results suggested that CDX2 siRNA could downregulate CDX2 mRNA and protein expression in SGC7901/DDP.

CDX2 siRNA reverses MDR

Although SGC7901/DDP cells were selected with the single anticancer drug cisplatin, they also displayed multiple resistances to other anticancer drugs. We studied the regulatory effects of CDX2 siRNA on the drug sensitivity of gastric cancer cells. MTT assay was used to detect the sensitivity of cells to one P-gp-related drug (doxorubicin) and two P-gp-non-related drugs (5-fluorouracil and cisplatin). As showed in Table 1, compared with SGC7901/DDP + siRNA-NC cells and SGC7901/DDP cells, SGC7901/DDP + siRNA-CDX2 exhibited significantly decreased IC50 values for cisplatin, doxorubicin and 5-fluorouracil ($P < 0.05$).

Effects of CDX2 siRNA on pump rate of doxorubicin

Pumping out chemotherapeutic agents is the key process in MDR[14]. We proposed that downregulation of CDX2 inhibited drug efflux in gastric cancer cells. To test this hypothesis, intracellular drug accumulation and retention were evaluated using doxorubicin as a probe. As shown in Figure 2A, compared with SGC7901/DDP + siRNA-NC cells and SGC7901/DDP cells, SGC7901/DDP + siRNA-CDX2 cells exhibited significantly increased accumulation and retention as well as a lower releasing index of doxorubicin (Figure 2B) ($P < 0.05$).

Effect of CDX2 siRNA on cell cycle control

We used flow cytometry to determine whether reversal of MDR by CDX2 siRNA in SGC7901/DDP cells was mediated, at least in part, through an effect on cell cycle progression. We found that the number of cells in G1 phase markedly increased, while those in S phase decreased in SGC7901/DDP + siRNA-CDX2 cells, compared with SGC7901/DDP + siRNA-NC cells and SGC7901/DDP cells (Figure 2C) ($P < 0.05$).

CDX2 siRNA induces apoptosis

Anti-apoptosis is an important mechanism of MDR, therefore, we investigated the effect of siRNA-CDX2 on cisplatin-induced gastric cancer cell apoptosis by calculating apoptosis index. Cells were stained with annexin V PE and 7-AAD, and then subsequently analyzed by flow cytometry. The dual parameter fluorescent dot plots showed that the viable cells were in the lower left quadrant, and the apoptotic cells were in the right quadrant. As shown in Figure 2E, compared with SGC7901/DDP + siRNA-NC cells and SGC7901/DDP cells, SGC7901/DDP + siRNA-CDX2 cells exhibited significantly increased apoptosis index (Figure 2F) ($P < 0.05$).

CDX2 siRNA influenced expression of c-Myc, survivin and cyclin D1

To investigate the mechanism by which CDX2 siRNA induces reversal of MDR in SGC7901/DDP cells, we detected expression levels of some well-known regulators of apoptosis (caspase-9, caspase-3, p53, bax, bcl-2, Survivin, and c-Myc), and an important cell cycle molecule (cyclin D1) by semiquantitative RT-PCR and Western blotting (Figure 3). The mRNA and protein expression level of c-Myc, survivin and cyclin D1 in SGC7901/DDP + siRNA-CDX2 cells was lower than that in SGC7901/DDP + siRNA-NC cells and SGC7901/DDP cells ($P < 0.05$). However, no significant difference in the expression level of caspase-9, caspase-3, p53, bax and bcl-2 was found in the cell models (data not shown).

Effect of CDX2 siRNA on reversing MDR of human gastric cancer in vivo

We examined the effect of CDX2 siRNA on growth of SGC7901/DDP cells *in vivo*, by implanting LV-siRNA-CDX2 and LV-siRNA-NC subcutaneously into the flanks of BALB/c nude mice. We detected expression levels of CDX2 *in vivo* by semi-quantitative RT-PCR and Western blotting. The mRNA (Figure 4A) and protein (Figure 4B) expression level of CDX2 in SGC7901/DDP + siRNA-CDX2 group was lower than that in SGC7901/DDP + siRNA-NC group and SGC7901/DDP group. Three weeks after implantation, TV in the SGC7901/DDP + siRNA-CDX2 group was significantly less than in the SGC7901/DDP and SGC7901/DDP + siRNA-NC groups ($P < 0.05$) (Figure 4D). The percentage of apoptotic tumor cells in SGC7901/DDP + siRNA-CDX2 cells was 7.2% ± 1.3%, which was more than the 3.1% ± 1.2% in SGC7901/DDP + siRNA-NC cells and 3.1% ± 1.4% in SGC7901/DDP cells, as determined by the HE staining and TUNEL assay (Figure 4C).

DISCUSSION

The development of MDR to cancer chemotherapy is a major obstacle to the effective treatment of gastric cancer[14]. However, the mechanism of MDR remains obscure. P-gp was the first molecule identified as a modulator of MDR. After that, various other molecules were

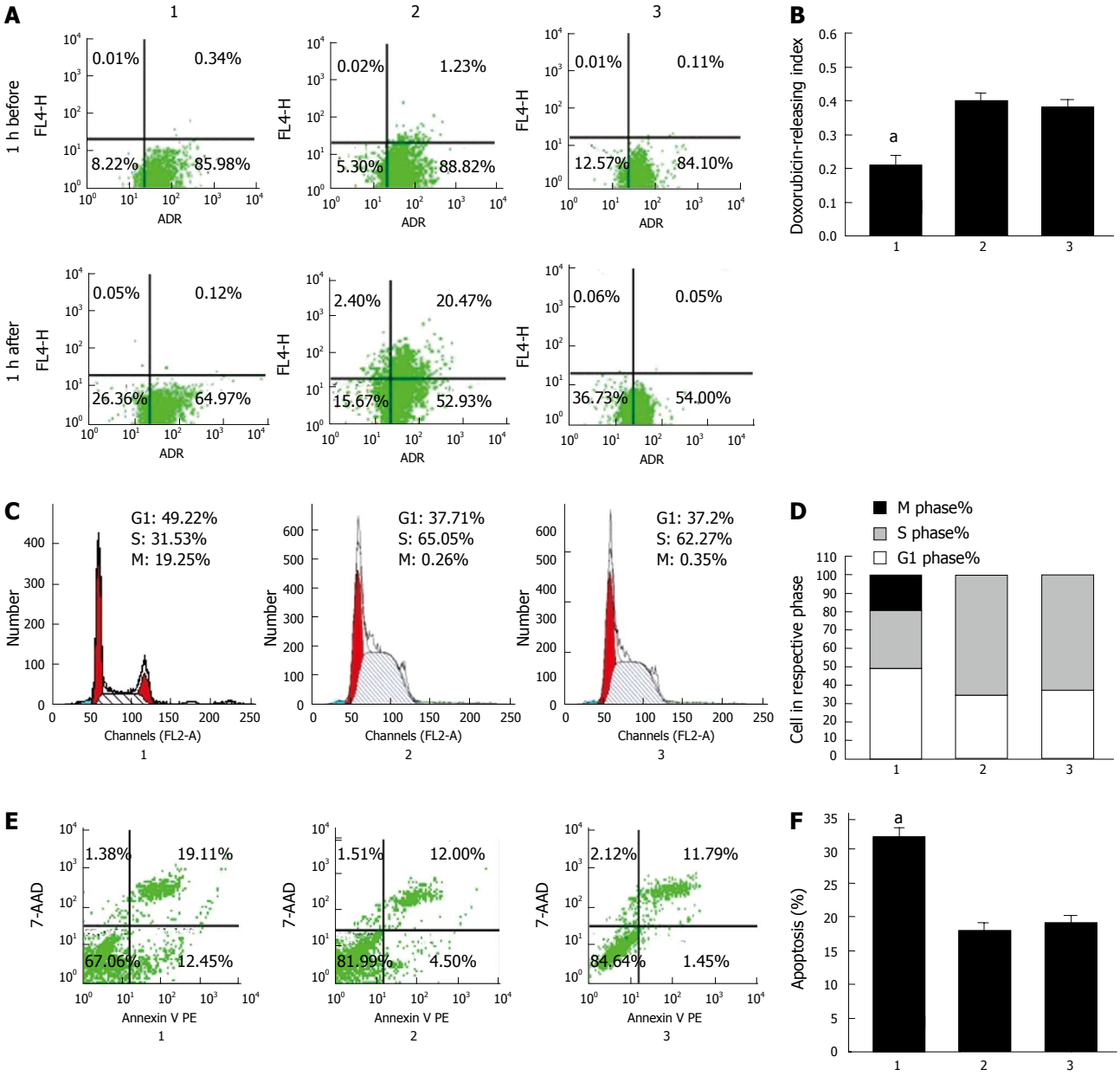
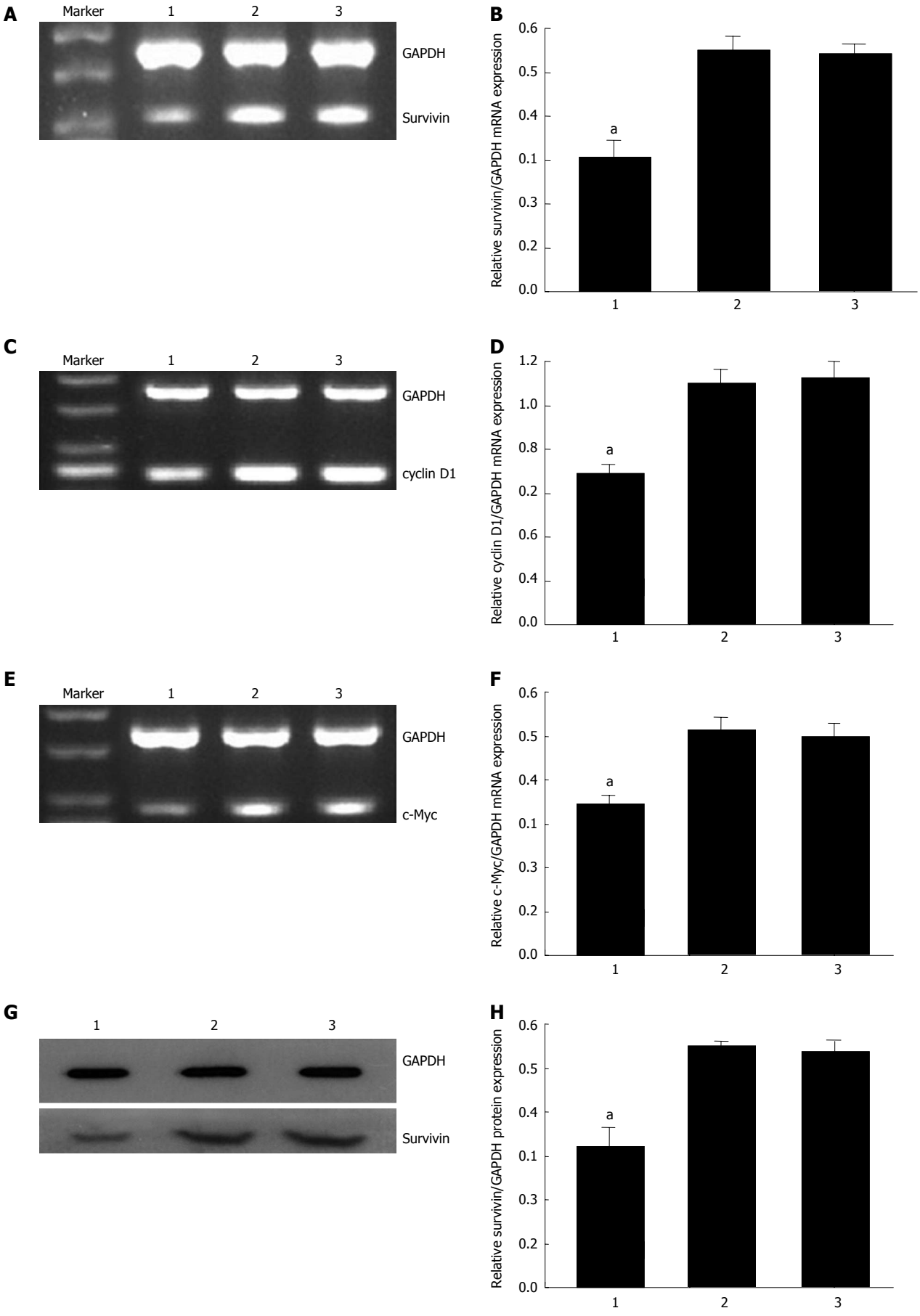


Figure 2 Effect of downregulation of CDX2 on cell pump rate of doxorubicin, cell cycle, and apoptotic rate in SGC7901/DDP cells after RNA interference. A, B: Pump rate of doxorubicin in SGC7901/DDP cells after RNAi was analyzed by flow cytometry; C, D: Cell cycle in SGC7901/DDP cells after RNAi was analyzed by flow cytometry; E, F: Apoptotic rate in SGC7901/DDP cells after RNAi was analyzed by flow cytometry. $^{a}P < 0.05$ for SGC7901/DDP + small interfering RNA (siRNA)-CDX2 cells *vs* SGC7901/DDP + siRNA-NC cells and SGC7901/DDP cells. Lane 1: SGC7901/DDP + siRNA-CDX2 cells; Lane 2: SGC7901/DDP + siRNA-NC cells; Lane 3: SGC7901/DDP cells.

shown to be involved, including transporters that eject anticancer drugs from cells, such as MDR-associated protein (MRP)[15], genes regulating apoptosis, such as p53[16], PKC[17], and Bcl-2 family[18]. Recently, the distribution of drugs in cancer cells was also considered to play a part in MDR[19]. According to our previous report, some classic molecules are involved in MDR, including caspase-3 (apoptosis-related cysteine peptidase) and caspase-9 (an initiator caspase, has been linked to the mitochondrial death pathway)[20,21], but there may be other mechanisms that control MDR of gastric cancer cells[12].

The CDX2 homeobox gene, which is homologous to the *Drosophila* gene caudal, has an essential role during early development[2], an important study by Ma *et al*[22] demonstrated that short interfering RNA-mediated knockdown of CDX2 resulted in reduced apical sodium-dependent bile acid transporter (ASBT) mRNA expression in intestinal cells. Overexpression of CDX2 in human colon cancer cells induces a less malignant phenotype, inhibiting proliferation, invasion, and migration[23]. Furthermore, CDX2 has a crucial role in the regulation of MDR1 gene expression in drug resistance[10]. However, the precise molecular mechanism of CDX2 in reversing MDR in gastric cancer cells is still poorly characterized. The present study is believed to be the first to correlate CDX2 with MDR of gastric cancer cells, and we found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Relative survivin/GAPDH mRNA expression
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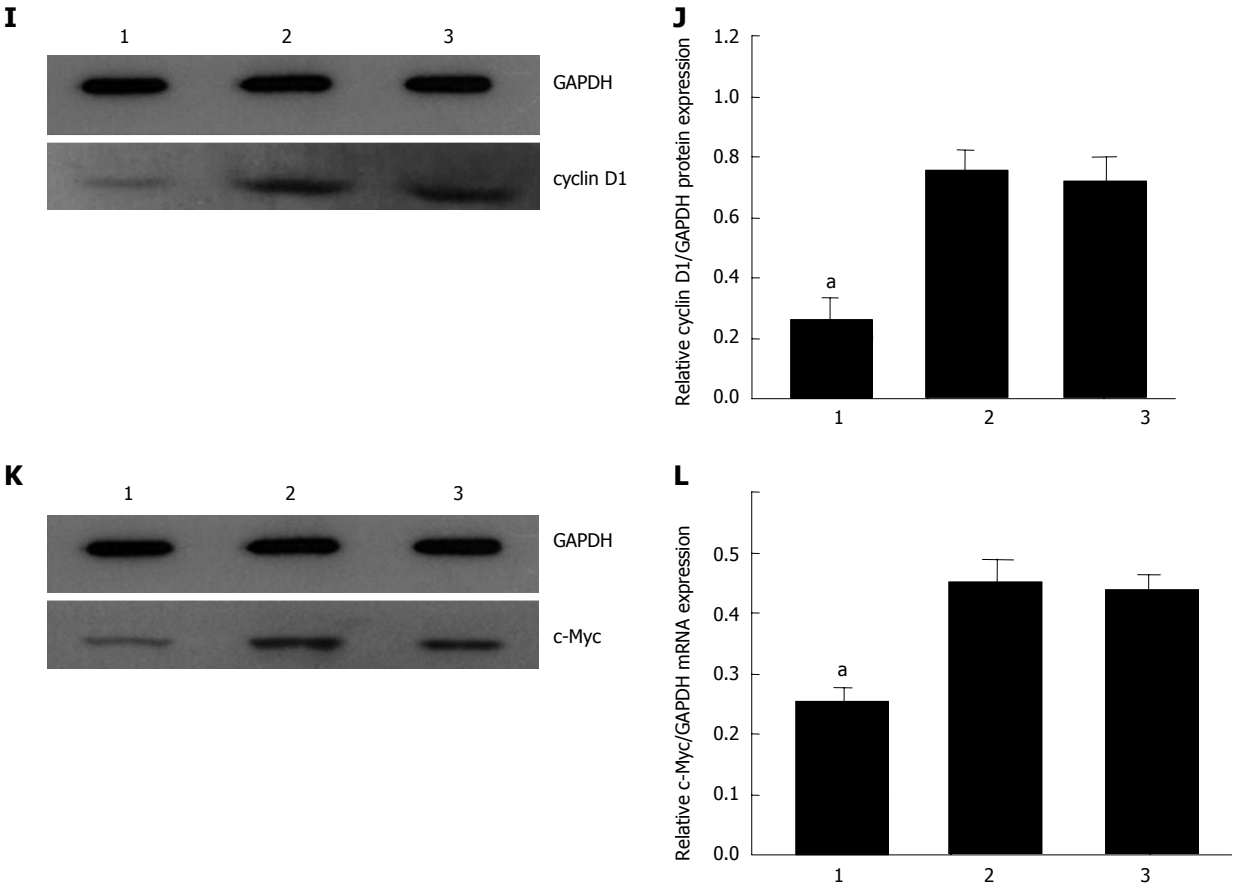

Figure 3 RNA interference-mediated inhibition of CDX2 decreased survivin, cyclin D1 and c-Myc mRNA and protein expression. mRNA expression levels of survivin (A), cyclin D1 (C), and c-Myc (E) were determined by semiquantitative reverse-transcriptase polymerase chain reaction. Protein expression levels of survivin (G), cyclin D1 (I), and c-Myc (K) were determined by western blotting. mRNA results were expressed as the ratio of survivin (B), cyclin D1 (D), and c-Myc (F) to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Western blotting results are expressed as the ratio of optical density of survivin (H), cyclin D1 (J) and c-Myc (L) bands to GAPDH bands. All values are mean ± SE. $^{a}P < 0.05$ for SGC7901/DDP + small interfering RNA (siRNA)-CDX2 cells *vs* SGC7901/DDP + siRNA-NC cells and SGC7901/DDP cells. Lane 1: SGC7901/DDP + siRNA-CDX2 cells; Lane 2: SGC7901/DDP + siRNA-NC cells; Lane 3: SGC7901/DDP cells.

that expression of CDX2 regulated drug efflux pumping, the cell cycle, and apoptosis. The multiple changes conferred by CDX2 on gastric cancer cells are not surprising, given the involvement of CDX2 in a wide range of biochemical reactions, and CDX2 is a homeobox transcription factor that contributes to reversing MDR.

Our study indicated that CDX2 siRNA led to marked downregulation of CDX2 mRNA and protein expression in SGC7901/DDP cells, caused cell cycle arrest in the G0/G1 phase, and induced apoptosis. Furthermore, downregulation of CDX2 in SGC7901/DDP cells enhanced sensitivity to cisplatin, 5-fluorouracil (P-gp-nonrelated drug), and doxorubicin (P-gp-related drug). The ability to pump doxorubicin was reduced significantly, moreover, a strong antitumor effect of CDX2 siRNA *in vivo* was observed, as tumor growth was suppressed and tumor apoptosis was increased in nude mice when CDX2 mRNA and protein were downregulated. These findings suggest that CDX2 siRNA reversed MDR of human gastric cancer cells.

Doxorubicin is a common substrate for P-gp, but SGC7901/DDP + siRNA-CDX2 cells also exhibited significantly decreased IC50 values for cisplatin and 5-fluorouracil. It should be noted that P-gp-mediated drug efflux was not the only mechanism involved in drug resistance. Previous studies have shown that the effect of P-gp on drug resistance is closely related to cell cycle distribution and apoptosis[24-26]. Cyclin D1 is a regulatory kinase of cell cycle distribution. Previously, overexpression of cyclin D1 in a human fibrosarcoma cell line has been shown to confer resistance to methotrexate[27], which suggests that cyclin D1 overexpression can contribute to the resistance of cancer cells to chemotherapeutic agents. Conversely, suppression of cyclin D1 levels has been shown to potentiate the response of human pancreatic cancer cells to cisplatinum, transfection and multidrug selection experiments have demonstrated that resistance to mitoxantrone can be associated with MDR1 and/or multidrug resistance-associated protein (MRP) overexpression[28]. Indeed, subsequent analysis of MDR1 and MRP expression has revealed that cyclin D1 suppression decreases MDR1 and MRP mRNA levels[24]. Besides regulation of cell cycle distribution, apoptosis is a common pathway that finally mediates the killing effects of anticancer drugs, which is an important cause of MDR. Mitochondria are known to play an active role in the apop-

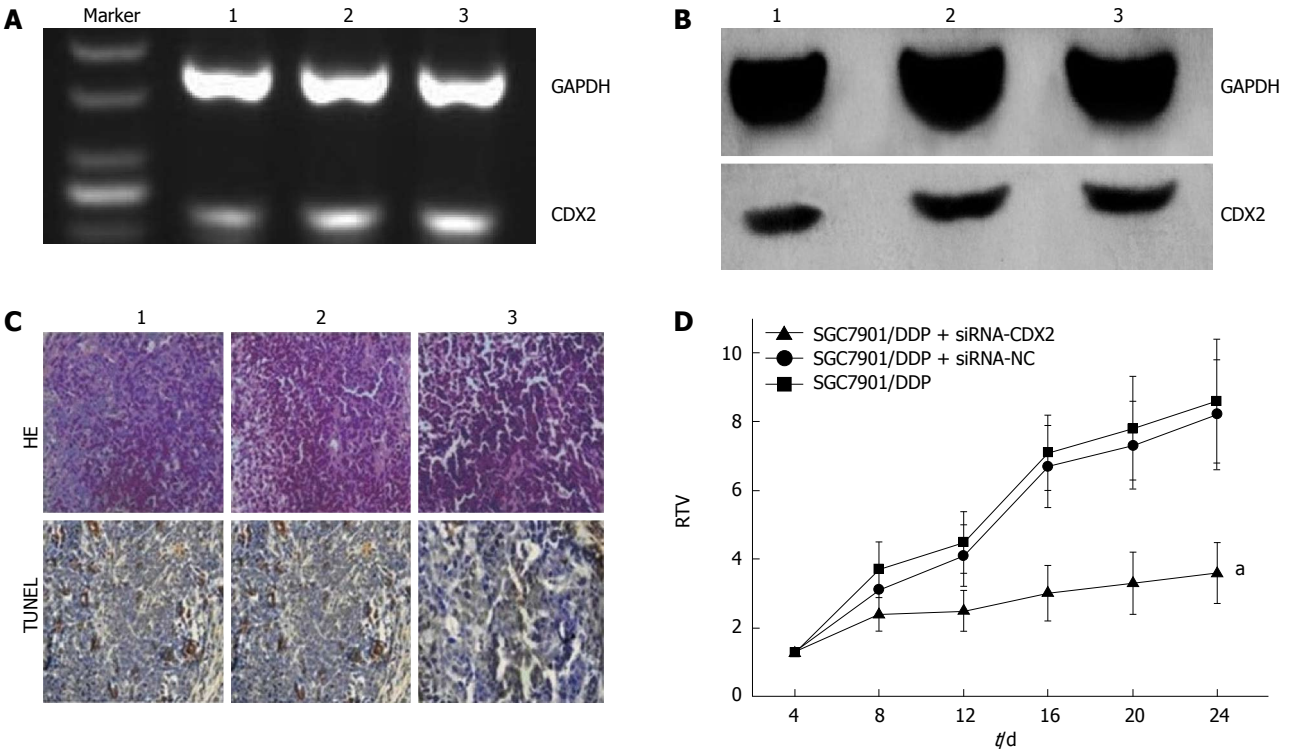

Figure 4 Effect of RNA interference-mediated inhibition of CDX2 mRNA and protein expression and downregulation of CDX2 on apoptosis *in vivo*. A: mRNA expression level of CDX2 was determined by semiquantitative reverse-transcriptase polymerase chain reaction; B: Protein expression level of CDX2 was determined by western blotting; C: Tumor cell apoptosis was assessed by HE staining and TUNEL assay. CDX2 siRNA induced more apoptosis of tumor cells (× 400); D: Relative tumor volume (RTV) of nude mice in each group is presented. Each time point represents the mean RTV for each group. RTV in the SGC7901/DDP + small interfering RNA (siRNA)-CDX2 group was smaller than that in control animals. [a]$P < 0.05$ for SGC7901/DDP + siRNA-CDX2 cells *vs* SGC7901/DDP + siRNA-NC cells and SGC7901/DDP cells. Lane 1: SGC7901/DDP + siRNA-CDX2 cells; Lane 2: SGC7901/DDP + siRNA-NC cells; Lane 3: SGC7901/DDP cells. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

totic process by various mechanisms, including release of caspase activators, disruption of electron transport and energy metabolism, and production of reactive oxygen species[29]. Survivin induces mitochondrial fragmentation and reduces mitochondrial respiration[30]. These data indicate that survivin is closely related to apoptosis. Therefore, in the present study, inhibition of CDX2 expression may have decreased cyclin D1 and survivin expression directly or indirectly, which was responsible for reversal of MDR in human gastric cancer cells *in vitro* and *in vivo*. Further studies are needed to confirm our results.

The term MDR was originally coined to define a condition enabling a disease-causing organism or cancer cells to resist distinct drugs or chemicals with a wide variety of structure and function, targeted at eradicating the organism/cancer cell. Much routine chemotherapy cannot achieve good therapeutic effects because of MDR. It is important to find a new way to reverse MDR. In this study, we showed that CDX2 plays a critical role in reversing MDR. Downregulation of CDX2 using RNAi reversed the progression of MDR in gastric cancer SGC7901/DDP cells *in vitro* and *in vivo*. In conclusion, this study lays the foundation for treatment of MDR in gastric cancer through manipulation of CDX2 expression.

COMMENTS

Background

The term multidrug resistance (MDR) was originally coined to define a condition enabling a disease-causing organism or cancer cells to resist distinct drugs or chemicals with a wide variety of structure and function, targeted at eradicating the organism/cancer cell. Much routine chemotherapy cannot achieve good therapeutic effects because of MDR. It is believed that MDR is the key factor in the failure of gastric cancer chemotherapy. It is important to find a new way to reverse MDR. The caudal-type homeobox gene, CDX2, plays an important role in intestinal metaplasia, and is a precursor of intestinal-type gastric carcinoma. However, the effect of CDX2 in reversing MDR is still not clear.

Research frontiers

CDX2 has a crucial role in the regulation of *MDR1* gene expression in drug resistance, but P-glycoprotein (P-gp)-mediated drug efflux is not the only mechanism involved in drug resistance. The CDX2 research hotspot is how it affects the reversal of MDR by other pathways.

Innovations and breakthroughs

This study is believed to be the first to demonstrate that downregulation of CDX2 causes cell cycle arrest in the G0/G1 phase, and induces apoptosis. Furthermore, downregulation of CDX2 in SGC7901/DDP cells enhances the sensitivity of SGC7901/DDP cells to cisplatin, 5-fluorouracil (P-gp-non-related drug), and doxorubicin (P-gp-related drug). The ability to pump doxorubicin was reduced significantly, moreover, a strong antitumor effect of CDX2 siRNA *in vivo* was observed, as tumor growth was suppressed and tumor apoptosis was increased in nude mice when CDX2 mRNA and protein were downregulated. CDX2 siRNA also decreased c-Myc, survivin and cyclin D1 expression as determined by semiquantitative reverse-transcriptase polymerase chain reaction and Western blotting.

Applications

This study lays the foundation for treatment of MDR in gastric cancer through manipulation of CDX2 expression.

Terminology

The transcription factor, CDX2, is a member of the caudal-related homeobox gene family, and is mainly expressed in the intestine. It is also known to be a key factor in reversing MDR by manipulation of MDR1 expression.

Peer review

This is a well-written manuscript and most of the experiments were properly controlled and clearly presented.

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BRIEF ARTICLE

Helicobacter pylori infection as a cause of iron deficiency anaemia of unknown origin

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Abstract

AIM: To assess the aetiological role of *Helicobacter pylori* (*H. pylori*) infection in adult patients with iron-refractory or iron-dependent anaemia of previously unknown origin.

METHODS: Consecutive patients with chronic iron-deficient anaemia (IDA) with *H. pylori* infection and a negative standard work-up were prospectively evaluated. All of them had either iron refractoriness or iron dependency. Response to *H. pylori* eradication was assessed at 6 and 12 mo from follow-up. *H. pylori* infection was considered to be the cause of the anaemia when a complete anaemia resolution without iron supplements was observed after eradication.

RESULTS: *H. pylori* was eradicated in 88 of the 89 patients. In the non-eradicated patient the four eradicating regimens failed. There were violations of protocol in 4 patients, for whom it was not possible to ascertain the cause of the anaemia. Thus, 84 *H. pylori* eradicated patients (10 men; 74 women) were available to assess the effect of eradication on IDA. *H. pylori* infection was considered to be the aetiology of IDA in 32 patients (38.1%; 95%CI: 28.4%-48.8%). This was more frequent in men/postmenopausal women than in premenopausal women (75% *vs* 23.3%; $P < 0.0001$) with an OR of 9.8 (95%CI: 3.3-29.6). In these patients, anaemia resolution occurred in the first follow-up visit at 6 mo, and no anaemia or iron deficiency relapse was observed after a mean follow-up of 21 ± 2 mo.

CONCLUSION: Gastric *H. pylori* infection is a frequent cause of iron-refractory or iron-dependent anaemia of previously unknown origin in adult patients.

Key words: *Helicobacter pylori*; Iron-deficiency anaemia; Iron refractoriness; Gluten-sensitive enteropathy; Menopause

Core tip: Data on the effect of *Helicobacter pylori* (*H. pylori*) eradication on adult patients with iron-refractory or iron-dependent anaemia of previously unknown origin are scarce, and thus the frequency of *H. pylori* infection as the cause of anaemia in that setting is unknown. Resolution of iron-deficient anaemia (IDA)

was observed in 32 out of the 84 *H. pylori* eradicated patients (38.1%). In all of them there was no relapse after a mean follow-up of 21 ± 2 mo. Thus, *H. pylori* infection was considered the aetiology of IDA in these cases. *H. pylori* infection as the aetiology of IDA was greater in men *plus* postmenopausal women than in premenopausal women (75.0% *vs* 23.3%, $P < 0.0001$).

Monzón H, Forné M, Esteve M, Rosinach M, Loras C, Espinós JC, Viver JM, Salas A, Fernández-Bañares F. *Helicobacter pylori* infection as a cause of iron deficiency anaemia of unknown origin. *World J Gastroenterol* 2013; 19(26): 4166-4171 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i26/4166.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i26.4166

INTRODUCTION

Iron-deficiency anaemia (IDA) occurs in 2%-5% of adult men and postmenopausal women in the developed world, with blood loss from the gastrointestinal tract being the most common cause[1-4]. In addition, IDA also occurs in 5%-12% of otherwise healthy premenopausal women[5]. IDA is a common cause of referral to gastroenterologists (4%-13% of referrals)[3], and for 5%-10% of patients with IDA without gastrointestinal bleeding the cause of the condition remains obscure in spite of extensive examination[6,7].

Helicobacter pylori (*H. pylori*) colonisation in gastric mucosa may impair iron uptake and increase iron loss, potentially leading to IDA. The speculative mechanisms by which *H. pylori* may produce IDA have recently been reviewed[8-10]. Four meta-analyses to assess the effect of *H. pylori* eradication combined with ferrous supplementation on the treatment of IDA have been published[11-14]. The conclusions suggest that *H. pylori* eradication therapy improves iron absorption, since *H. pylori* eradication combined with iron administration was more effective than iron administration alone for the treatment of IDA. However, there was no follow-up of patients after oral iron therapy was completed, and relapse of IDA after *H. pylori* eradication was not evaluated; thus, it was not established whether *H. pylori* infection was the cause of IDA. Most of the intervention trials have been performed in geographical areas where both IDA and *H. pylori* infection are highly prevalent, and where the aetiology of IDA may be multifactorial (malnutrition, vitamin deficiencies, chronic parasitic infections, malaria). In western countries there are only some uncontrolled intervention studies showing recovery from anaemia after *H. pylori* eradication[15,16]. In light of the above-mentioned studies, *H. pylori* infection has been considered as a risk factor for IDA. The British Society of Gastroenterology recommends eradication of *H. pylori* infection in patients with IDA and normal colonoscopy and oesophagogastroduodenoscopy (Grade of recommendation, C)[1], and the Maastricht guidelines suggest to eradicate *H. pylori* in patients with IDA (Grade of recommendation, A)[17]. However, data on the effect of *H. pylori* eradication on adult patients with iron-refractory or iron-dependent IDA of previously unknown origin are scarce, and thus the frequency of *H. pylori* infection as the cause of IDA in that setting is unknown. Therefore, the aim of the present study was to assess the aetiological role of *H. pylori* infection in such patients, in a geographical background where concomitant causes of IDA are unusual.

MATERIALS AND METHODS

Patients

Consecutive patients with unexplained chronic IDA or isolated iron deficiency (ID) referred to the Gastroenterology Department from January 2007 to December 2010 were prospectively evaluated.

Patients were included if they were older than 18 years of age with all the following: (1) chronic IDA defined as haemoglobin < 10.5 g/dL in women and < 11.5 g/dL in men, and serum ferritin < 13 μg/L or ID defined as only serum ferritin < 13 μg/L; (2) gastric *H. pylori* infection; (3) iron refractoriness or iron dependency (see below for definition); (4) negative faecal immunochemical tests for occult blood (at least three negative samples); (5) negative coeliac serology [both serum immunoglobulin A (IgA)-antiendomysial an IgA-human anti-tissue transglutaminase antibodies], although patients diagnosed with coeliac disease in whom IDA persisted in spite of being on a strict gluten-free diet with negative coeliac serology and no villous atrophy were included; (6) normal gastroscopy and full colonoscopy; (7) normal physical examination, blood analysis (including routine blood biochemistry, C reactive protein, folate and vitamin B_{12} levels), and urinalysis; and (8) normal gynaecological examination.

Patients with the following conditions were excluded from the study: (1) frequent (three times a week or more) use of non-steroidal anti-inflammatory drugs or salicylates during the previous 6 mo; (2) use of dicumarinics; (3) other conditions which cause anaemia or interfere with erythropoiesis including malignancy, haematological diseases, connective tissue disease, chronic diseases such as chronic renal failure, chronic liver disease, severe cardiac and respiratory disease, and previous gastrointestinal surgery; (4) pregnancy or lactation; (5) history of alcoholism or drug addiction; (6) heavy menstrual flow (cycles > 5 d, associated with passage of clots after the three first days) and/or metrorrhagia; (7) obvious blood loss (melena, haematochezia, haematuria, recurrent epistaxis); (8) adherence to vegetarian or iron-deficient diet; and (9) expected lack of cooperation.

Capsule endoscopy was not routinely performed since one inclusion criterion was that repeated faecal immunochemical tests for occult blood were negative. In individual cases (12 patients), it was performed by the decision of the physician at charge, yielding in all cases normal results.

All patients had received iron supplements and all of them fulfilled the criteria of either iron refractoriness or iron dependency. Iron refractoriness was defined as an inappropriate increase in haemoglobin levels (< 2 g/dL) after completion of a 5000 mg dosing cycle of ingested elemental iron over one month or longer[18]. Iron dependency was defined as the patient's requiring daily oral iron supplementation (ferrous sulphate, 100-200 mg daily of elemental iron) to maintain adequate haemoglobin levels.

Study design

The following tests were prospectively performed in all included patients: (1) two endoscopic biopsies from both gastric body and antrum, and four biopsies from distal duodenum; (2) histological examination of antral biopsies and/or ^{13}C-urea breath test to assess *H. pylori* infection; and (3) human leukocyte antigen (HLA)-DQ2 and HLA-DQ8 haplotypes of predisposition to coeliac disease.

In all patients *H. pylori* was eradicated using a standard 7-d triple regimen with omeprazole 20 mg *bid*, amoxicillin 1 g *bid*, and clarithromycin 500 mg *bid*. Two-week quadruple regimens were used as a rescue therapy for patients failing the first-line eradication therapy, and in some cases a levofloxacin-based third-line rescue therapy was used. *H. pylori* eradication was evaluated by histological examination of antral biopsies and/or ^{13}C-urea breath test. Analytical response to *H. pylori* eradication was assessed at 6 and 12 mo of follow-up. In cases with associated lymphocytic duodenitis (LD), histological follow-up was also performed at 6 and 12 mo.

Final diagnosis

H. pylori infection was considered to be the cause of IDA or ID when after eradication a complete response (*i.e.*, IDA or ID recovery, with normal serum ferritin levels), without iron supplements, was observed at 12 mo of follow-up. A diagnosis of gluten-sensitive enteropathy was performed in patients with LD and positive coeliac genetics (HLA-DQ2 and/or HLA-DQ8) without response after achieving *H. pylori* eradication, when there was a sustained (at 12 mo follow-up) complete clinical and histological response on a strict gluten-free diet[19]. The cause of either IDA or ID was considered to be unknown in patients without response to both *H. pylori* eradication and gluten-free diet (when indicated).

Histological studies

Four endoscopic biopsies from the 2nd-3rd portions of the duodenum and two biopsies from both body and antrum were obtained in the index endoscopy. *H. pylori* infection was investigated in gastric antral mucosal samples by standard histopathological assessment[20]. Duodenal samples were processed using haematoxylin/eosin staining and CD3 immunophenotyping, and these were blindly evaluated by an expert gastrointestinal pathologist (Salas A). LD was defined as 25 or more intraepithelial lymphocytes per 100 epithelial nuclei and normal villous architecture, as suggested in recent literature[21]; the cut-off value was validated in our laboratory[19]. This cut-off value was also selected to define LD due to gluten-sensitive enteropathy, which corresponds to the Marsh 1 type lesion of the coeliac disease spectrum[21].

Helicobacter pylori status

Patients were classified as having *H. pylori* infection when either histology or ^{13}C-urea breath test was positive. ^{13}C-urea breath test was performed on those patients with negative histology who either were taking proton-pump inhibitors or had antral intestinal metaplasia at the index endoscopy, as previously described[20]. In the case of proton-pump inhibitors, the breath test was performed 4 wk after discontinuation.

Ethics

The protocol was approved by the Ethics Committee of the Hospital Universitari Mútua Terrassa, and all participants provided informed consent.

Statistical analysis

Results are expressed as mean ± SE and as percentages plus their 95%CI. Chi-square statistics were used to assess significant associations between qualitative variables. The OR and 95%CI of the significant associations were computed.

RESULTS

One hundred thirty-six consecutive adult patients fulfilled the inclusion criteria during the study period. Twenty-two were excluded due to presence of exclusion criteria, 7 patients had previously unrecognised non-steroidal anti-inflammatory drug intake, and 15 had other causes of anaemia (1 infection by intestinal parasites, 2 recurrent rectal bleeding attributed to haemorrhoids, 1 chronic renal failure, 2 small bowel Crohn's disease, 1 gastrinoma, and 8 pernicious anaemia). Twenty-five additional patients (18.4%) were lost in follow-up before achieving a definite diagnosis of their anaemia. Thus, 89 patients were finally included in the study (10 men; 79 women; mean age: men, 54.0 ± 15.8 years; premenopausal women, 44.0 ± 8.6 years; postmenopausal women, 59.0 ± 9.8 years). There were no significant differences in demographic data or frequency of menopause, *H. pylori*-related chronic gastritis, associated enteropathy (LD) or coeliac genetics between included patients and those lost in follow-up (Table 1).

H. pylori was eradicated in 88 of the 89 patients. In the non-eradicated patient the four eradicating regimens failed. There were violations of protocol in 4 patients, for whom it was not possible to ascertain the cause of the anaemia. Thus, 84 *H. pylori*-eradicated patients were available to assess the effect of eradication on IDA.

Resolution of IDA or ID was observed in 32 out of the 84 *H. pylori*-eradicated patients (38.1%; 95%CI: 28.4-48.8). In all of them, IDA or ID recovery was observed at the 6-month follow-up visit after *H. pylori*-erad-

Table 1 Comparison of demographic, clinical, and biological data between included patients and those lost in follow-up

	Included patients (n = 89)	Loss of follow-up patients (n = 25)
Age (yr), mean ± SE	46.0 ± 11.8	41.0 ± 14.2
Sex (M/F)	10/79	4/21
Postmenopausal women	17.90%	16.00%
Chronic antral gastritis	81.80%	78.30%
Chronic body gastritis	83.00%	78.30%
Associated enteropathy	59.50%	60.00%
HLA-DQ2 and/or DQ8+	48.30%	40.00%

There were no significant differences in any parameter. HLA: Human leukocyte antigen; M/F: Male/female.

ication, and there was no relapse after a mean follow-up of 21 ± 2 mo. Therefore, *H. pylori* infection was considered the aetiology of IDA in these cases. Frequency of *H. pylori* infection as the aetiology of IDA was greater in men (8 of 10, 80%) *plus* postmenopausal women (10 of 14, 71.4%) than in premenopausal women (14 of 60, 23.3%) (75.0% *vs* 23.3%, $P < 0.0001$) with an OR of 9.8 (95%CI: 3.3-29.6). There were no differences in the frequency of *H. pylori* infection as the cause of IDA between patients with and those without associated enteropathy (18 of 49, 36.7%; with LD and 14 of 35, 40%, without LD).

In addition, a gluten-free diet was offered to 13 patients in whom IDA persisted after *H. pylori* eradication. Gluten-sensitive enteropathy was the aetiology of IDA in 4 (men, 1 of 10, 10%; premenopausal women, 1 of 14, 7.1%; postmenopausal women, 2 of 60, 3.3%) of the 84 *H. pylori* eradicated patients (4.8%; 95%CI: 1.8-11.6). In all of them LD was detected, and a clinical (IDA recovery without iron supplementation) and histological remission after a gluten-free diet was observed. There was no relapse of IDA or ID after the 12 mo of follow-up.

The final diagnosis of IDA in the remaining 48 patients, who were mainly premenopausal women, was unknown. Despite *H. pylori* eradication there was a need to maintain iron supplementation during follow-up, with persistent iron-dependent IDA. A relation with menstrual blood loss was observed in 30 of the premenopausal women since anaemia recovered after either entering menopause or starting hormonal contraceptive therapy. As previously mentioned, none of them had heavy menstrual blood loss at inclusion, and for all of them the gynaecologic examination had been normal.

DISCUSSION

Results of the present study suggest that *H. pylori* infection is a frequent cause of IDA in adult patients with iron refractoriness or iron dependency in whom the standard diagnostic work-up is negative. In 38% of such patients *H. pylori* eradication was associated with both IDA resolution without the need for more iron supplementation and an absence of IDA relapse after nearly two years mean follow-up. These observations argue in favour of causality of *H. pylori* infection. In addition, the efficacy of *H. pylori* eradication to recover from IDA in such patients was compared between men *plus* postmenopausal women and premenopausal women. There was IDA recovery in 75% and 23% of them, respectively, with highly significant differences. In fact, the OR of *H. pylori* infection as the cause of IDA was almost 10 times higher in the first group than the second.

We have to take into account that the *H. pylori* re-infection rate after cure in our geographical area is low, around 1% patient-year; this implies that the present results might not be extrapolated to other regions with higher re-infection rates.

Results of the present study on premenopausal women are in disagreement with previous results of Annibale *et al*[15], since they showed recovery from anaemia at 12 mo of follow-up after *H. pylori* eradication in 92% of patients, mainly premenopausal women. The discrepancies revolve around the definition of response. In our study, response to *H. pylori* eradication was defined as anaemia recovery with normalisation of serum ferritin levels. In Annibale's study, however, ferritin levels returned to normal in only 17% of the patients despite recovery from anaemia, which is a figure similar to that of the 23% obtained in our study. Taking into account the meta-analysis data mentioned in the introduction[11-14], and the results of the present study in men and postmenopausal women, *H. pylori* infection may also be a contributing factor to IDA in premenopausal women, and *H. pylori* eradication is indicated to improve iron absorption. In this sense, it would be of interest to assess whether iron requirements change after *H. pylori* eradication in these patients. Regrettably, iron requirements were not sufficiently well recorded in the present study to allow for this evaluation.

Other factors may contribute to IDA in otherwise healthy premenopausal women such as menstrual loss, increased iron demands on pregnancy and breast feeding, and dietary deficiency[22]. Hormonal contraceptive therapy may reduce menstrual blood loss by approximately 50%, even in women with average or slightly above-average blood loss[23]. In the present study, this type of therapy was effective for IDA recovery in those premenopausal women in whom increased iron requirements persisted after *H. pylori* eradication.

H. pylori infection may be a cause of LD, which may disappear after eradication of the infection[19,24]. However, whether *H. pylori*-infected patients with LD are more prone to developing IDA is unknown. The present study shows that the frequency of *H. pylori* infection as the final diagnosis of IDA is similar for patients with and without associated enteropathy. Therefore, these data argue against a pathophysiological role for this mild enteropathy in the development of IDA in *H. pylori*-infected patients.

In 4.5% of the included patients the final diagnosis was gluten-sensitive enteropathy. These results agree with

a previous study by our group showing that a subgroup of patients with IDA of previously unknown origin and positive coeliac genetics presented a gluten-sensitive mild enteropathy with negative coeliac serology[25].

In conclusion, the results of the present study show that *H. pylori* infection is a frequent cause of IDA in men and postmenopausal women with either iron refractoriness or iron dependency, in whom other causes of IDA have been previously ruled out. *H. pylori* eradication therapy produces long-term resolution of IDA in such patients. Also, *H. pylori* infection may be a contributing factor to IDA in otherwise healthy premenopausal women without heavy menstrual blood loss, and it is the aetiology of IDA in almost 25% of them.

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COMMENTS

Background

Iron deficiency anaemia (IDA) is a common cause of referral to gastroenterologists (4%-13% of referrals), and for 5%-10% of patients with IDA without gastrointestinal bleeding the cause of the condition remains obscure in spite of extensive examination. Previous data suggest that *Helicobacter pylori* (*H. pylori*) eradication therapy improves iron absorption, since *H. pylori* eradication combined with iron administration was more effective than iron administration alone for the treatment of IDA. The Maastricht guidelines suggest to eradicate *H. pylori* in all patients with IDA.

Research frontiers

H. pylori eradication may improve iron absorption, but how often is gastric *H. pylori* infection the cause of IDA? Data on the effect of *H. pylori* eradication on adult patients with iron-refractory or iron-dependent anaemia of previously unknown origin are scarce, and thus, the frequency of *H. pylori* infection as the cause of IDA in that setting is unknown.

Innovations and breakthroughs

This study is performed in a large prospective series of consecutive patients using very strict inclusion criteria, with a long follow-up after *H. pylori* cure. Resolution of anaemia was defined as both haemoglobin and iron stores normalization, which was long-term maintained without requiring iron supplements. Frequency of IDA resolution was compared between men/post-menopausal women and pre-menopausal women.

Applications

Gastric *H. pylori* infection may be a frequent cause of iron-refractory or iron-dependent anaemia of previous unknown origin in adult patients, mainly in men and post-menopausal women. In these patients, *H. pylori* infection eradication produced the cure of IDA. In addition, *H. pylori* infection may be a contributing factor to IDA in otherwise healthy premenopausal women without heavy menstrual blood loss, being the aetiology of IDA in almost a 25% of them.

Peer review

The authors in this article have focused on the possible role of *H. pylori* infection in causation of IDA. They have stated that 38% of the patients may have the anaemia duo to the infection by *H. pylori*.

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BRIEF ARTICLE

Single endoscopist-performed percutaneous endoscopic gastrostomy tube placement

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Abstract

AIM: To investigate whether single endoscopist-performed percutaneous endoscopic gastrostomy (PEG) is safe and to compare the complications of PEG with those reported in the literature.

METHODS: Patients who underwent PEG placement between June 2001 and August 2011 at the Baskent University Alanya Teaching and Research Center were evaluated retrospectively. Patients whose PEG was placed for the first time by a single endoscopist were enrolled in the study. PEG was performed using the pull method. All of the patients were evaluated for their indications for PEG, major and minor complications resulting from PEG, nutritional status, C-reactive protein (CRP) levels and the use of antibiotic treatment or antibiotic prophylaxis prior to PEG. Comorbidities, rates, time and reasons for mortality were also evaluated. The reasons for PEG removal and PEG duration were also investigated.

RESULTS: Sixty-two patients underwent the PEG procedure for the first time during this study. Eight patients who underwent PEG placement by 2 endoscopists were not enrolled in the study. A total of 54 patients were investigated. The patients' mean age was 69.9 years. The most common indication for PEG was cerebral infarct, which occurred in approximately two-thirds of the patients. The mean albumin level was 3.04 ± 0.7 g/dL, and 76.2% of the patients' albumin levels were below the normal values. The mean CRP level was high in 90.6% of patients prior to the procedure. Approximately two-thirds of the patients received antibiotics for either prophylaxis or treatment for infections prior to the PEG procedure. Mortality was not related to the procedure in any of the patients. Buried bumper syndrome was the only major complication, and it occurred in the third year. In such case, the PEG was removed and a new PEG tube was placed *via* surgery. Eight patients (15.1%) experienced minor complications, 6 (11.1%) of which were wound infections. All wound infections except one recovered with antibiotic treatment. Two patients had bleeding from the PEG site, one was resolved with primary suturing and the other with fresh frozen plasma transfusion.

CONCLUSION: The incidence of major and minor complications is in keeping with literature. This finding may be noteworthy, especially in developing countries.

Key words: Gastrostomy; Gastric feeding tube; Enteral nutrition; Enteral feeding; Endoscopy; Gastrointestinal

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INTRODUCTION

Percutaneous endoscopic gastrostomy (PEG) has been used widely for the enteral feeding of patients who have a functioning gastrointestinal tract but are unable to consume adequate nutrition orally. Patients with cerebro-

vascular diseases, Parkinson's disease, dementia and head injury and those suffering from head and neck cancer and upper digestive tract cancer are candidates for PEG[1,2]. A PEG tube can be placed using one of four methods: push (Sachs-Vine), pull (Ponsky), introducer (Russell) or versa (t-fastener). The pull and push techniques are preferred because they offer greater safety and efficacy[3,4]. Both minor and major complications may occur during PEG placement. Major complications associated with PEG include peritonitis, gastric perforation, esophageal perforation, gastrocolocutaneous fistula, gastric outlet obstruction, necrotizing fasciitis and buried bumper syndrome. Minor complications include pneumoperitoneum, temporary ileus, hematoma, hemorrhage, wound infection, aspiration, tube dislodgement, gastroesophageal erosion, and gastric ulcer. Other gastrointestinal problems include gas distension, nausea, emesis, constipation and diarrhea[5-7]. In general practice, a PEG is placed by two endoscopists[1,8]. The aim of this study is to evaluate whether single endoscopist-performed PEG is safe and to compare the major and minor complications of PEG with those reported in the literature.

MATERIALS AND METHODS

This study was approved by the Baskent University Institutional Review Board and Ethics Committee (Project No: KA12/150) and supported by the Baskent University Research Fund. Patients who underwent PEG placement between June 2001 and August 2011 at the Baskent University Alanya Teaching and Research Center were evaluated retrospectively. Patients whose PEG was placed for the first time by a single endoscopist were enrolled in the study. For all patients, the PEG was placed using the "pull method". All of the patients were evaluated for indications for PEG, major and minor complications of PEG, nutritional status (prealbumin and albumin levels), C-reactive protein (CRP) levels and antibiotic treatment or antibiotic prophylaxis prior to PEG placement. Comorbidities and the rates, time and reasons for mortality were also evaluated, as were the reasons for PEG removal and the duration of PEG placement. The patients' first-degree relatives were telephoned and interviewed about the complications associated with the PEG and the patients' outcomes.

In our medical center, the standard PEG procedure was performed by single endoscopist. Before the procedure, permission for PEG placement was obtained from the patients' first-degree relative. The procedure was performed in the intensive care unit. Lidocaine spray was administered to the throat for local anesthesia. Midazolam and/or propofol-based sedation were administered intravenously by an anesthesiologist. An upper endoscopy was performed at the beginning of the procedure to exclude severe gastric ulceration, varices and outlet obstruction. After the stomach was insufflated with air through scope, the best location for the PEG placement was determined by pressing a finger slightly against the abdominal wall. The best location was indicated by the clear indentation

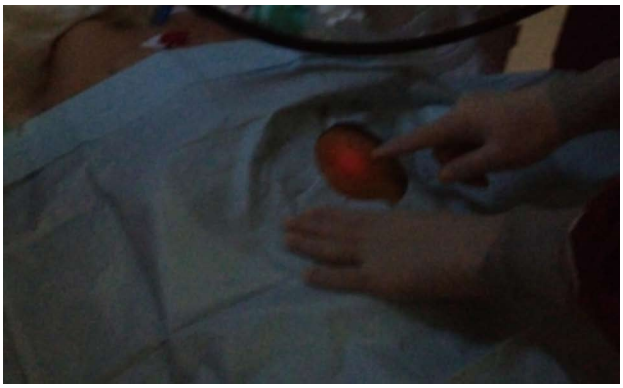

Figure 1 The best location was indicated by the clear indentation of the finger observed inside the stomach and the illumination of the abdominal wall.

of the finger observed inside the stomach on the greater curvatures and the illumination of the abdominal wall (Figure 1). The nurse was then given the scope. The sterile-dressed endoscopist cleaned the abdominal wall using a povidone-iodine solution. A one-centimeter incision was made after local anesthetic was applied to the planned location. The nurse filled the patient's stomach with air, and then the endoscopist inserted the needle of the PEG set through abdominal wall into the fully insufflated stomach. After removing the trocar, the endoscopist passed the guide wire through the needle. The nurse then caught the guide wire by the snare which was inserted through the endoscope, the endoscopist then withdrew the guide wire and the endoscope from the patient's mouth. After the endoscopist redressed, attached the guide wire to the PEG tube and the wire was pulled out of the abdominal wall, moving the PEG tube down the esophagus. Control endoscopy was performed to optimally place the PEG tube tip. The PEG tube was turned to locate the appropriate position and was fixed with an external device, leaving a 5-mm distance between the external device and the abdominal wall. This site was cleaned with povidone-iodine solution and dressed with gauze. Enteral feeding began 24 h after the procedure and ensuring that no local wound infection was present. The patient was inspected for erythema, induration and discharge at the PEG site and was assessed using the scoring system developed by Jain *et al*[9] for PEG infection. The patient was also followed by the nutrition team for other complications and nutritional status until discharge. The patient's family was asked to inform the nutrition team about possible complications.

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences software program (Version 11.0, SPSS Inc., Chicago, IL, United States).

RESULTS

Between June 2001 and August 2011, a total of 82 patients underwent PEG placement. Twenty patients un-

Table 1 Demographic and laboratory characteristics of the study subjects *n* (%)

Age, yr	69.9 ± 16.3
Gender, M:F	30 (55.6):24 (46.3)
Comorbidities	
Diabetes mellitus	19 (35.2)
Chronic obstructive pulmonary disease	6 (11.1)
Coronary artery disease	11 (20.4)
Cardiac arrhythmia	4 (7.4)
Hypertension	20 (37.0)
Chronic renal failure	3 (5.6)
Hyperlipidemia	2 (3.7)
Other[1]	4 (7.4)
Antimicrobial therapy prior to PEG	40 (74.1)
Laboratory findings	
Leukocytosis (> 11 kg/mm^3)	22 (40.7)
CRP elevation (> 8 mg/dL)	48 (90.6)
High CRP (> 80 mg/dL)	22 (41.5)
Low albumin levels (< 3.5 g/dL)	40 (76.9)

[1]Hydrocephaly, breast cancer, meningioma, chronic liver disease. M: Male; F: Female; PEG: Percutaneous endoscopic gastrostomy; CRP: C-reactive protein.

derwent PEG replacement and were excluded from the study. Sixty-two patients underwent the PEG procedure for the first time. Eight of these procedures were performed by 2 endoscopists and were excluded from the study. A total of 54 patients were enrolled in the study. Indications were cerebral infarct in 39 patients (72.2%), cardiac arrest and cerebral ischemia in 4 patients (7.4%), dementia in 7 patients (12.9%), head trauma in 3 patients (5.6%), and cancer in 1 patient (1.9%).

Of the patients whose PEG was placed for the first time, 24 (46.3%) were women and 30 (55.6%) were men. The mean age was 69.9 years. The comorbidities accompanying the patients' primary disease were hypertension, diabetes mellitus, cardiac arrhythmia, coronary artery disease, chronic obstructive pulmonary disease, chronic renal disease, hyperlipidemia and hydrocephaly. The mean albumin levels were 3.04 ± 0.7 g/dL, and 76.2% were below normal values. The mean CRP level was high in 90.6% of patients prior to the procedure (Table 1). In our study, 74.1% of the patients received antibiotics either for prophylaxis or for treatment for infections prior to the PEG procedure. The demographic, clinical and laboratory characteristics of the study subjects are shown in Table 1.

After hospitalization, the mean time past until PEG placement was 22 ± 15.6 d. Buried bumper syndrome was the only one major complication (1.6%), and it occurred in the third year in one patient. In that case, the PEG was removed, and a new PEG tube was placed surgically. Eight patients (15.1%) experienced minor complications, 6 (11.1%) of which were wound infections and 2 of which (3.7%) were bleeding. All wound infections except for 1, which resulted in the removal of the PEG, recovered with antibiotic treatment. Two patients experienced bleeding from the PEG site; one patient was receiving anticoagulation therapy. One case resolved with primary suture, and the other resolved with fresh frozen plasma transfusion.

The first-degree relatives of all of the patients were interviewed by phone. The family members of 6 of the 54 PEG patients could not be reached by telephone, so we do not have long-term follow-up results for these patients. In our study, 1 mo survival was 85.4%, and three-month survival was 41.7%. Twenty-nine patients died during follow-up. The PEG indications for the patients who died were as follows: 14 had cerebral infarct, 3 had head trauma, 2 had cardiac arrest and cerebral ischemia and 1 had cancer. Mortality was not related to PEG placement in any of the patients and mainly depended on the underlying medical problems. The PEG tube was withdrawn in seven patients after they regained swallowing function and in one patient with an uncontrolled local wound infection. As of this writing, eleven patients live with the PEG tube, and 6 of them underwent PEG replacement during follow-up. To date, their relatives have not mentioned any problem related to the PEG in follow-up telephone interviews.

DISCUSSION

Although PEG is usually a safe procedure, certain complications can occur that may cause mortality, especially in patients with comorbidities. In our study, no mortality was associated with the PEG procedure. Buried bumper syndrome was the only major complication, and it occurred in only one patient (1.9%) in the third year of PEG placement. Minor complications occurred in 15.1% of patients, and most of these complications were wound infections.

Survival is an important endpoint in PEG studies. One-month survival is approximately 80% to 90% in most reports[10-12]. Similar to our study, the most frequent indication for PEG insertion was a neurological condition, and several studies reported that stroke was the most common indication[12-14]. In our study, one-month survival was 85.4%, and three-month survival was 41.7%. Buried bumper syndrome is an uncommon but severe complication of the procedure. It usually occurs after four months of PEG placement; however, it has also been reported to occur as late as 7 years after placement[15-17]. Rino *et al*[5] reported this complication as early as 5 d after the procedure. Finocchiaro *et al*[10] reported that one hundred twenty-eight patients were followed long-term for more than 31 d; major complications occurred in 3% of the patients, 2 of whom had buried bumper syndrome. Other major complications included 1 case of aspiration pneumonia and 1 case of subcutaneous abscess. In our study, buried bumper syndrome was the only observed major complication, and it occurred 3 years after the procedure. In the patient with buried bumper syndrome, the PEG tube was successfully surgically removed, and a new PEG tube was placed *via* the same procedure.

As in our study, the most common complication of

PEG was infection, which sometimes results in the removal of the PEG tube[18,19]. In a prospective study in which antibiotic prophylaxis was not given, the rate of peristomal infection was 33.6%[7]. Another study reported wound infections rates of up to 18%, and antibiotic prophylaxis was shown to reduce the rate to nearly 3%[19]. In a prospective, randomized, double-blind, placebo-controlled study by Jain *et al*[9], antibiotic prophylaxis with cefazolin was associated with decreased local PEG site infection. In a meta-analysis by Jafri *et al*[20], antibiotic prophylaxis before the PEG procedure was effective in reducing postprocedure local infection rates. In our study, 74.1% of the patients received antibiotics for either prophylaxis or the treatment of infections prior to the PEG procedure. The local wound infection rate was 11.1%, which is comparable to the rates reported by other studies in the literature. Only one patient developed a PEG site infection that did not resolve with antibiotic therapy; in this case, the PEG was removed. The other minor complication in our study was bleeding from the PEG puncture site, which occurred in two patients. One patient was treated with fresh frozen plasma, and the other was treated *via* primary suture of the abdominal wall vessel. Bleeding from the puncture site occurs as a result of a puncture of the abdominal wall vessel soon after the procedure. It can also be treated by tightening the outside apparatus of the PEG tube. Singh *et al*[21] reported that gastrointestinal bleeding after PEG placement occurred in 3.3% of patients, and bleeding directly attributed to PEG was noted in 0.4%.

Many factors contribute to PEG complications. The PEG tube placement team's experience, the PEG tube size, underlying malignancy and the institution in which the PEG procedure is performed are risk factors for wound infection. Low albumin levels and high CRP levels, age over 65 years and low BMI have also been associated with increased mortality risk[7,8,22-26]. In our study, the mean age was 69.9 years. High CRP levels were found in 41.5% of the patients, and low albumin levels were found in 76.9%. Although these unfavorable parameters existed prior to the procedure, there was no evidence of mortality related to PEG.

The nonrandomized and retrospective nature of our study are its restrictions. A prospective and randomized study might better define the safety and appropriateness of the single endoscopist-performed procedure.

In conclusion, the major and minor complications of single-endoscopist PEG are consistent with those reported in the literature for PEG procedures performed by two endoscopists. This finding may be noteworthy, especially in developing countries.

COMMENTS

Background

Percutaneous endoscopic gastrostomy (PEG) is lifesaving for patients who cannot feed orally for certain reasons. PEG is routinely placed by two endoscopists and carried inherent complications, some of which are life-threatening. It is not known whether PEG placement performed by a single endoscopist is safe or appropriate.

Research frontiers

The complications of PEG are significant. No study has reported the single-endoscopist PEG procedure or its related complications.

Innovations and breakthroughs

Although this study is retrospective and lacks the advantages of prospective and randomized trials, it provides important information indicating that PEG procedures can be applied by a single endoscopist, and the complications encountered are similar to those reported in other studies of PEG performed by two endoscopists.

Applications

Single endoscopist-performed PEG may be an appropriate and safe method for performing the procedure, especially in developing countries.

Peer review

This is a well-written retrospective study about the PEG procedure, which is performed here by a single endoscopist. The results show that it may be safe and appropriate for a single endoscopist to perform PEG. This study may lead to prospective and randomized trials in this field.

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BRIEF ARTICLE

Comparison of double pants with single pants on satisfaction with colonoscopy

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Abstract

AIM: To increase satisfaction and diminish anxiety and shame during colonoscopy, we developed novel double pants (NDP) which consist of doubled fabrics with an inner hole. The aim of study was to compare satisfaction, anxiety and shame between NDP and conventional single pants (CSP).

METHODS: Total 160 consecutive examinees were randomly divided into NDP and CSP group. Before colonoscopy, questionnaires identifying state and trait anxiety were completed. After colonoscopy, questionnaires for overall satisfaction (Group Health Association of America 9) and pants-specific satisfaction (5-20), state anxiety (20-80), and shame (6-24) were interviewed.

RESULTS: Pants-specific satisfaction scores regarding willingness to repeat colonoscopy using same pants (3.3 ± 0.8 *vs* 2.1 ± 0.9, $P < 0.001$) and recommendation of same pants to other people (3.3 ± 0.7 *vs* 2.0 ± 1.0, $P < 0.001$) were significantly higher in NDP than CSP groups. State anxiety (33.0 ± 7.0 *vs* 35.4 ± 6.9, $P = 0.028$) and shame (6.6 ± 1.5 *vs* 8.1 ± 3.2, $P = 0.001$) after colonoscopy was lower in NDP group compared with CSP group.

CONCLUSION: The NDP contribute to increase satisfaction and decrease anxiety and shame after colonoscopy.

Key words: Pants; Colonoscopy; Satisfaction; Shame; Anxiety

Core tip: We developed novel double pants (NDP) those are consisted of double fabrics with an inner hole. We compared the satisfaction, anxiety and shame between NDP and conventional single pants (CSP). The examinees in NDP group responded higher pants specific satisfaction, lower state anxiety after colonoscopy and lower shame score compared to those in CSP group. NDP developed in our institute may contribute to increase satisfaction and decrease anxiety and shame after colonoscopy.

Chung SH, Park SJ, Hong JS, Hwang JY, Lee SA, Kim KR, Lee HS, Hong SP, Cheon JH, Kim TI, Kim WH. Comparison of double pants with single pants on satisfaction with colonoscopy. *World J Gastroenterol* 2013; 19(26): 4177-4184 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i26/4177.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i26.4177

INTRODUCTION

Colonoscopy has recently increased in importance worldwide due to its use in screening for colon polyps and colorectal cancer[1]. Even in institutions where conscious sedated colonoscopy is available, colonoscopy without sedation is still performed due to patient comorbidities, the economic burden of sedation, and examinee preference. There is considerable global variation in the prevalence of sedative endoscopy[2]. Most colonoscopies in the United States are performed as sedated procedures, but some countries rarely use sedation in colonoscopy[3,4].

Consideration of the factors affecting satisfaction during colonoscopy has also recently increased because satisfaction may represent an important quality indicator for colonoscopy[5,6]. Moreover, the satisfaction of examinees may be reduced by anxiety, shame, discomfort, and embarrassment[7-11]. Many factors have been identified which determine satisfaction, anxiety, and shame, including the circumstance of the endoscopy room, the clothing for the procedure, endoscopist's skill, and the unfamiliarity of the medical staff with whom examinees interact during the procedure.

During colonoscopy at our center, examinees previously wear conventional single pants (CSP), where there is no hole for insertion of the scope. Examinees remove the CSP to below the level of the buttocks and expose their buttocks area during colonoscopy. Exposing the buttocks during colonoscopy can make examinees feel shameful and anxious, which can diminish their satisfaction with the colonoscopy. To decrease the shame and anxiety induced by exposing the buttocks of examinees wearing CSP during colonoscopy, we developed novel double pants (NDP). Examinees wearing NDP can undergo colonoscopy without taking off the inner pants. The smaller area of the buttock exposed by using NDP could minimize shame and anxiety during colonoscopy. Therefore we hypothesized that NDP could decrease shame and anxiety and increase satisfaction. We aimed to assess satisfaction, anxiety, and shame of patients wearing NDP compared with patients wearing CSP during colonoscopy through a prospective randomized single-center study. We also investigated the factors associated with patient satisfaction, anxiety, and shame during colonoscopy.

MATERIALS AND METHODS

Participants and study design

The study included examinees over 20 years old who agreed to undergo colonoscopy without sedation in the endoscopy unit of Severance Hospital, Yonsei University College of Medicine, Seoul, South Korea from January 2012 and July 2012. All of included people were the patients who visited the clinic for routine health check-up or for evaluating their mild gastrointestinal symptoms. Because we tried to minimize the selection bias of tertiary referral medical center, we excluded the patients who were referred by the physicians of primary or secondary medical center and needed more special care due to their objective medical problems. Before they signed at written informed consent, the nurse in outpatient clinic explained the purpose of the study. They got the information about not only the purpose of the study, but also the study design, the types of pants, the possible adverse events of procedure, and the contents of interview. Examinees under 20 years of age, those who had undergone colonoscopy within the past three years, those who were treated with an emergent colonoscopic procedure, those with a history of bowel resection surgery, inflammatory bowel disease (IBD), cancer, colostomy, ileostomy, psychotic disease including depression, anxiety disorder, or obsessive compulsive disorder, women who were pregnant or lactating, illiterate patients, foreigners, or examinees with American Society of Anesthesiologists (ASA) Scores of ⩾ 3 were excluded. We used our preliminary survey data to perform a power calculation due to the lack of the prior published reports to guide this analysis. We calculated that a sample size of 80 participants was sufficient to detect an effect value of 0.5 (mean difference/common SD) at a significance level of 0.05% (two-sided) with 80% power and 20% drop-out rate. A total of 185 colonoscopy examinees were enrolled in this study between February 2012 and July 2012. Eight examinees were excluded because of colon cancer found during the colonoscopy, ten examinees were excluded because of psychologic disorder, three examinees were excluded because of a history of bowel resection, and four examinees were excluded because of ASA scores ⩾ 3, as shown in Figure 1. Finally a total of 160 consecutive examinees were randomly divided into NDP and CSP groups. Participants in this study were randomly assigned to a "CSP group" or a "NDP group" using a permuted four-block randomization method. Biostatistician made permuted four-block randomization table and calculated the numbers of participants. All random code was contained in a closed box. The nurse in outpatient clinic enrolled the examinees. Before colonoscopy, participants, outcome assessors, and care providers (endoscopists and nurses) were blinded to assignment to

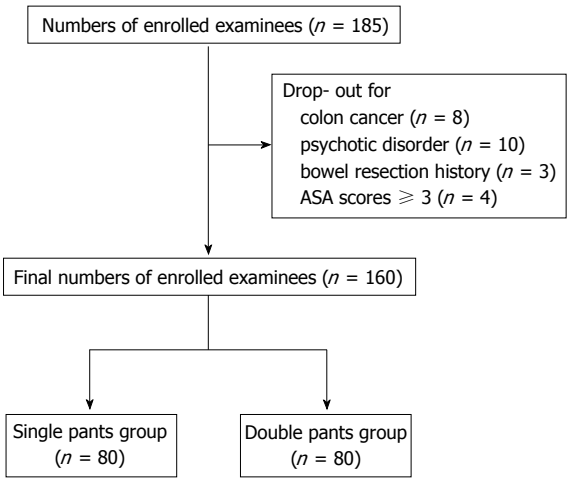

Figure 1 Consort diagram. ASA: American Society of Anesthesiologists.

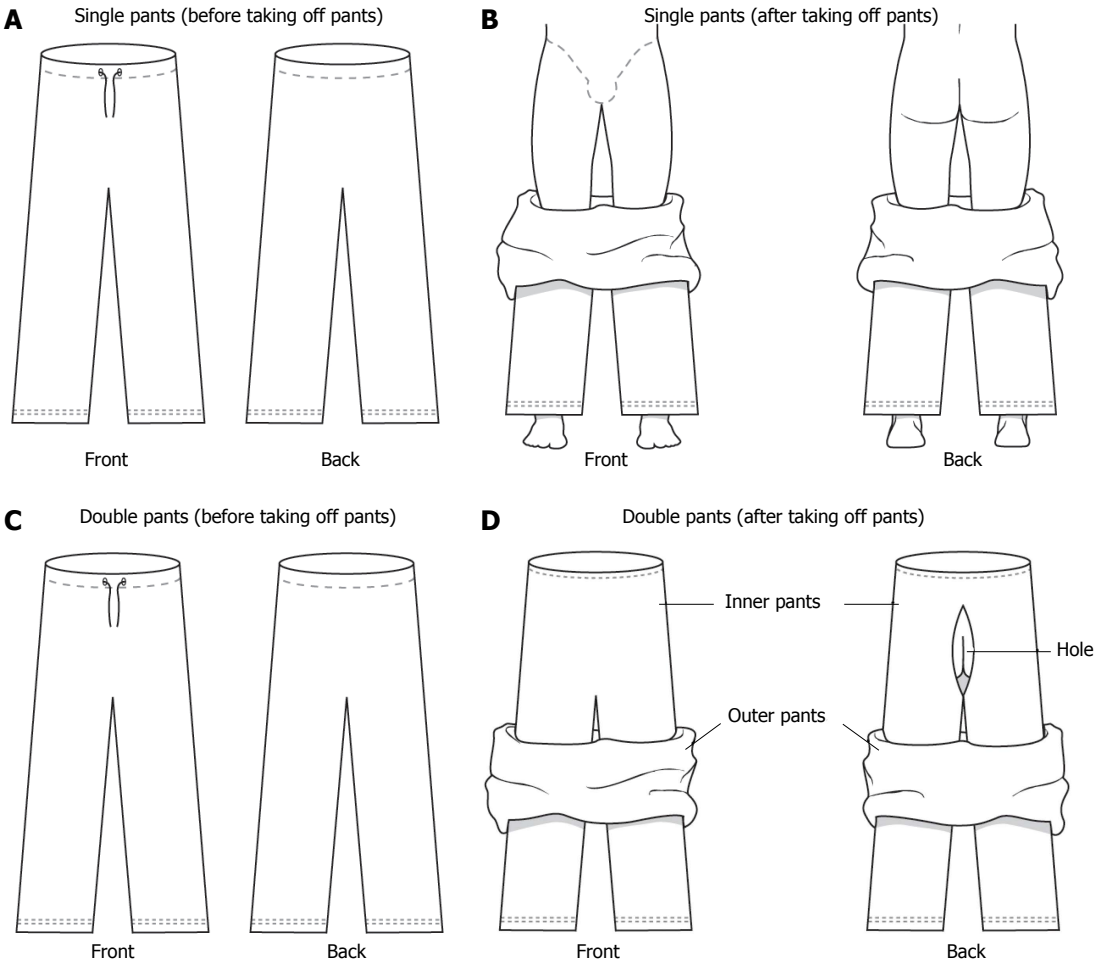

Figure 2 Drawings of the conventional single pants and novel double pants. A: The conventional single pants were composed of a single layer of fabric; B: If the examinees wearing conventional single pants dropped the outer pants below hips, the total area of the hip is exposed; C: The novel double pants were composed of a double layer of fabric from the hip to the thigh and a single layer of fabric below the thigh; D: In the inner layer of the double pants, there is a hole for insertion of scope in the back. If the examinees wearing novel double pants dropped the outer pants below hips, the only buttock area is exposed through the hole (25 cm × 15 cm) for insertion of scope.

the allocation because the third party examiner allocated the enrolled subjects regarding to the prepared permuted-block randomization table. Written informed consent was provided by all participants in the study.

Pants

In CSP, there is no hole for insertion of the scope. Examinees remove the CSP to below the level of the buttocks and expose their buttocks area during colonoscopy, as shown in Figure 2A and B. NDP consist of single fabric only below the thigh, and doubled fabric from the hip to the thigh with a hole in the inner pants at the level of the buttocks. The hole is 25 cm wide and 15 cm long. Examinees wearing NDP can undergo colonoscopy without taking off the inner pants, as shown in Figure 2C and D. We supplied the 80 NDP (Bobo trading, Seoul, South Korea) to NDP group and 80 CSP (Seodaemun uniform, Seoul, South Korea) to CSP group. All patients got the pants in the hospital just before the procedure according to the randomized allocation by the nurse in outpatient clinic. The pants had been prepared with marking "A type" (CSP) and "B type" (NDP).

Interview

In this study the only one nurse, as the third party examiner, interviewed the patients before and after the procedure to exclude interviewer's influence on the answers in this study. Before colonoscopy, examinees underwent a one-on-one interview in a quiet, separated room with a third-party examiner in which they completed questionnaires identifying state and trait anxiety, marital status, education, and residence. After colonoscopy, examinees had a similar one-on-one interview to complete the questionnaires, which included the Group Health Association of America 9 (GHAA 9) scales and questions regarding pant-specific satisfaction, state anxiety, and shame. After colonoscopy, examinees' pain during colonoscopy was assessed after the procedure and scored from 0 to 10 using a face pain scale (0-very happy, no pain, 2-hurts just a little bit, 4-hurts a little more, 6-hurts even more, 8-hurts a lot, 10-hurts as much as you can imagine; don't have to be crying to feel this much pain)[12].

Colonoscopy

Professional endoscopists who had performed more than 1000 colonoscopies performed all of the study colonoscopy procedures with a standard colonoscope (CF Q240L, CF Q240I, CF H260AI, CF Q260AI; Olympus Optical Co, Ltd, Tokyo, Japan). The indication for colonoscopy, ASA status, Ottawa quality scale, procedural time, number of polyps, number of examinees with polyps, method of polyp removal, rates of adverse events after polyp removal, rates of successful cecal approach, and gender difference between examinees and endoscopists in the NDP and CSP groups were investigated.

Outcome measurements

Satisfaction: The questionnaires measuring satisfaction included 14 questions regarding the examinees' colonoscopy experience. Nine of the questions regarding overall satisfaction were derived from a previously validated GHAA 9 satisfaction survey[13]. These questions used a 5-point scale to grade satisfaction (1: poor, 2: fair, 3: good, 4: very good, 5: excellent). A score of more than 3 was considered a favorable response. Five questions were asked to elicit pants-specific satisfaction as related to the following: difficulty of defecation wearing the pants; difficulty in position change during the colonoscopy; worriness about the exposed buttock area; willingness to try the same pants for the next exam; and recommendation of the same pants to other people. The five pants-specific satisfaction questions used a 4-point scale to grade satisfaction (1, not at all; 2, somewhat; 3, moderately so; 4, very much so). Three of the five questions were negative questions about satisfaction and thus scored in reverse. Scores on pants-specific satisfaction ranged between 5 and 20.

Anxiety: The trait anxiety questionnaire has been shown to reflect the general disposition of patients or stable tendency for anxiety, while the state questionnaire reflects a patient's anxiety related to a particular set of circumstances[9,14]. The trait and state questionnaires each consists of 20 statements, and all answers were graded using a 4-point scale (1, not at all; 2, somewhat; 3, moderately so; 4, very much so). Seven of the 20 questions from the STAI−Trait anxiety and 10 of the 20 questions from the STAI-State anxiety were negative questions and scored in reverse. The scores of the trait and state questionnaire range between 20 and 80, and higher scores reflected higher anxiety.

Shame: Among the experienced shame scale, 4 questions were used to measure body shame during colonoscopy[15]. We designed another 2 questions to measure shame following the exposure of the body during position change for the colonoscopy and exposure of the buttock area while walking to the colonoscopy room. In total, 6 different questions were used to measure shame. All answers were graded using a 4-point scale (1, not at all; 2, somewhat; 3, moderately so; 4, very much so). Scores on the body shame questionnaire ranged between 6 and 24, and higher scores reflected higher level of shame.

Ethical considerations

Informed consent was obtained from each patient included in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008) as reflected in a prior approval by the institution's human research committee. The study protocol was approved by the ethical committee of Yonsei University College of Medicine. The study protocol was also approved by clinicaltrial.gov (NCT 01524042).

Statistical analysis

All differences in CSP and NDP were examined using SPSS Statistics (version 18.0.0, IBM Corp., Armonk, NY, United States). Continuous variables were compared using Student's t test, while categorical data were analyzed using the χ^2 test (Fisher's exact test) between two groups. Univariate and multivariate linear regression analysis were used to assess independent predictive factors associated with satisfaction, state anxiety after colonoscopy, and shame. For multivariate analysis, variables with $P < 0.1$ by univariate analysis were included. All statistical tests were two-tailed and considered statistically significant with a P value < 0.05.

RESULTS

Baseline characteristics and endoscopic characteristics of the study population

There was no significant difference in baseline characteristics including age, gender, marital status, education, and residence in the study population and in the NDP and CSP groups in Table 1. There were no significant differences in the endoscopic characteristics of study population including indication for colonoscopy, Ottawa quality scale, procedural time, number of polyps, number of examinees with polyps, method of polyp removal, rates of adverse events after polyp removal, rates of successful cecal approach, pain scale, and gender difference between examinees and endoscopists in the NDP and CSP groups, as shown in Table 2.

Outcomes of satisfaction, state anxiety, trait anxiety and shame

There was no significant difference in GHAA9 score (Likert scale, 0-5) between the NDP and CSP groups. There were high favorable response rates (FRRs) which were greater than 90% for waiting time, waiting on procedure day, personal manner of physician and support staff, technical skills, adequacy of explanation, overall rating of the visit, and willingness to have the procedure repeated by the same physician and at the same facility in Table 3. In terms of pants-specific satisfaction in Table 3, the CSP group worried more about exposing the buttock area during the colonoscopy than did the NDP group (3.3 ± 0.7 *vs* 2.9 ± 0.7, $P < 0.001$). The NDP group was more willing to wear same pants when they undergo their

Table 1 Baseline characteristics of study population *n* (%)

	CSP (*n* = 80)	NDP (*n* = 80)	*P* value
Age (yr)	59.1 ± 11.4	59.4 ± 11.7	0.847
20 ⩽ age < 40	3 (3.8)	5 (6.2)	
40 ⩽ age < 60	38 (47.5)	43 (53.8)	
age ⩾ 60	39 (48.7)	32 (40)	
Gender			0.210
Male	41 (51.3)	45 (56.3)	
Female	39 (48.7)	35 (43.7)	
Marital status			> 0.999
Married	75 (93.8)	76 (96.0)	
Single or divorced	5 (6.2)	4 (4.0)	
Education			0.320
Middle school	20 (25.0)	13 (16.2)	
High school	31 (38.8)	28 (35.0)	
University	22 (27.5)	32 (40.0)	
Graduate school	7 (8.7)	7 (8.8)	
Residence			0.609
Urban	73 (91.3)	70 (87.5)	
Rural	7 (8.7)	10 (12.5)	
ASA status			0.896
Class Ⅰ	32 (40)	36 (45.0)	
Class Ⅱ	48 (60)	44 (55.0)	
Class Ⅲ	0 (0.0)	0 (0.0)	
Class Ⅳ	0 (0.0)	0 (0.0)	
Class Ⅴ	0 (0.0)	0 (0.0)	

CSP: Conventional single pants; NDP: Novel double pants; ASA: American Society of Anesthesiologists.

Table 2 Endoscopic characteristics of study population *n* (%)

	CSP (*n* = 80)	NDP (*n* = 80)	*P* value
Indication of colonoscopy			0.260
Abnormality on other study	2 (2.5)	4 (5.0)	
Stool occult blood positive	9 (11.2)	13 (16.2)	
Screening	47 (58.8)	42 (52.5)	
Anemia	0.0 (0)	1.0 (1.2)	
Diarrhea	3(3.8)	4 (5.0)	
Surveillance after polyp removal	6 (7.5)	11 (13.8)	
Abdominal pain	13 (16.2)	5 (6.3)	
Ottawa Quality Scale	3.9 ± 2.5	4.3 ± 2.8	0.382
Procedural time (min)			
Insertion	10.7 ± 8.5	8.6 ± 5.5	0.059
Withdrawal	12.8 ± 6.2	11.8 ± 5.5	0.309
Number of polyp	1.1 ± 1.8	0.8 ± 1.5	0.266
Number of examinees with polyps	47 (58.8)	42 (52.5)	0.525
Method of polyp removal			0.521
Biopsy	25 (52.1)	22 (56.4)	
Snaring polypectomy	15 (31.3)	8 (20.5)	
Endoscopic mucosal resection	8 (16.6)	9 (23.1)	
Adverse events			
Bleeding	0 (0.0)	0 (0.0)	-
Perforation	0 (0.0)	0 (0.0)	-
Cecal approach	80 (100)	80 (100)	-
Pain scale[1]	3.5 ± 2.7	3.7 ± 2.4	0.719
Genders between examinee and endoscopist			0.525
Same gender	42 (52.5)	47 (58.8)	
Different gender	38 (47.5)	33 (41.2)	

Data are expressed as absolute numbers (percentage) or mean ± SD. [1]Examinees' pain during colonoscopy was assessed after the procedure and scored from 0 to 10 using a face pain scale (0-very happy, no pain, 2-hurts just a little bit, 4-hurts a little more, 6-hurts even more, 8-hurts a lot, 10-hurts as much as you can imagine; don't have to be crying to feel this much pain). CSP: Conventional single pants; NDP: Novel double pants.

next colonoscopy than the CSP group (3.3 ± 0.8 *vs* 2.1 ± 0.9, $P < 0.001$). The NDP group was also more willing to recommend other people wear the same pants when they undergo their own colonoscopies than the CSP group (3.3 ± 0.7 *vs* 2.0 ± 1.0, $P < 0.001$). A significantly lower shame score was estimated in the NDP group compared with CSP group (6.6 ± 1.5 *vs* 8.1 ± 3.2, $P < 0.001$), which is shown in Table 3.

Predictive factors for satisfaction, state anxiety, and shame after colonoscopy

To investigate the predictive factors related to satisfaction, state anxiety, and shame after colonoscopy, univariate and multivariate regression analysis was performed (Table 4). In the multivariate analysis of pants-specific satisfaction, unmarried examinees had less pants-specific satisfaction than married examinees [B (SE) = -1.82 (0.61), $P = 0.004$], and the NDP group had higher pants-specific satisfaction than the CSP group [B (SE) = 2.72 (0.28), $P < 0.001$]. In multivariate analysis of state anxiety after the colonoscopy, female participants had higher state anxiety score after the procedure than males [B (SE) = 3.52 (1.14), $P = 0.002$]. Unmarried examinees had higher state anxiety score after colonoscopy than married examinees [B (SE) = 4.45 (2.22), $P = 0.047$]. Urban examinees had higher state anxiety score after colonoscopy than rural examinees [B (SE) = 4.68 (1.70), $P = 0.007$]. The NDP group had a tendency of lower state anxiety score after colonoscopy than the CSP group [B (SE) = -1.80 (1.04), $P = 0.086$]. In the multivariate analysis of shame, female examinees had higher shame score than male examinees [B (SE) = 1.25 (0.37), $P = 0.001$]. Unmarried examinees also had higher shame score than married examinees [B (SE) = 2.78 (0.81), $P = 0.001$]. The NDP group had lower shame score than the CSP group [B (SE) = -1.37 (0.37), $P = 0.001$].

DISCUSSION

In this study, we compared the satisfaction, anxiety and shame between NDP and CSP with prospective randomized control trial. The examinees in NDP group responded with higher pants-specific satisfaction, lower state anxiety after colonoscopy, and lower shame scores compared to those in CSP group. Thus, the NDP developed at our institution may contribute to increased satisfaction and decreased anxiety and shame after colonoscopy.

Although there have been a wide range of studies regarding satisfaction[1,16-20] and anxiety[9,21-23] during colonoscopy, our study was unique because it specifically addressed colonoscopic pants and investigated the differences in emotional change by the type of colonoscopic pants participants wore. Colonoscopic pants were designed considering the maximization of the efficiency of colonoscopies and hygiene aspects. Various types of colonoscopic pants have been used at different institutions. In some centers, examinees wear CSP or pants with

Table 3 Comparison of satisfaction rated by Group Health Association of America 9 survey by favorable response rate and likert scale, pants specific satisfaction, state anxiety and shame after colonoscopy between novel double pants and conventional single pants

	FRR, *n* (%)			Likert scale (mean ± SD)		
	CSP	NDP	*P* value	CSP	NDP	*P* value
GHAA9						
Appointment wait time	73 (91.3)	72 (90.0)	> 0.999	3.7 ± 0.7	3.6 ± 0.9	0.351
Waiting on procedure day	74 (92.5)	76 (95.0)	0.746	3.8 ± 0.8	3.9 ± 0.8	0.649
Personal manner of physician	78 (97.5)	79 (98.8)	> 0.999	4.4 ± 0.6	4.4 ± 0.6	0.712
Technical skills of physician	74 (92.5)	76 (95.0)	0.746	4.1 ± 0.8	4.1 ± 0.7	0.564
Personal manner of support staff	77 (96.3)	78 (97.5)	> 0.999	4.3 ± 0.7	4.0 ± 0.5	0.546
Adequacy of explanation of what was done	74 (92.5)	78 (97.5)	0.276	4.0 ± 0.6	4.0 ± 0.5	0.902
Overall rating of visit	75 (93.8)	74 (92.5)	> 0.999	3.9 ± 0.6	3.9 ± 0.6	0.806
Yes/No questions						
Would have procedure by same physician: Yes	78 (97.5)	77 (96.3)	> 0.999			
Would have procedure at same facility: Yes	79 (98.8)	79 (98.8)	> 0.999			
Pants specific satisfaction						
Difficulty in defecation				3.7 ± 0.5	3.8 ± 0.4	0.106
Difficulty in position change				3.7 ± 0.5	3.8 ± 0.3	0.175
Worriness about exposing buttock area in procedure				3.3 ± 0.7	2.9 ± 0.7	< 0.001
Will to wear same pants at next colonoscopy				2.1 ± 0.9	3.3 ± 0.8	< 0.001
Will to recommend to other people to wear same pants				2.0 ± 1.0	3.3 ± 0.7	< 0.001
Before colonoscopy						
Total scores of trait anxiety				39.2 ± 8.8	39.4 ± 8.2	0.904
Total scores of state anxiety				37.9 ± 8.2	39.9 ± 8.8	0.144
After colonoscopy						
Total scores of state anxiety				35.4 ± 6.9	33.0 ± 7.0	0.028
Total scores of shame				8.1 ± 3.2	6.6 ± 1.5	< 0.001
Change of state anxiety before and after the procedure				-2.4 ± 7.6	-6.9 ± 8.4	0.001

The scores on pants-specific satisfaction ranged between 5 and 20, and the scores of the trait and state anxiety questionnaire range between 20 and 80. The scores on the body shame questionnaire ranged between 6 and 24. A score of more than 3 of Likert scale was considered a FRR (Excellent: 5; Very Good: 4; Good: 3; Fair: 2; Poor: 1). Likert scale: Excellent: 5; Very Good: 4; Good: 3; Fair: 2; Poor: 1. Scores of pants specific satisfaction, Not at all: 1; A little: 2; Moderately: 3; Very much: 4. CSP: Conventional single pants; NDP: Novel double pants; GHAA9: Group Health Association of America 9; FRR: Favorable response rate.

Table 4 Univariate and multivariate analysis of pants specific satisfaction after colonoscopy

Predictive factors	Outcome (pants specific satisfaction)				Outcome (state anxiety after colonoscopy)				Outcome (shame after colonoscopy)			
	Univariate		Multivariate		Univariate		Multivariate		Univariate		Multivariate	
	B (SE)	*P* value	B (SE)	*P* value	B (SE)	*P* value	B (SE)	*P* value	B (SE)	*P* value	B (SE)	*P* value
Sex												
Male	Ref.				Ref.		Ref.		Ref.		Ref.	
Female	0.04 (0.36)	0.913			4.29 (1.06)	0.913	3.52 (1.14)	0.002	1.31 (0.40)	0.001	1.25 (0.37)	0.001
Age	-0.02 (0.01)	0.104			0.01 (0.04)	0.104			-0.01 (0.01)	0.928		
Marital status												
Married	Ref.		Ref.		Ref.		Ref.		Ref.		Ref.	
Unmarried	-1.6 (0.7)	0.033	-1.82 (0.61)	0.004	4.48 (2.40)	0.033	4.45 (2.22)	0.047	2.79 (0.87)	0.002	2.78 (0.81)	0.001
Education												
Middle school	Ref.				Ref.				Ref.			
High school	-0.04 (0.49)	0.931			-1.81 (1.53)	0.931			-0.01 (0.57)	0.998		
University	0.27 (0.50)	0.506			-2.64 (1.55)	0.506			0.22 (0.58)	0.697		
Graduate school	0.57 (0.73)	0.430			-2.50 (2.24)	0.430			0.15 (0.84)	0.853		
Type of pants												
CSP	Ref.		Ref.		Ref.		Ref.		Ref.		Ref.	
NDP	2.70 (0.29)	< 0.001	2.72 (0.28)	< 0.001	-2.43 (1.10)	< 0.001	-1.80 (1.04)	0.086	-1.47 (0.39)	< 0.001	-1.37 (0.37)	0.001
Residence												
Rural area	Ref.				Ref.		Ref.		Ref.			
Urban area	-0.46 (0.58)	0.430			4.2 (1.7)	0.430	4.68 (1.70)	0.007	0.93 (0.67)	0.165		
Genders between examinee and endoscopist												
Same gender	Ref.				Ref.		Ref.		Ref.			
Different gender	-0.36 (0.36)	0.308			3.90 (1.07)	< 0.001	1.45 (1.15)	0.209	0.65 (0.41)	0.115		

CSP: Conventional single pants; NDP: Novel double pants.

a hole in the buttock area with a movable flap, which can potentially expose buttock area during walking. In other centers, examinees wear single pants with hole in the buttocks and outer gown to hide the hole, which is likely inferior to the NDP we describe in terms of repair and maintenance expenses.

In our study there was no significant difference in GHAA 9 patient satisfaction between NDP and CSP groups as the contents of GHAA 9 were not closely connected with satisfaction regarding colonoscopic pants. However, in the survey of pants-specific satisfaction, the NDP group showed increased satisfaction than the CSP group in terms of their willingness to wear the same pants in the next time and to recommend the same pants to other people. High patient satisfaction during colonoscopy may result in a higher rate of compliance with screening and clinical surveillance programs[1]. The American Society for Gastrointestinal Endoscopy and the American College of Gastroenterology recommends assessment of patient satisfaction during colonoscopy to evaluate the quality of colonoscopy[5,6].

The sensitivity related to the psychological aspects including anxiety and shame might partly depends on the different characteristics including age, gender, behavior, social environment and sensitivity of the society. As example lower anxiety scores was reported to be associated with older age, male sex, lower income, experience of previous colonoscopy and lower education[9]. During gastrointestinal endoscopic procedures, female examinees have been reported to have higher state and trait anxiety levels than male examinees[10,22]. In our study, female, unmarried, urban examinees and CSP group had more state anxiety after colonoscopy than that of their male, married, rural examinees and NDP group counterparts. The differences of shame and state of anxiety after colonoscopy between the two groups were moderate in most of the items. But, the difference in change of state of anxiety before and after the procedure might be clinically significant. Because all participants are healthy persons in psychological aspect at baseline, even moderate change of anxiety level after colonoscopy could result in decreased satisfaction of the procedure and reduced compliance to next examinations in clinical practice.

There were some limitations to our study. Endoscopists could not be completely blinded to the types of colonoscopic pants worn because they ultimately saw which pants they were wearing during the colonoscopic procedure. However, the outcome assessors were blinded during the total period of study. In addition, all the endoscopists who performed the colonoscopy were excluded from the outcome assessors. And there was no questionnaire for endoscopists in this study. Examinees with cancer or IBD were suspected to have higher anxiety levels during colonoscopy, and thus were excluded. Therefore understanding the satisfaction, anxiety, and satisfaction of these particular examinees would require further investigation. Most of examinees in this study were over 40 years old and more than half of examinees underwent colonoscopy for screening purposes; the number of young unmarried examinees was very small in this study, and the level of anxiety, shame and satisfaction in this group may be significantly different. For a more ideal comparision of anxiety, shame and satisfaction during colonoscopy between the NDP and CSP group, it would be better for one examinee to wear single pants and double pants during colonoscopy, but this was not logistically feasible. To compensate for this limitation in state anxiety we measured the change in state anxiety before and after colonoscopy in the same examinees.

In conclusion, the examinees in the NDP group had higher pants-specific satisfaction and lower state anxiety and lower shame after colonoscopy compared to CSP group. Therefore NDP could help to increase satisfaction and decrease anxiety and shame after colonoscopy. Future studies should continue to investigate factors for anxiety, shame and satisfaction.

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COMMENTS

Background

Exposing buttocks during colonoscopy can make examinees feel unsatisfied, anxious, and shameful. To increase satisfaction and diminish anxiety and shame during colonoscopy, the authors developed novel double pants (NDP) which consist of doubled fabrics with an inner hole.

Research frontiers

Pants-specific satisfaction scores regarding willingness to repeat colonoscopy using same pants and recommendation of same pants to other people were significantly higher in NDP than conventional single pants (CSP) groups. State anxiety and shame after colonoscopy was lower in NDP group compared with CSP group.

Innovations and breakthroughs

Although there have been a wide range of studies regarding satisfaction and anxiety during colonoscopy, this study was unique because it specifically addressed colonoscopic pants. The authors developed NDP, which consist of single fabric only below the thigh, and doubled fabric from the hip to the thigh with a hole in the inner pants at the level of the buttocks. Examinees wearing NDP can undergo colonoscopy without taking off the inner pants.

Applications

Through these findings, the NDP developed at our institution may contribute to increased satisfaction and decrease anxiety and shame after colonoscopy.

Terminology

NDP are single fabric only below the thigh, and doubled fabric from the hip to the thigh with a hole in the inner pants at the level of the buttocks. The hole is 25 cm wide and 15 cm long.

Peer review

Satisfaction studies are important in relationship with the compliance of colorectal cancer screening programs and less so in the group of patients with specific suspicion of diseases affecting the anus, rectum or colon. This complex study is well designed and analyzed. The topic of the manuscript is interesting and the work performed is ambitious.

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BRIEF ARTICLE

Cap polyposis: A rare cause of rectal bleeding in children

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Abstract

AIM: To evaluate the clinicopathological features and treatment outcomes of cap polyposis in the pediatric population.

METHODS: All pediatric patients with histologically proven diagnosis of cap polyposis were identified from our endoscopy and histology database over a 12 year period from 2000-2012 at our tertiary pediatric center, KK Women's and Children's Hospital in Singapore. The case records of these patients were retrospectively reviewed. The demographics, clinical course, laboratory results, endoscopic and histopathological features, treatments, and outcomes were analyzed. The study protocol was approved by the hospital institutional review board. The histological slides were reviewed by a pediatric histopathologist to confirm the diagnosis of cap polyposis.

RESULTS: Eleven patients were diagnosed with cap polyposis. The median patient age was 13 years (range 5-17 years); the sample included 7 males and 4 females. All of the patients presented with bloody stools. Seven patients (63%) had constipation, while 4 patients (36%) had diarrhea. All of the patients underwent colonoscopy and polypectomies (excluding 1 patient who refused polypectomy). The macroscopic findings were of polypoid lesions covered by fibrinopurulent exudates with normal intervening mucosa. The rectum was the most common involvement site (n = 9, 82%), followed by the rectosigmoid colon (n = 3, 18%). Five (45%) patients had fewer than 5 polyps, and 6 patients (65%) had multiple polyps. Histological examination of these polyps showed surface ulcerations with a cap of fibrin inflammatory exudate. Four (80%) patients with fewer than 5 polyps had complete resolution of symptoms following the polypectomy. One patient who did not consent to the polypectomy had resolution of symptoms after being treated with sulphasalazine. All 6 patients with multiple polyps experienced recurrence of bloody stools on follow-up (mean = 28 mo).

CONCLUSION: Cap polyposis is a rare and under-recognised cause of rectal bleeding in children. Our study has characterized the disease phenotype and treatment outcomes in a pediatric cohort.

Key words: Cap polyposis; Polyps; Rectal bleeding; Pediatrics; Inflammatory bowel disease

Core tip: Cap polyposis is a rare and under-recognized condition with distinct clinical, endoscopic and histopathological features. All children with cap polyposis invariably present with rectal bleeding. Awareness of this diagnosis is important as its clinical and endoscopic features can mimic inflammatory bowel disease result-

ing in prolonged and inappropriate treatment. This article evaluates the clinicopathological features and treatment outcomes in a series of children with cap polyposis. Complete polypectomy should be performed where possible in combination with medical therapy. Prognosis is good for children with few polyps although recurrence rate is high in those with multiple polyps at diagnosis requiring further surgical intervention.

Li JH, Leong MY, Phua KB, Low Y, Kader A, Logarajah V, Ong LY, Chua JHY, Ong C. Cap polyposis: A rare cause of rectal bleeding in children. *World J Gastroenterol* 2013; 19(26): 4185-4191 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i26/4185.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i26.4185

INTRODUCTION

Cap polyposis (CP) is a rare and under-recognized condition with distinct clinical, endoscopic and histopathological features. It was first described by Williams *et al*[1] in 1985. CP is characterized by inflammatory polyps that are usually located from the rectum to the distal descending colon. Histologically, these polyps consist of elongated, tortuous, and often distended crypts covered by a "cap" of inflammatory granulation tissues. Macroscopic findings include dark red, sessile polyps that are commonly situated on the apices of transverse mucosal folds, with normal intervening mucosa.

Characteristic symptoms in adults include mucous diarrhea, tenesmus and rectal bleeding[2]. CP may be confused with other inflammatory conditions of the large intestine, in particular inflammatory bowel disease (IBD), due to their similarities in clinical and endoscopic features. The pathogenesis of CP is unknown, and no specific treatment has yet been established.

CP has been rarely described in the pediatric population. We report a case series of 11 pediatric patients diagnosed with CP and characterized their clinical, endoscopic, and histological features.

MATERIALS AND METHODS

All pediatric patients with histologically proven diagnosis of CP were identified from our endoscopy (total number = 1905) and histology database over a 12 year period from 2000-2012 at our tertiary pediatric center, KK Women's and Children's Hospital in Singapore. The case records of these patients were retrospectively reviewed. The demographics, clinical course, laboratory results, endoscopic and histopathological features, treatments, and outcomes were analyzed. The study protocol was approved by the hospital institutional review board (Singhealth Centralised Institutional Review Board). The histological slides were reviewed by a pediatric histopathologist to confirm the diagnosis of CP.

RESULTS

Patients

There were 11 pediatric patients diagnosed with cap polyposis from 2000 and 2012. The clinical features of these patients are summarized in Table 1. There were 7 males and 4 females, with a median age of 13 years (range 5-17 years). The racial distributions included 5 Malays, 4 Chinese, and 2 Indian patients.

Clinical features

Common presenting features of these patients included per-rectal bleeding, constipation and straining, diarrhea, and abdominal pain (Table 1). All 11 patients presented with blood in the stools. Seven patients (63%) had constipation and/or straining, and 4 patients (36%) had diarrhea. Abdominal pain was a presenting complaint in 6 (54%) patients. Digital rectal examinations revealed rectal polypoid masses in 7 (63%) patients and 1 patient had perianal fissure.

The median hematological values at diagnosis were hemoglobin 10.9 (IQR 12.6-14) g/dL, white cell counts 6.7 × 10^9/L (IQR 4-11)10^9/L and platelet counts of 342 (IQR 150-400) × 1000/μL. All of the patients had normal coagulation profiles. Eight of the 11 patients had normal serum albumin measured with a median value of 38 g/L (IQR 35-45). Only 2 patients (IDs 8 and 10) had hypoalbuminemia. Inflammatory markers C-Reactive protein/Erythrocyte Sedimentation Rate were measured in 6 patients (ID 2, 5, 7, 8, 9, and 10), and they were normal.

Endoscopic features and histology

All patients underwent colonoscopy with macroscopic findings of polyps or polypoid lesions. These were mainly small, red and sessile polyps covered by a thick layer of fibrinopurulent exudates predominantly found on the apices of the mucosa folds. The intervening mucosa was normal both macroscopically and on histological examination (Figure 1A and B). The polyps were most commonly located in the rectum only (n = 9, 82%). Two patients (18%) had polyps in the rectum and sigmoid colon. The number of polyps ranged from 1 to more than 10. Five (45%) patients had fewer than 5 polyps, and 6 (65%) patients had multiple (> 5) polyps on initial colonoscopy.

On histological examination, these colonic polyps showed a variable degree of surface ulceration associated with a cap of fibrin inflammatory exudates and granulation tissue. Focally, the surface epithelium is preserved but attenuated. The crypts within the polyps showed crypt elongation and luminal epithelial serration. In some cases, the crypts were mildly distended towards the surface. The lamina propria contained a variably increased number of acute and chronic inflammatory cells (Figure 2). These histological features were consistent with inflammatory cap polyps. The mucosa surrounding these polyps was normal.

Three patients presented with abdominal pain and

Table 1 Clinical features and treatment outcomes of cap polyposis patients

ID	Age (yr)	Sex	Diarrhea	C + S	Abdo pain	PR bleeding	No. of polyps	Site	Antibiotics	Stool softeners	Recur	Follow up (mo)
1	5	M	No	No	No	Yes	1	R	No	Yes	No	24
2	13	M	No	Yes	No	Yes	4	R	No	Yes	No	4
3	8	M	NA	Yes	No	Yes	1	R	No	Yes	No	3
4	8	M	No	Yes	No	Yes	1	R	Metro	No	No	3
5	15	F	Yes	No	No	Yes	3	R	No	No	No	24
6	15	M	Yes	Yes	Yes	Yes	$n > 5$	R	Metro	Yes	Yes	36
7	10	M	Yes	No	Yes	Yes	$n > 5$	R	Metro	Yes	Yes	5
8	11	F	No	Yes	Yes	Yes	$n > 5$	R	Metro	Yes	Yes	72
9	13	F	No	Yes	Yes	Yes	$n > 5$	R	No	Yes	Yes	72
10	14	F	No	Yes	Yes	Yes	$n > 5$	R + S	No	Yes	Yes	36
11	17	M	Yes	No	No	Yes	$n > 5$	R + S	No	Yes	NA	Lost
												Mean = 28

C + S: Constipation or straining; R: Rectum; S: Sigmoid; Metro: Metronidazole; M: Male; F: Female.

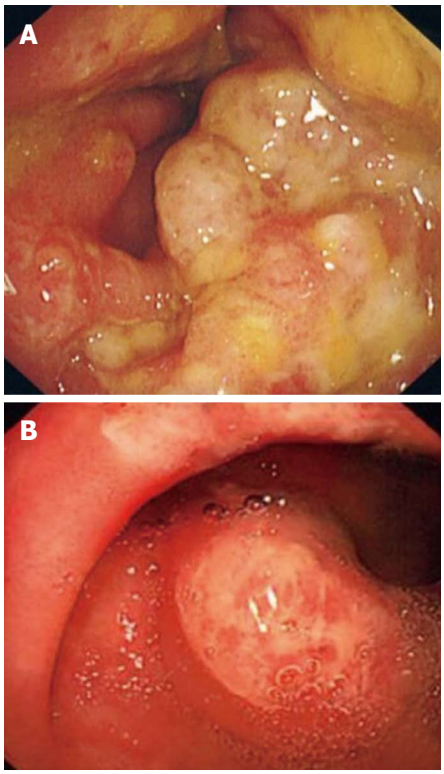

Figure 1 Colonoscopy images of 2 patients with cap polyposis. A: Colonoscopy image of a patient showing multiple small sessile polypoid lesions with mucous exudates of cap polyposis (CP) in the rectum; B: Colonoscopy image of a patient showing a single sessile red polypoid lesion located on the transverse folds with normal intervening mucosa.

underwent simultaneous upper gastrointestinal (GI) endoscopies; the histological findings showed mild gastritis but no evidence of *Helicobacter pylori* (*H. pylori*). There were no polypoid lesions noted in the stomach of these 3 patients.

Management and outcomes

All but one patient underwent polypectomies. Patients 1-5 had fewer than 5 polyps detected by the initial colonoscopy. Patients 1-4 had polypectomy performed and were subsequently treated with stool softeners. Patient 4 also received a course of metronidazole. All 4 of these patients had complete resolution of their symptoms with no further rectal bleeding at mean follow-up period of 28 mo. Patient 5 had 3 small sessile polyps and did not consent for polypectomy but had complete resolution of symptoms at 18 mo after being treated with sulphasalazine.

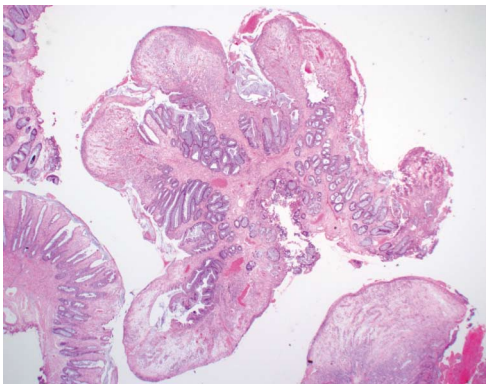

Figure 2 Histology of a sessile colonic polyp from a patient with cap polyposis. The polyp was comprised of granulation tissue and focally distended crypts with a slightly serrated luminal surface. Surface ulceration with fibrinomucoid exudates was also present.

Six patients with multiple polyps (Patients 6-11) underwent colonoscopy and polypectomies. One patient was lost to follow-up (Patient 11). The other 5 patients were given stool softeners and 3 patients were given metronidazole. All of the patients experienced a recurrence of symptoms, mainly blood in the stools at subsequent follow-up (mean follow-up period of 28 mo). These patients required repeated colonoscopies with polypectomies and continued to have intermittent per rectal bleeding. Patient 8 eventually had a resolution of his symptoms after 6 colonoscopies with multiple polypectomies at follow-up of 6 years. Patient 11, whose polyps were confined to the recto-sigmoid area, had persistent rectal bleeding that required recurrent blood transfusions and will require more extensive surgical resection in the future. All of the 5 patients with multiple polyps were otherwise well-thrived and had normal inflammatory markers at subsequent follow-up.

DISCUSSION

CP is a rare but distinct disorder with characteristic endoscopic and histological features first described by Williams[1]. Although CP was first described more than 20 years ago, this disease is still not well recognized by physicians. Only approximately 60 cases have been reported in the English language medical literature, mainly as case series or case reports. Due to its rarity, CP is often underrecognized and misdiagnosed as inflammatory bowel disease IBD, leading to prolonged and inappropriate treatment.

To our knowledge, this report is the first and largest case series describing CP in the pediatric population. Shimuzu *et al*[3] have previously described CP in a 12 year old girl. Previous adult studies have found that CP occurs more frequently in females than males[3]. In contrast, there were more boys than girls (7 males and 4 females) in our study, and their median age was 13 years old. We also noted a slightly higher proportion of Malay patients (approximately 40%) in our cohort.

Common presenting symptoms of previously described cases of CP include mucous and/or bloody diarrhea, habitual straining with defecation, chronic constipation and abdominal pain[4]. Rectal bleeding occurred universally in all 11 patients in our study. In total, 65% of our patients had chronic straining and/or constipation, while 35% of the patients had diarrhea. Almost half of our patients presented with abdominal pain, particularly those with multiple polyps. Anemia was a predominant feature in our cohort, with median Hb of 10.9 g/dL.

Protein-losing enteropathy has been reported to be associated with CP[5-9]. Shiomi *et al*[9] reported a case of CP in which protein loss from the lesions was confirmed *via* technetium 99m-labeled diethylenetriaminepentaacetic acid complexed to human serum albumin. There have also been reports of lower limb edema resulting from protein-losing enteropathy from CP[5,7-10]. In addition, laboratory investigations often reveal low total protein and serum albumin levels in CP patients. Symptoms of pre-tibial edema and low protein levels have been shown to normalize with resolution of cap polyposis[5,6-9]. None of the patients in our study presented with lower limb edema. Moreover, hypoalbuminemia was not a predominant feature in our cohort, with only 2 patients having documented low albumin levels.

Endoscopic and histological features

CP is characterized by polyps covered with fibrinopurulent exudates on the surface. The polyps range in size from several millimeters to as large as 7 cm[11,12]. The number of polyps varies from 1 to a few hundred, and the polyps are typically located at the apices of mucosal folds. These polyps have varying morphologies, including polypoid, ulcerative, and flat types[13]. The intervening mucosa has been described as normal or covered with white specks[13], although the significance of this morphology has yet to be ascertained. Initial edematous, flushed mucosa with subsequent development of polyps at the same area, has been reported with serial endoscopic studies[7,14]. The rectum and rectosigmoid colon are the most commonly affected sites, although pan-colonic and gastric involvement has been described[4,6,15]. In agreement with previous findings, all patients in our series had polyps localized to the rectum or rectosigmoid region with normal intervening mucosa.

Histologically, cap polyps consist of elongated, tortuous and hyperplastic crypts that are attenuated towards the mucosal surface. The surfaces of these polyps are ulcerated and covered by a thick layer of fibrinopurulent exudates, hence the term "cap polyps". The lamina propria also contain inflammatory cells[5]. These histological features were reported in our patients.

Role of abnormal colonic motility

The exact etiology and pathogenesis of inflammatory cap polyps are unclear. Various possible causes, including infection[6,14], mucosal ischemia[2], inflammation[16], abnormal bowel motility, and repeated trauma to the colonic mucosa caused by straining[8] have been proposed. Campbell *et al*[2] proposed that abnormal colonic motility may lead to prolapse of redundant mucosa at the apices of transverse mucosal folds. The resultant local ischemia produces characteristic histological appearances of fibromuscular obliteration of the lamina propria; superficial erosion associated with granulation tissue; and elongated, tortuous, hyperplastic glands. These histological features are also present in other conditions, such as prolapsing mucosal polyps, solitary rectal ulcers, inflammatory cloacogenic polyps, and gastric antral vascular ectasia. These findings, along with the fact that CP predominantly affects the rectosigmoid and has a circumferential involvement of the colonic mucosa, have led many researchers to attribute CP to abnormal colonic motility and repeated trauma to the colonic mucosa caused by straining during defecation. Hence, CP has been considered part of a spectrum of "mucosal prolapse syndromes"[5,17,18].

However, aberrant motility of the distal large bowel may only partially contribute to the development of cap polyposis. Although many CP patients present with a history of straining at defecation, these individuals usually lack a typical mucosal prolapse that involves the anterior wall of the lower rectum[18]. There have also been reports of CP developing in patients without evidence or history of abnormal colonic motility. Géhénot *et al*[14] and Konishi *et al*[16] reported the differentiating features between and mucosal prolapse syndrome, where there was significant thickening of the second layer on endoscopic ultrasound in cap polyposis, as opposed to smooth, diffuse thickening of the third layer and minimal thickening of the second layer in mucosal prolapse. Furthermore, avoidance of straining alone has not been reported as an effective treatment modality. In our cohort of patients, stool softeners were prescribed in 8 of 11 patients to avoid straining on defaecation but the symptoms still recurred in those with multiple polyps.

The role of inflammation and infection

In recent years, the role of infectious organisms, such as *H. pylori*[5,6,7,19,20], and inflammation[14] has been proposed. Three of our patients underwent an upper GI endoscopy, but none of them showed any evidence of *H. pylori* on in their gastric mucosa biopsies.

Konishi *et al*[21] described a patient in whom CP was noted to progress along the surgical anastomotic line after a laparoscopic sigmoid colectomy was performed, leading to the hypothesis that local inflammation plays a role in the development of cap polyposis. The effectiveness of infliximab in 2 case reports[22,23] also supports the role of inflammation in the pathogenesis of CP. However, molecular studies of the abnormal mucus in CP have proven inconclusive. Buisine *et al*[24] found a predominance of non-sulphated mucins in cap polyps, compared to both non-sulphated and sulphated mucins expressed in normal colonic mucosa. This finding has been associated with a wide range of pathological conditions, including colorectal carcinomas, ulcerative colitis, and familial polyposis, and there has been a lack of data to show a direct involvement of mucins in the initial pathogenesis of cap polyposis.

Treatment of cap polyposis

Different treatment modalities have been trialed including steroids, aminosalicylates, infliximab, *H. pylori* eradication, endoscopic and surgical resection with variable clinical outcomes. The clinical course of CP has been reported to range from spontaneous remission[19,22] to a disease course requiring surgical resection of the affected bowel segments.

Metronidazole has been used widely in the treatment of CP, often in combination with other modalities[5,7,8,25-27]. Shimizu *et al*[3] reported improvement of symptoms and stromal infiltration on colonic biopsies in a patient treated with metronidazole after failed treatment with mesalamine and levofloxacin, leading to the hypothesis that the role of metronidazole may be related to its anti-inflammatory effects rather than antibiotic action against specific pathogens. It has been postulated that by acting as a radical scavenger, metronidazole can inhibit leukocyte emigration and adherence[5]. In our series, 4 patients received a course of metronidazole together with polypectomy. Of these 4 patients, 3 patients had a recurrence of symptoms. This result suggests that metronidazole may have a limited role in the treatment of patients who have multiple cap polyps.

Limited reports of treatment with infliximab have yielded varying results[22,23,28]. Kim *et al*[26] reported a resolution of symptoms and endoscopic lesions after a single infusion of infliximab, but Maunory *et al*[27] reported a case of recurrence despite 2 infusions of infliximab.

The effective eradication of *H. pylori* has been reported to play a role in the treatment of CP. A review of the English language medical literature has identified 6 reports[6,7,15,19,20,25] in which 9 CP patients were treated with *H. pylori* eradication treatment, 8 of whom showed a complete resolution of symptoms and cap polyps (88.9%) and 1 patient experienced a partial improvement of symptoms. Interestingly, all 9 patients tested positive for *H. pylori*, and the patient who showed only a partial improvement in symptoms had persistent *H. pylori* infection despite eradication therapy.

The possible role of *H. pylori* in extragastric diseases, including idiopathic thrombocytopenic purpura, iron deficiency anemia, chronic urticaria and ischemic heart disease, has been suggested[28-30]. Various mechanisms, including the release of inflammatory mediators, molecular mimicry and a systemic immune response, have been postulated[20]. Although *H. pylori* has not been detected in the colonic mucosae of CP patients, these results suggest that *H. pylori* infection may indirectly play a part in the etiology of CP. Similar histological features between Menetrier's disease (in which *H. pylori* has been postulated to play a role) and CP, such as elongated tortuous crypts, have been highlighted by Akamatsu *et al*[7]. Testing for *H. pylori* in all CP cases and subsequent eradication therapy, if necessary, has been recommended by various authors[7,20]. In our series of pediatric patients, 3 patients underwent upper endoscopy, but none of these patients had *H. pylori* detected in their gastric mucosal biopsies. One patient received triple therapy but continued to have recurrence of symptoms. The role of *H. pylori* eradication therapy needs to be further evaluated in the pediatric population.

Steroids and aminosalicylates have been used with varying results[8,25]. Chang *et al*[25] reported a series of 7 patients, 2 of whom maintained clinical response after 2 courses of systemic steroids: Symptoms persisted in 1 patient despite 2 courses of steroids and aminosalicylates; 3 patients experienced spontaneous remission, and 1 patient showed partial improvement after *H. pylori* eradication therapy. None of our patients received steroid therapy, although one patient was successfully treated with aminosalicylic acid. All of our patients for whom inflammatory markers were measured had normal values for these markers.

Polypectomy and surgical removal of the affected colon have produced inconsistent results, with Ng *et al*[4] reporting recurrence in 2 of 5 patients receiving polypectomy and recurrence in 2 of 4 patients who underwent surgical resection of the affected colon[7,31]. In our series, 10 of 11 patients underwent polypectomies. However, only half of these patients achieved complete remission at mean follow-up of 28 mo. These were patients with fewer than 5 polyps at presentation. Those patients with persistent symptoms despite polypectomies were more likely to have multiple polyps at presentation.

Clinical course

The clinical course and long-term prognosis of CP remain largely unknown. A self-limiting course has been reported, despite whether polypectomy or surgery has been performed[32,33]. A polypectomy will, however, provide a definitive histological diagnosis and may also be war-

ranted when the patient presents with significant lower gastrointestinal bleeding. A complete polypectomy was effective in several studies[4,5], and this approach is recommended whenever possible. Our findings suggest that patients with multiple polyps at diagnosis are more likely to experience symptom recurrence.

In conclusion, CP is a rare cause of rectal bleeding in children. Awareness of this diagnosis is important as the clinical and endoscopic features of CP can mimic Inflammatory Bowel Disease[32] and a misdiagnosis can result in prolonged and inappropriate treatment. CP polyps are distinctive inflammatory polyps covered by a cap of fibrinopurulent exudates normally located at the apices of the mucosal folds with normal intervening mucosa both macroscopically and on histological examination. Although the pseudopolyps in IBD have granulation tissues, the intervening mucosa is usually associated with inflammatory changes, such as superficial ulcerations, granularity and/or friability with crypt abscesses[34]. CP is mainly localized to the rectum and sigmoid, whereas the pseudopolyps in IBD may involve the entire colon. Clinically, CP patients are also more likely to have normal inflammatory markers with no extraintestinal manifestations, such as weight loss, oral ulcers, joint pain *etc*.

The clinical course of CP has not been well described. CP may in some instances, be a self-limiting condition. A complete colonoscopy should be performed as polyps have been described throughout the colon[35,36] when possible, a total polypectomy is recommended. Patients with predominant straining/constipation symptoms can be treated with laxatives and advised to avoid straining. Medical treatment including antibiotics (*e.g.*, metronidazole) and eradication therapy for *H. pylori* has been shown to be effective in some reports. There is currently no good evidence for using aminosalicylic acid or immunosuppressive therapy for treatment of CP. Surgical resection may be indicated if symptoms persist despite medical therapy, although recurrence has been described post-operatively.

In summary, CP is a rare cause of rectal bleeding in children. Awareness of this diagnosis is important as its clinical and endoscopic features can mimic inflammatory bowel disease and a misdiagnosis can result in prolonged and inappropriate treatment. Response to medical treatment has been shown to be inconsistent and unsatisfactory. Endoscopic or surgical excision can be curative, but the recurrence rates are high, particularly if numerous polyps are present. Longer term studies are necessary to understand the natural course of this condition.

COMMENTS

Background

Cap polyposis (CP) is a rare and under-recognized condition with distinct clinical, endoscopic and histopathological features first described by Williams *et al*. Little is known of CP in the pediatric population.

Research frontiers

The pathogenesis of CP is unknown and no specific treatment has been established. CP has been rarely described in the paediatric population. The research hotspot is to better define the clinical features and course of CP in children, and identify effective treatment modalities.

Innovations and breakthroughs

The study is the first case series in available literature to characterise the disease phenotypes and treatment outcomes in a group of paediatric patients.

Applications

Cap polyposis is a rare condition especially in children. It is commonly misdiagnosed as inflammatory bowel disease subjecting patients to unnecessary immunosuppressive therapy. The clinical course and long-term prognosis of cap polyposis remain largely unknown. A case series describing the treatment modalities and clinical course of CP in children will raise the awareness of this rare condition amongst paediatricians and gastroenterologists, as well as improve treatment outcomes.

Terminology

Cap polyposis is characterised by inflammatory polyps located in the rectum to distal descending colon. Histologically, these polyps consist of elongated, tortuous crypts covered by a "cap" of inflammatory granulation tissue.

Peer review

The manuscript is interesting and the main importance of the research is represented by the help offered to clinicians and endoscopists in recognizing and treating a rare pathology that could be misdiagnosed as Inflammatory Bowel Disease. These series of children demonstrate the importance of cap polyposis in young patients. Manuscript is well presented and well written.

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BRIEF ARTICLE

Transcatheter arterial chemoembolization followed by immediate radiofrequency ablation for large solitary hepatocellular carcinomas

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Author contributions: Wang ZJ contributed to the design of the project, development of the combined strategy and preparation of the paper; Wang MQ contributed to conception of the project and administrative support; Duan F, Song P, Liu FY, Chang ZF and Li K assisted in the performance of the procedure; Wang Y and Yan JY collected and assembled the data.

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Abstract

AIM: To assess the technical safety and efficacy of transcatheter arterial chemoembolization (TACE) combined with immediate radiofrequency ablation (RFA) for large hepatocellular carcinomas (HCC) (maximum diameter ⩾ 5 cm).

METHODS: Individual lesions in 18 patients with HCCs (mean maximum diameter: 7.5 cm; range: 5.1-15.5 cm) were treated by TACE combined with percutaneous RFA between January 2010 and June 2012. All of the patients had previously undergone one to four cycles of TACE treatment. Regular imaging and laboratory tests were performed to evaluate the rate of technical success, technique-related complications, local-regional tumor responses, recurrence-free survival time and survival rate after treatment.

RESULTS: Technical success was achieved for all 18 visible HCCs. Complete response (CR) was observed in 17 cases, and partial response was observed in 1 case 1 mo after intervention. The CR rate was 94.4%. Local tumors were mainly characterized by coagulative necrosis. During follow-up (2-29 mo), the mean recurrence-free survival time was 16.8 ± 4.0 mo in 17 cases of CR. The estimated overall survival rate at 6, 12, and 18 mo was 100%. No major complications were observed. Levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the blood of 17 patients transiently increased on the third day after treatment (ALT 200.4 ± 63.4 U/L *vs* 24.7 ± 9.3 U/L, $P < 0.05$; AST 228.1 ± 25.4 U/L *vs* 32.7 ± 6.8 U/L, $P < 0.05$). Severe pain occurred in three patients, which was controlled with morphine and fentanyl.

CONCLUSION: TACE combined with immediate RFA is a safe and effective treatment for large solitary HCCs. Severe pain is a major side effect, but can be controlled by morphine.

Key words: Large hepatocellular carcinoma; Transcatheter arterial chemoembolisation; Radiofrequency ablation; Combination therapy; Synchronism

Core tip: Transcatheter arterial chemoembolization (TACE) immediately followed by radiofrequency ablation (RFA) under digital subtraction angiography-computed tomography is used to treat large hepatocellular carcinomas. This technology can improve the synergistic treatment effects of TACE and RFA, as well as reduce the need for repeated treatments and amount of radiation exposure. Furthermore, different treatment technologies are fused into one machine, thereby simplify-

ing the operational process. TACE immediately followed by RFA enhances tumor inactivation ability, decreases recurrence rates, prolongs patient survival time and improves prognosis.

Wang ZJ, Wang MQ, Duan F, Song P, Liu FY, Chang ZF, Wang Y, Yan JY, Li K. Transcatheter arterial chemoembolization followed by immediate radiofrequency ablation for large solitary hepatocellular carcinomas. *World J Gastroenterol* 2013; 19(26): 4192-4199 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i26/4192.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i26.4192

INTRODUCTION

Hepatocellular carcinoma (HCC), the sixth most malignant tumor worldwide, is the third most common tumor leading to death; unfortunately, only 10%-54% of all patients with HCC are suitable for surgery[1-3]. Transcatheter arterial chemoembolisation (TACE) is one of the modalities used to treat unresectable HCC; however, its low complete tumor necrosis rate results in tumor recurrence and metastasis and influences long-term efficacy[1,3-6]. In addition, the effect of TACE is influenced by tumor size which decreases inactivation ability, especially for HCCs with diameters larger than 5 cm[7,8]. Compared with TACE, the combination of TACE with radiofrequency ablation (RFA) shows enhanced efficacy against HCC and prolonged survival in patients[7-11]. RFA is usually performed 1-4 wk after TACE[3,11-13]. However, the combination of TACE with immediate synchronous RFA for unresectable and large HCCs has not yet been reported. We retrospectively summarized 18 patients treated between January 2010 and June 2012 to assess the technical safety and efficacy of TACE combined with immediate synchronous RFA as a treatment modality for HCC.

MATERIALS AND METHODS

Patients

This retrospective study was approved by the Ethics Committee of the People's Liberation Army (PLA) General Hospital, and all patients signed informed consent forms. A total of 18 patients were admitted to the Department of Interventional Radiology of the PLA General Hospital between January 2010 and June 2012 and were diagnosed with HCC by ultrasound, computed tomography (CT), magnetic resonance imaging (MRI) and α-fetoprotein (AFP) blood test or pathological examination according to the diagnostic criteria for HCC established by the National Association for the Study of Liver Cancer. All patients, including 16 males and 2 females, with an average age of 55 ± 8.0 years (47-63 years) underwent TACE followed immediately by synchronous RFA. The AFP level was higher than 20 ng/mL in 10 cases. On the basis of the Child-Pugh score, 13 cases were classified as Grade A and 5 cases were classified as Grade B.

Patients were allowed to receive the combination therapy if: (1) various imaging examinations (ultrasound, CT, MRI and intra-procedure imaging in TACE) showed one lesion in the liver with a maximum diameter larger than 5 cm and the patients had no surgical indications or refusal to surgery, and (2) the Child-Pugh score was Grade A or B. Patients were excluded from the treatment if they had: (1) cancer embolus in the main portal vein and its left and right portal veins, arteriovenous fistula formation, biliary invasion and extrahepatic metastases and (2) severe coagulation disorders, such as prothrombin activity < 40% and platelet concentration < 30×10^9/L. All the RFA lesions were located 1 cm away from the gall bladder, intestinal canal, bile duct and major blood vessels. All patients underwent regular physical examination and tests (routine blood test, hepatorenal function, electrolytes, blood coagulation and tumor markers), as well as other relevant examinations (liver CT, ultrasound or MRI, lung and brain CT and bone ECT). The maximum diameter of the lesion was determined on enhanced CT or MRI. TACE was performed in these 18 patients one to three times prior to the procedure.

Methods

TACE: All the interventional procedures were performed *via* INNOVA4100 IQ digital subtraction angiography (DSA) (GE Company, United States) by an interventional radiologist with 8-10 years of experience at the Department of Interventional Radiology. After the right femoral artery was punctured by Seldinger's technique, a 4F catheter (RH, Terumo Corporation, Japan) was used for celiac artery and superior mesenteric artery angiography, as well as selective hepatic arteriography if necessary. Chemoembolisation was then conducted with a 3F microcatheter (Progreat, Terumo Corporation, Japan) on the feeding arteries of the tumor. Three to four of the following drugs were administered during the procedure: epirubicin (30-50 mg), cisplatin (40-60 mg) or oxaliplatin (100-150 mg), mitomycin (10-14 mg), 5-FU (500-750 mg), calcium folinate (200-300 mg) and hydroxycamptothecine (10-14 mg). Each drug powder was mixed with lipiodol to form an emulsion and liquid chemotherapy drugs for target vessel perfusion through a microcatheter. When the branch of the portal vein had developed or blood flow had obviously slowed down after chemoembolisation induced by the lipiodol emulsion, gelatine sponge particles were used to perform embolisation. Patients with large lesions or a large number of blood vessels around the tumor were administered the same drugs, but with the addition of 500-700 μmol/L polyvinyl alcohol (Cook Medical, Bloomington, IN, United States). Collateral artery embolisation was conducted if branches such as the phrenic artery and internal thoracic artery were involved in the tumor blood supply.

Immediate synchronous RFA: Percutaneous RFA was immediately performed under general anaesthesia after TACE with the guidance of a C-arm cone beam CT

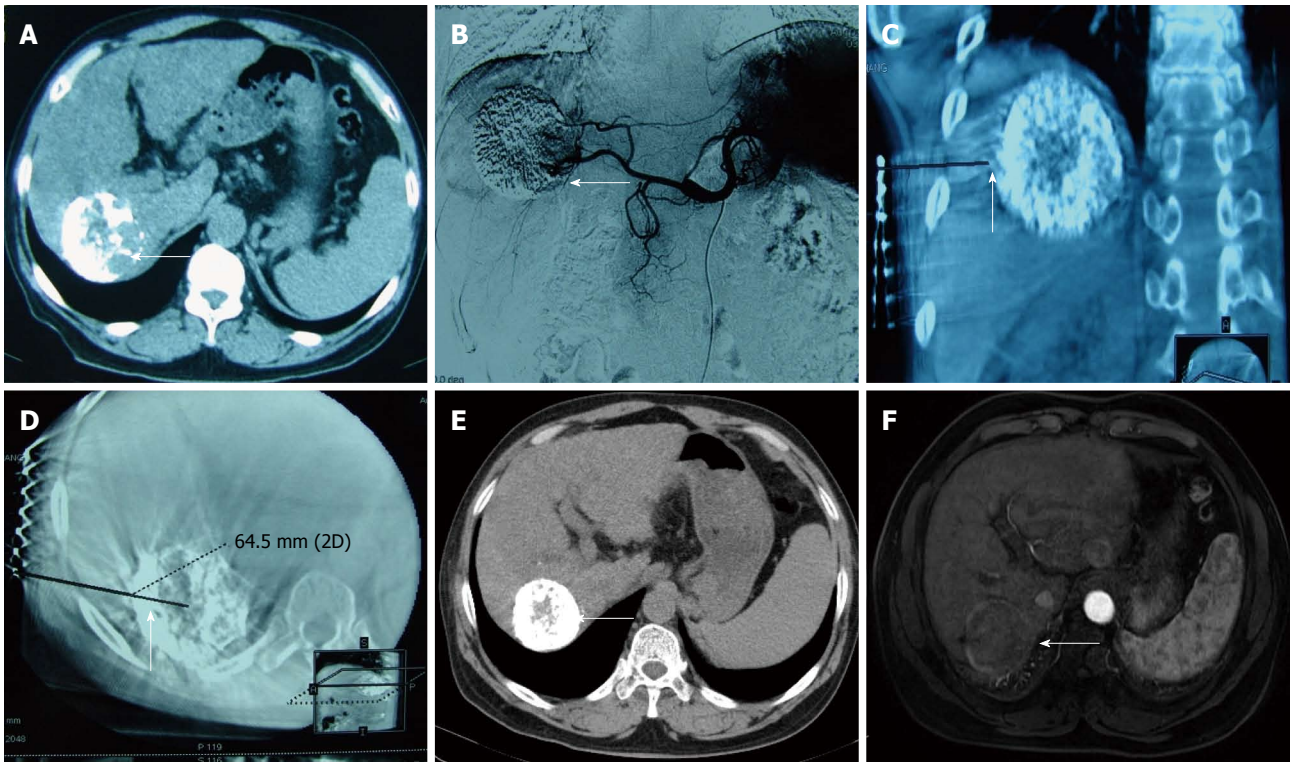

Figure 1 A male patient aged 60 years with hepatocellular carcinoma. Computed tomography (CT) scan showed a residual lesion in the liver after the first interventional therapy. Thus, transcatheter arterial chemoembolisation (TACE) immediately followed by radiofrequency ablation (RFA) was performed under the guidance of digital subtraction angiography (DSA)-CT. A: Liver CT scan showed a partial lipiodol deposit (arrow) after first interventional therapy; B: Angiogram performed before RFA showed a lipiodol deposit in the liver and staining of the delay phase around the lipiodol (arrow); C, D: INNOVA4100 IQ DSA-CT was used to obtain the coronal section and cross section of the reconstructed image to design the puncture path and angle (arrow); E, F: Liver CT scan 23 mo after combination therapy showed good lipiodol deposits without enhancement (arrow).

and DSA. Three-dimensional (3D) CT navigation with INNOVA4100 IQ DSA was used. To establish 3D CT images, the radiofrequency (RF) puncture path and its parameters, 6 × 6 square metal grid lines (diameter: 1 cm) were placed in parallel on the right side of the 8^{th}-10^{th} costal margin or below the xiphoid. Then, a 3D CT scan of the target lesion and image reconstruction (Figure 1) were conducted. The corresponding site was located on the body surface instead of the ribs, and the puncture path was identified through the surface point. The target lesion was determined to avoid important organs, such as the intestinal canal, gallbladder and lung. The puncture path was calibrated to one (*i.e.*, bull's eye configuration), and various parameters, such as the angle of the head, lateral position of the tube ball and needle depth, were determined. The device was then switched on to automatically set the system, which adjusted the tube ball to the correct position. To puncture and localize the target lesion under the perspective, the RF needle was inserted toward the target, and the bull's eye graphic converged on the target. When the puncture needle was in the correct position, the multipolar RF needle was switched on and the tube ball was rotated 70°-90° toward the right and left lateral positions to verify whether the RFA needle was located in the target lesion. For the RFA parameter setting, different RFA needles and RFA parameters were selected according to the tumor position, size and shape. A multipolar RF needle (RITA Company, Cristal Lake, IL, United States) with a maximum ablation diameter of 5 cm and needle length of 15 to 25 cm was used in all cases due to the presence of large HCCs in some patients. The following settings were used: power, 150 to 200 W; ablation time, 6 min (3 cm), 8 min (4 cm) and 15 min (5 cm) and target temperature, 105 ℃. RFA was performed twice as routine and three times if necessary. After ablation, final solidification of the puncture path and inactivation of tumor tissue were conducted to avoid bleeding and tumor implantation metastasis.

Post-procedure treatment and follow-up: Local pressure (RF puncture site and puncture site in right femoral artery) was applied after RFA. To alleviate pain, an analgesic pump was used continuously for 3 d which injected 0.2 mg/kg fentanyl with 10 mg of tropisetron hydrochloride and normal saline at a total volume of 80 mL. Electrocardiographic monitoring was performed for 24 h. The following procedures were also conducted: anti-infection, improvement of damaged liver function, nutritional support and defaecation. About 3 d to 1 wk after the procedure, routine blood examination, hepatorenal function and electrolytes were examined. Exactly 1 mo after the procedure, the patients underwent CT, ultrasound or enhanced MRI scan, and examinations for hepatorenal function and tumor markers (AFP, CA199

and carcinoembryonic antigen). If the tumor was well controlled, subsequent reviews were arranged at 2-3 mo intervals. All the images were analysed by a radiologist with over 8 years of experience. The efficacy of the combination therapy on local tumors was assessed according to the evaluation method recommended by the European Association for the Study of the Liver. Complete response (CR) was defined as the absence of signs of intensified lesions in and around the tumor. Partial response (PR) referred to a minimum of 50% reduction in size of the enhancing tumor. Progressive disease (PD) described the presence of new lesions or at least one lesion with 25% reduction in the size of the enhancing tumor. Stable disease (SD) referred to the presence of a stable lesion between PR and SD. During follow-up, the complete inactivation rate, duration and necrosis characteristics of the local tumor and survival condition of the patients were assessed. The appropriate treatment (combination therapy, close observation or single RFA or TACE) of the patients was determined on the basis of clinical conditions, such as the characteristics of new or residual lesions. Major complications were evaluated on the basis of bleeding and injuries of the intestinal canal, bile duct and gallbladder. Minor complications were assessed using several indicators, including changes in hepatorenal function after combination therapy, and changes in syndrome symptoms after embolisation, such as pain.

Statistical analysis

Quantitative data were analysed using CHISS2004 software. The *t* test was employed to compare liver function (Child-Pugh Grade) before and after intervention.

RESULTS

Technical evaluation and clinical efficacy

All patients were tolerant of the concurrent combination therapy. A total of 18 lesions were confirmed by previous images and successfully labelled with lipiodol deposit in TACE. One-off RFA puncture was successfully conducted with two to three RFA needles per lesion (Figures 2, 3). The success rate of the combination therapy was 100%. For 9 cases with lesions near the diaphragm, the puncture avoided normal lung tissue under the perspective and entered the lesions for RFA treatment.

One month after the intervention, all 18 patients underwent routine imaging (ultrasound, enhanced CT or MRI of liver) and AFP examination. Local lesions were mainly characterized by coagulative necrosis without liquefactive necrosis. Increased AFP was discovered in 10 patients before intervention, which significantly decreased 1 mo after surgery. CR was observed in 17 cases, and PR was observed in 1 case. During follow-up, the mean recurrence-free survival time of the 18 cases was 16.8 ± 4.0 mo (2-29 mo). The estimated overall survival rate at 6, 12 and 18 mo was 100%.

Complications after intervention

No TACE and percutaneous RFA-related complications, such as bleeding or necrosis of the gallbladder, bile duct, intestinal canal, pneumothorax or hepatapostema or liver/kidney failure, occurred after the intervention. Pain was completely alleviated by injection of the fentanyl mixture in 16 patients using the analgesic pump, and by 3-4 mg of morphine once every 24 h in 3 patients in addition to the analgesic pump. All patients received oral central analgesics (such as oxycodone hydrochloride) 4 d after treatment to relieve pain. Different degrees of constipation, low fever (37.3-38.4 ℃) and nausea and vomiting were observed in 5, 7 and 5 cases, respectively, and relieved with medication. The ALT (200.4 ± 63.4 U/L) and AST (228.1 U/L ± 25.4 U/L) levels in all patients transiently increased ($P = 0.00 < 0.05$) on the third day after treatment relative to levels before surgery (24.7 ± 9.3 and 32.7 ± 6.8 U/L, respectively). ALT and AST levels decreased to 29.8 ± 11.5 U/L and 36.8 ± 10.2 U/L ($P = 0.15$, $P = 0.16 > 0.05$), respectively, on the 7th day after treatment. No statistical differences in bilirubin and albumin were found before and after treatment.

DISCUSSION

Large solitary HCCs are a special type of liver cancer, the prognosis of which is better than that of the multinodular type after complete inactivation by chemoembolisation or resection[14-17]. Although surgery is still the primary treatment mode for HCC, it has several disadvantages resulting from the large size or location of the carcinoma, such as difficulty in complete resection, heavy bleeding, high incidence of complications and high recurrence rates after surgery[16,17]. Aside from surgery, many other non-surgical treatments such as TACE and RFA are used to cure solitary HCCs. However, TACE or RAF can only inactivate local carcinomas with diameters smaller than a specific value. For instance, TACE treatment requires repetition and a large dose of lipiodol, involves a high likelihood of collateral formation after multiple embolisations and has low inactivation rates after the procedure and adverse effects on long-term liver function and prognosis[17]. In our study, all the patients underwent one to three cycles of TACE before the combination therapy and were reviewed for the presence of residual or new tumors. The results indicate that a single technique cannot completely inactivate HCC and that recurrence rate of the cancer was high after the procedure.

The combination of TACE and RFA is one of the major treatments used to enhance the inactivation rate of local tumors, decreasing short- or long-term recurrence rates and extending patient survival[3-12,17-20]. However, the current combination therapy is mainly performed separately or several times and the local recurrence rate increases as tumor diameter increases[21,22]. The interval between TACE and RFA is longer than 1 or 4 wk, during which recanalization after embolisation, collateral formation and elimination of lipiodol-chemotherapeutant may occur. Therefore, this method is not strictly concurrent, and the effects of TACE and RFA are not fully synergistic.

To increase local tumor inactivation rates, prelimi-

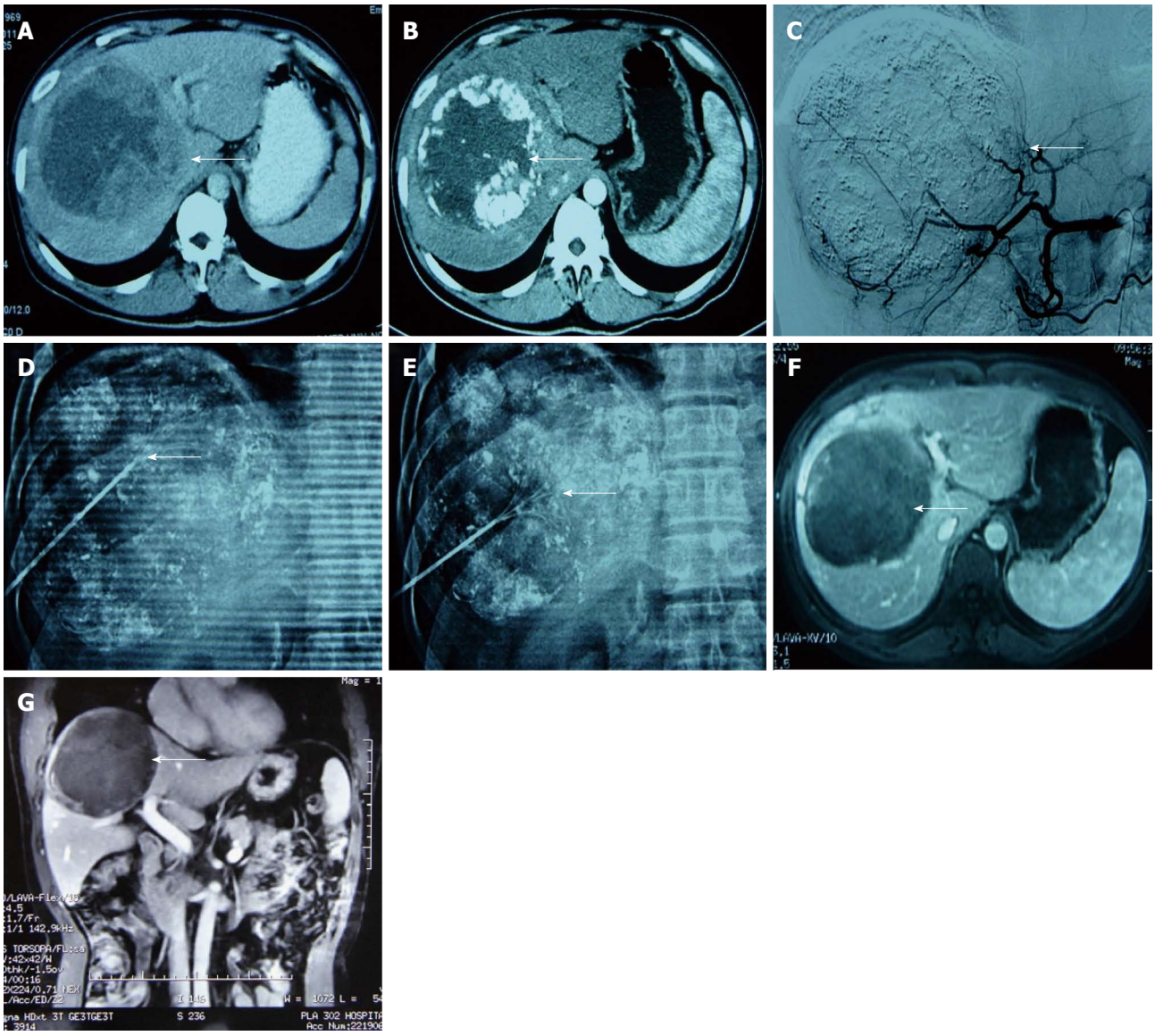

Figure 2 A male patient aged 42 years with poorly differentiated hepatocellular carcinoma. Computed tomography (CT) scan showed a residual lesion in the liver after first interventional therapy with α-fetoprotein (345 μg/mL). Thus, transcatheter arterial chemoembolisation combined with radiofrequency ablation (RFA) was performed. A: CT scan showed a large solitary tumor in the right hepatic lobe before interventional therapy (arrow); B: A lipiodol deposit appeared around the liver tumour after the first procedure. CT revealed an enhanced residual lesion in the artery phase (arrow); C: Hepatic artery angiogram before RFA showed staining of the residual lesion around the lipiodol in the liver (arrow); D, E: A multipolar probe with a maximum extended diameter of 5 cm, which could cover the residual lesion, was designed to perform RFA in the region labelled by lipiodol and the original lesion (lipiodol deposition area). A diaphragmatic dome was involved, and the puncture tunnel detoured the lung tissue under the fluoroscope (arrow); F, G: The hepatic lesion was well controlled, and no recurrence was found after 15 mo follow-up (arrow).

nary studies on the safety and effectiveness of TACE combined with immediate RFA in the treatment of liver tumors (diameter, ≤ 5 cm) have been conducted. Gadaleta *et al*[23] used immediate combination therapy to treat HCC and liver metastatic tumors of different sizes and achieved a success rate of 100% with an 88% CR. Kang *et al*[18] treated HCC (diameter, ≤ 5 cm) by TACE combined with immediate RFA. Approximately 1 mo later, the complete necrosis rate of tumors successfully labelled by TACE was 100%, and the cumulative incidence of local tumor progression at 1 year and 3 years was 1.8% and 9.4%. These results demonstrate the positive effects of immediate combination therapy. We studied the application of immediate combination therapy for large solitary HCCs (diameter, > 5 cm) and showed the favorable clinical efficacy of this method. The clinical efficiency was 100% in 18 patients, with 17 cases of CR and 1 case of PR. Compared with single TACE, combination therapy increased the local inactivation rate and prolonged recurrence-free survival. During follow-up, the estimated overall survival rate at 6, 12 and 18 mo was 100%. Based on the literature and clinical practice, as well as comparisons with conventional sequential therapy, the advantages of immediate combination therapy are as follows: (1) Single TACE or RFA cannot completely inactivate the tumor, especially the tissue on the tumor border, resulting in recurrence. In immediate combination therapy, lipiodol precipitation in the lesion wraps around

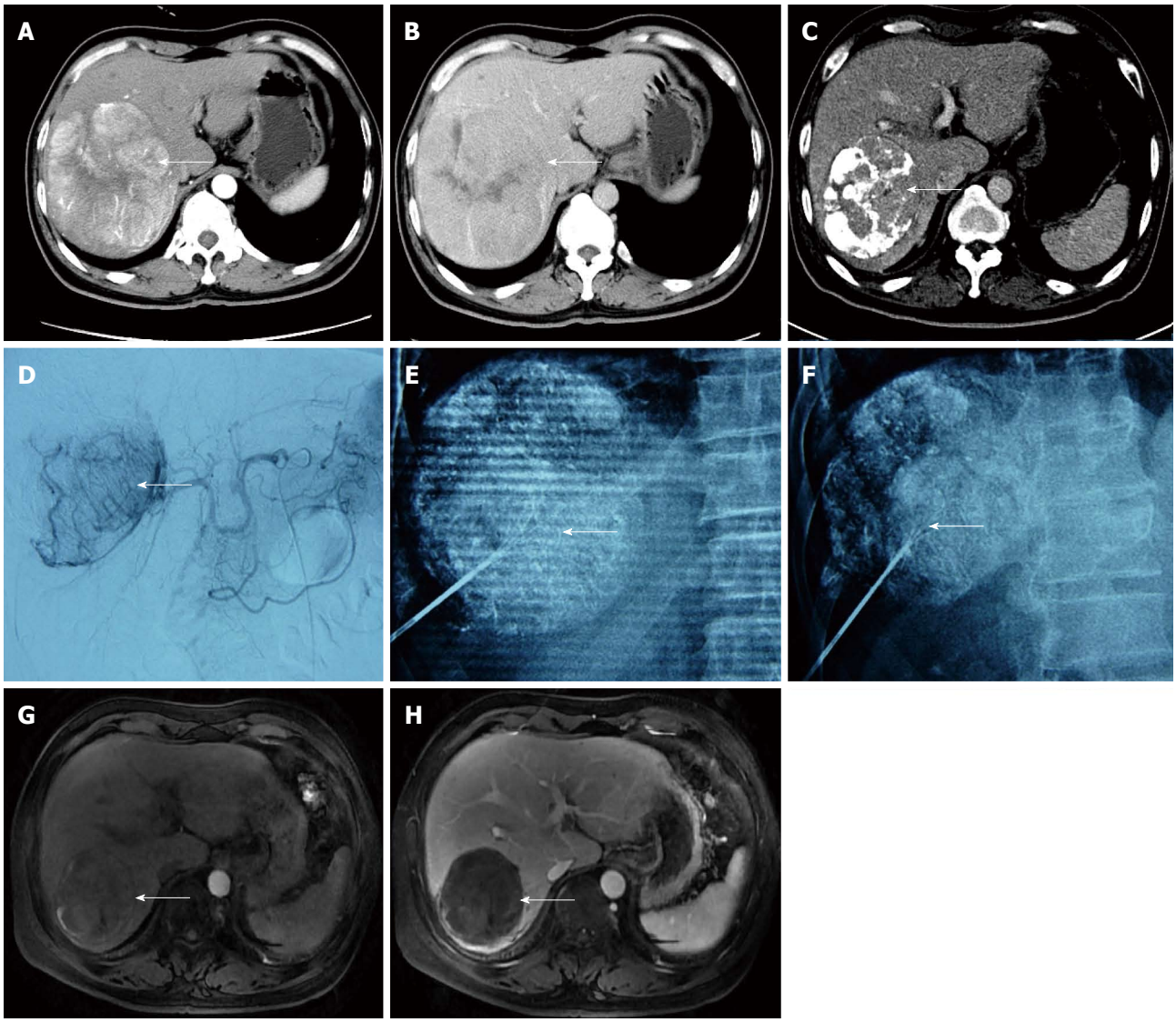

Figure 3 A male patient aged 53 years with a large lesion in the right lobe of the liver. A residual lesion was found after three cycles of transcatheter arterial chemoembolisation (TACE). TACE combined with radiofrequency ablation (RFA) was performed under the guidance of digital subtraction angiography-computed tomography. A, B: A large hypervascular hepatocellular carcinomas was observed in the right lobe (arrow); C, D: A residual lesion around the lipiodol deposit was still visible after three cycles of TACE (arrow); E, F: The lesion was successfully labelled after TACE. A multipolar needle was opened and rotated 70° to the left (E) and right (F) sides to verify whether the RF needle was in the center of the residual lesion (arrow); G, H: The large lesion was well controlled, and no recurrence was observed by magnetic resonance imaging during 13 mo follow-up after combination therapy (arrow).

and inactivates the surrounding tissue of the tumor, thereby preventing recurrence from residual tumors[3,24]; (2) Immediate combination therapy also fully enhances the synergistic effects of chemotherapeutics and thermal ablation. In TACE, lipiodol, which is the carrier of chemotherapeutics, is uncontrolled and unstable. Lipiodol cannot be released slowly with sustained high concentrations. Chemotherapeutics in lipiodol are eliminated over time if no other embolisation agents (such as sponge) are added to the drug. However, in TACE with immediate RFA, chemotherapeutics, such as adriamycin, can inhibit tumors due to their high accumulative concentration in and around the lesions[25,26]; and (3) TACE accurately locates and labels new and residual lesions, as well as lesions that cannot be observed by conventional CT and B ultrasound, through lipiodol precipitation. Thus, RFA targets are more specific. The labelling of lesions by lipiodol precipitation is more effective, especially in lesions that have become relatively complicated after several cycles of TACE because previous necrotic and new or residual lesions are labelled[27,28].

TACE combined with RFA is a safe treatment with 4%-6% incidence of various complications; severe complications are rare[3,18,23,28]. Kang *et al*[18] reported two cases of serious liver damage and one case of hemorrhage from a ruptured hepatic artery in combination therapy. Only one case of hepatic function damage without other complications, such as severe liver failure, was found in our series; this complication may have resulted from the application of a super-selective embolisation technique and bypassing of normal liver tissue by the RFA needle. Most patients only experienced post-embolisation syn-

drome (pain, low fever, *etc.*), transient liver dysfunction and constipation after surgery, all of which were relieved with medication. Constipation may be associated with the continuous application of analgesics, and low fever may be related to absorption after tumor necrosis. Transient liver dysfunction is related to combination therapy. Given that concurrent TACE with RFA increases pain in patients, RFA was performed immediately after TACE under general anaesthesia and tracheal intubation. An analgesic pump was utilized for 3 d to relieve pain. However, three patients still experienced intense local pain, which was alleviated by the addition of analgesics. Therefore, instead of the conventional combination of TACE and RFA, we recommend the application of an analgesic pump for 3 d after immediate combination therapy. Compared with the liquefactive necrosis induced by single TACE or RFA, the tumors were characterized by coagulative necrosis, which has a lower risk for local secondary hepatapostema[5].

Our study has several limitations: (1) The small sample size in this retrospective research may have influenced the results to some extent; and (2) the follow-up period was too short to accurately evaluate long-term efficacy. More patients should be included in comparative studies and further assessment of the advantages of combination therapy.

In conclusion, TACE immediately followed by RFA is a safe and effective treatment for large HCCs. To prevent pain after the procedure, an analgesic pump should be used for 3 d. The long-term efficacy of this combination therapy requires further assessment.

COMMENTS

Background

Transcatheter arterial chemoembolisation (TACE) is the main treatment method for unresectable primary hepatocellular carcinomas (HCC). However, simple TACE has low tumor inactivation and high recurrence rates. In addition, with increasing tumor size, the tumor inactivation rate significantly decreases.

Research frontiers

Combination therapy [mainly TACE combined with radiofrequency ablation (RFA)] is one of the main modalities for treating unresectable HCC. However, RFA is often performed 1 to 2 wk after TACE, which considerably reduces the synergistic effects of the combination therapy, especially for large HCCs.

Innovations and breakthroughs

The combination of TACE with immediate RFA under digital subtraction angiography-computed tomography (CT) guidance is applied to treat large single HCCs. With this technique, different treatment technologies are fused into one angiographic machine, which improves their synergistic effects (*e.g.*, heat treatment with epirubicin and embolisation with thermal ablation), thereby enhancing the inactivation rate of large HCCs and reducing the radiation dose applied to patients and the risk of repeated treatment. Preliminary results show that combination therapy has obvious advantages and can help improve the long-term survival of patients. This technique also has significance in the treatment of other metastatic and hypervascular HCCs.

Applications

TACE immediately followed by RFA is a safe and effective treatment for large HCCs. This technology can improve the synergistic treatment effects of TACE and RFA, as well as reduce the need for repeated treatments and amount of radiation exposure. Furthermore, different treatment technologies are fused into one machine, thereby simplifying the operational process. TACE immediately followed by RFA enhances tumor inactivation ability, decreases recurrence rates, prolongs patient survival time and improves prognosis.

Terminology

TACE: Transcatheter arterial chemoembolisation with lipiodol and chemical drugs. Immediate RFA: RFA procedure is performed immediately after TACE. A three-dimensional CT image, radiofrequency puncture path and parameters are first established to target the lesion and avoid non-target lesions. This combination therapeutic modality requires general anesthesia. Post-procedure pain management is required for 2 to 3 d. The combined therapeutic modality can be used for HCCs and single hypervascular metastatic tumors.

Peer review

In this manuscript, the authors summarised 18 patients treated between January 2010 and June 2012 to assess the technical safety and efficacy of TACE combined with immediate synchronous RFA as a means of treating HCC. The manuscript is very interesting. It is a very good study about the technical safety and efficacy of the combined therapy for large hepatocellular carcinomas.

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BRIEF ARTICLE

Metabonomic studies of pancreatic cancer response to radiotherapy in a mouse xenograft model using magnetic resonance spectroscopy and principal components analysis

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Abstract

AIM: To investigate the metabolic profiles of xenograft pancreatic cancer before and after radiotherapy by high-resolution magic angle spinning proton magnetic resonance spectroscopy (HRMAS ^{1}H NMR) combined with principal components analysis (PCA) and evaluate the radiotherapeutic effect.

METHODS: The nude mouse xenograft model of human pancreatic cancer was established by injecting human pancreatic cancer cell SW1990 subcutaneously into the nude mice. When the tumors volume reached 800 mm^3, the mice received various radiation doses. Two weeks later, tumor tissue sections were prepared for running the NMR measurements. ^{1}H NMR and PCA were used to determine the changes in the metabolic profiles of tumor tissues after radiotherapy. Metabolic profiles of normal pancreas, pancreatic tumor tissues, and radiation- treated pancreatic tumor tissues were compared.

RESULTS: Compared with ^{1}H NMR spectra of the normal nude mouse pancreas, the levels of choline, taurine, alanine, isoleucine, leucine, valine, lactate, and glutamic acid of the pancreatic cancer group were increased, whereas an opposite trend for phosphocholine, glycerophosphocholine, and betaine was observed. The ratio of phosphocholine to creatine, and glycerophosphocholine to creatine showed noticeable decrease in the pancreatic cancer group. After further evaluation of the tissue metabolic profile after treatment with three different radiation doses, no significant change in metabolites was observed in the ^{1}H NMR spectra, while the inhibition of tumor growth was in proportion to the radiation doses. However, PCA results showed that the levels of choline and betaine were decreased with the increased radiation dose, and conversely, the level of acetic acid was dramatically increased.

CONCLUSION: The combined methods were demonstrated to have the potential for allowing early diagnosis and assessment of pancreatic cancer response to radiotherapy.

Key words: High-resolution magic angle spinning proton magnetic resonance spectroscopy; Principal components analysis; Pancreatic cancer; Radiotherapy

Core tip: In the present study, for the first time to our knowledge, high-resolution magic angle spinning proton magnetic resonance spectroscopy and principal components analysis were combined to highlight metabolite profiles of pancreatic cancer after radiotherapy,

by analyzing the correlation between radiotherapy effect and metabolic change, and optimizing the therapeutic scheme. The results showed that metabolic profile changes of pancreatic cancer after radiotherapy were closely correlated with therapeutic effect. The outcome of the study is both interesting and beneficial to pathological research, early diagnosis, and therapy evaluation of pancreatic diseases.

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INTRODUCTION

Pancreatic cancer is a malignant tumor with very poor prognosis, and surgery has been considered as the only radical therapy. However, about 85% of newly diagnosed cases have developed distant metastasis, and only 5%-25% of pancreatic head cancer and less than 10% of pancreatic body cancer can be treated with surgical excision, and the postoperative recurrence rate is high. Therefore, radiation therapy has become the predominant treatment method for locally advanced pancreatic cancer[1-3]. Therapeutic evaluations of radiotherapy are mainly: remission from the symptoms of pain and jaundice, solid tumor size and its survival time, and the lack of a specific targeted method. During the last three decades, there has been ongoing magnetic resonance spectroscopy (MRS) research in malignant diseases. These studies provided valuable data on the biochemistry and metabolism of tumors, and on the effects of nutrients, hormones, and growth factors[4,5]. The mechanisms of action of anticancer drugs and the acquired resistance to these agents were delineated[6,7]. MRS was also used for monitoring the response to therapy[8,9].

High-resolution magic angle spinning proton magnetic resonance spectroscopy (HRMAS ^{1}H NMR) is a well-recognized technique in metabonomics studies *in vitro*, by which biopsy or postmortem samples of intact tissues are spun at the magic angle, resulting in a significant improvement in the resolution of the spectrum obtained for some of the line-broadening factors, such as dipole–dipole interactions and chemical shift anisotropy, and magnetic field inhomogeneities are averaged out[10,11]. This approach requires minimal sample preparation and, unlike convenient ^{1}H NMR spectroscopy of tissue extracts, both aqueous and lipid-soluble metabolites can be observed simultaneously *in situ*. In addition, information about the metabolic environment of the tumor can also be obtained. Therefore, HRMAS ^{1}H NMR has proved to be an efficient method for studying a wide variety of cancers, including breast cancer[12], cervical cancer[13], kidney cancer[14], prostate cancer[15], malignant lymph nodes[16], and liposarcoma[17] of animals and humans. However, so far, there are very few metabonomic studies in cancer therapeutics by the application of HRMAS ^{1}H NMR.

HRMAS ^{1}H NMR spectra obtained from tissues reflect the dynamic biological systems and processes that contribute to the overall metabolic status of an organism. It is not possible to isolate the effects of any single metabolite signal in a spectrum and, furthermore, the manual analysis of even a small number of such spectra is a laborious and complex task. Therefore, metabonomists utilize data reduction and multivariate analysis techniques, such as principal components analysis (PCA), to facilitate automated NMR pattern recognition[18,19]. Moreover, our previous study demonstrated that using ^{1}H NMR and PCA could discriminate pancreatic cancer from chronic pancreatitis accurately[20]. In the present study, HRMAS ^{1}H NMR and PCA were combined to highlight metabolite profiles of pancreatic cancer after radiotherapy, in order to analyze the correlation between radiotherapy effects and metabolic changes, and to optimize the therapeutic scheme. The study has an important implication for reference guides on therapeutic evaluation by nuclear magnetic resonance spectroscopy on pancreatic cancer *in vivo*.

MATERIALS AND METHODS

Animals and experiment schedule

Six- to eight-week-old female nude mice were obtained from the Planned Parenthood Research Institute, Shanghai, People's Republic of China. All animals in this study were housed under pathogen-free conditions and maintained in accordance with the guidelines of the Committee on Animals of the Second Military Medical University, Shanghai, China. Human pancreatic cancer cell line SW1990 in mid-log-growth phase was harvested by trypsinization. Single-cell suspensions (5×10^{6} cells in 0.1 mL HBSS) were injected subcutaneously into the nude mice. The tumors were measured every 4 d with a caliper, and the diameters were recorded. Tumor volume was calculated by the formula: $a^{2}b/2$, where a and b are the two maximum diameters. When tumors reached 2.0 cm × 2.0 cm, the duration of survival was recorded and the mouse euthanized.

For the radiotherapy experiment, when the tumor volume reached 800 mm^{3}, the mice were divided into four groups. Group A mice were used as untreated controls. Groups B, C, and D received 10, 20, and 30 Gy radiation doses, respectively. Tumor size was measured as described above. Two weeks later, tumor tissue sections were prepared for histological tests or for running the NMR measurements.

NMR spectroscopy

HRMAS ^{1}H NMR experiments were carried out using a DRX-500 spectrometer (1H frequency at 500.13 MHz;

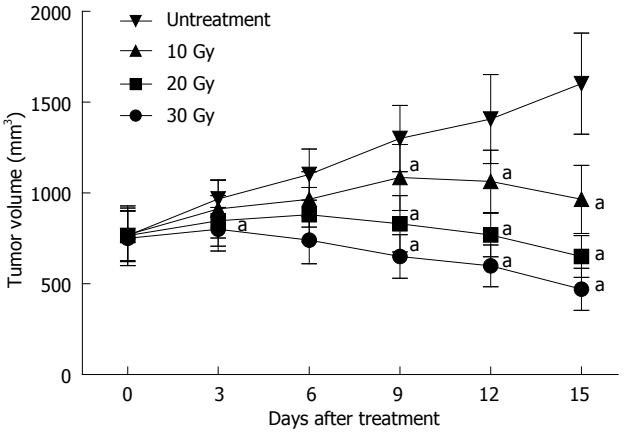

Figure 1 Effect of radiotherapy on the growth of human pancreatic tumor in nude mouse. Mice received a subcutaneous injection of SW1990 cells. When the tumor volume reached about 800 mm^3, the mice were divided into four groups. Group A mice were used as untreated controls. Groups B, C, and D received 10, 20, and 30 Gy radiation doses, respectively. Tumor size was measured for two weeks. $^{a}P < 0.05$ *vs* the untreated group.

Bruker Biospin, Rheinstetten, Germany). Tissue samples were rinsed three times with D_2O and placed into a 4-mm zirconium oxide MAS rotor with drops of D_2O (deuterium lock reference). Spectra were acquired at 300.0 K using single-pulse and CPMG pulse sequences, both with water presaturation during the relaxation delay of 2 s. CPMG pulse sequence was applied as a T2 filter to suppress signals from the molecules with short T2 values (such as macromolecules and lipids) using a total TE of 320 ms. The main parameters used for ^{1}H NMR spectra were: SW = 15 kHz; TD = 64 k; NS = 256; and MAS rate = 5 kHz. Spectral assignments were confirmed by 2-dimensional ^{1}H-^{1}H TOCSY and ^{1}H-^{1}H COSY (data not shown), together with values obtained from the literature[10,21].

The stability of tissue samples was evaluated by repeating a 1-dimensional NMR experiment after overall acquisition. No biochemical degradation was observed for any of the tissue samples.

Principal components analysis

Spectral data were phased and baseline-corrected using XWINNMR (Bruker Biospin). All FID were multiplied by an exponential function equivalent to a 0.3-Hz line broadening factor prior to Fourier transformation. Each HRMAS ^{1}H NMR spectrum was segmented into 211 regions of equal width (0.04 ppm) over the region 0.00-10.00, and the signal intensity in each region was integrated using AMIX version 3.6 (Bruker, Biospin). The region 4.50-5.00 was removed to eliminate baseline effects of imperfect water saturation. Prior to PCA, each integral region was normalized by dividing by the sum of all integral regions for each spectrum[12,14]. In order to exclude the effects of lipids and concentrate on the impacts of LMW metabolites in the CCM region, PCA was again done for ^{1}H CPMG NMR spectra over the range 0.7-4.70, each 0.04 ppm wide. PCA was used to calculate a new, smaller set of orthogonal variables from linear combinations of the intensity variables, while retaining the maximum variability present within the data. These new variables are the derived principal components, and the distribution of their values (scores) permits the simple visualization of separation or clustering between samples. The weightings (loadings) given to each integral region in calculating the principal components allows for the identification of those spectral regions of greatest influence to the separation and clustering and, hence, the deduction of biomarkers of pancreatic cancer.

Statistical analysis

Continuous variables are expressed as mean ± SD. Statistical analysis of data was done by Student's *t* test using SigmaPlot software. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Radiotherapy of human pancreatic tumor-bearing nude mouse

One week after SW1990 tumor cell inoculation, tumor size was measured and tumor volume recorded weekly. All 32 nude mouse models generated tumor tissues, and the success rate of model construction was 100% (32/32). Tumor volume in the control group (untreated), and the three groups which were given 10, 20, and 30 Gy radiation are shown in Figure 1. The transplanted tumor volume before treatment was 0.8 cm^3 on average, increasing with breeding period in the control group. Compared with the control group, the tumor volume of the treatment groups reduced significantly, with the most obvious being the 30 Gy dose treatment group. These data showed that radiotherapy could effectively suppress the growth of pancreatic cancer in the nude mice. The changes in the morphological levels are expected to be accompanied with observable changes in the tissue biochemical composition which can be accessed with HRMAS ^{1}H NMR spectroscopy *ex vivo*.

Metabolic profiles of normal pancreas and pancreatic tumor tissues

Using ^{1}H NMR spectroscopy, components such as Cho, taurine (Tau), betaine (Bet), glutamic acid (Glu), glycerophosphocholine, and choline phosphate (GPC + PC), acetic acid (Ace), alanine (Ala), and lactic acid (Lac) were detected and identified in the normal pancreas and isolated transplanted tumor tissues in the nude mouse by their spectrum peaks. The literature was referred to before (18-20) and 2-D spectrum estimation (J-res, COSY, TOCSY) (Figure 2A). Score plots of PCA based on ^{1}H NMR spectra were performed on 8 normal and 8 tumor samples, in which the spectra region was δ = 0.70-4.70, and the minimal region δ = 0.04 (Figure 2B). As shown in the loading plots, the main factors that differentiated the samples were δ 0.90-0.86, δ 1.34-1.26, δ 1.50-1.46, δ 3.30-3.18, δ 3.46-3.38, and δ 3.70-3.66, which were con-

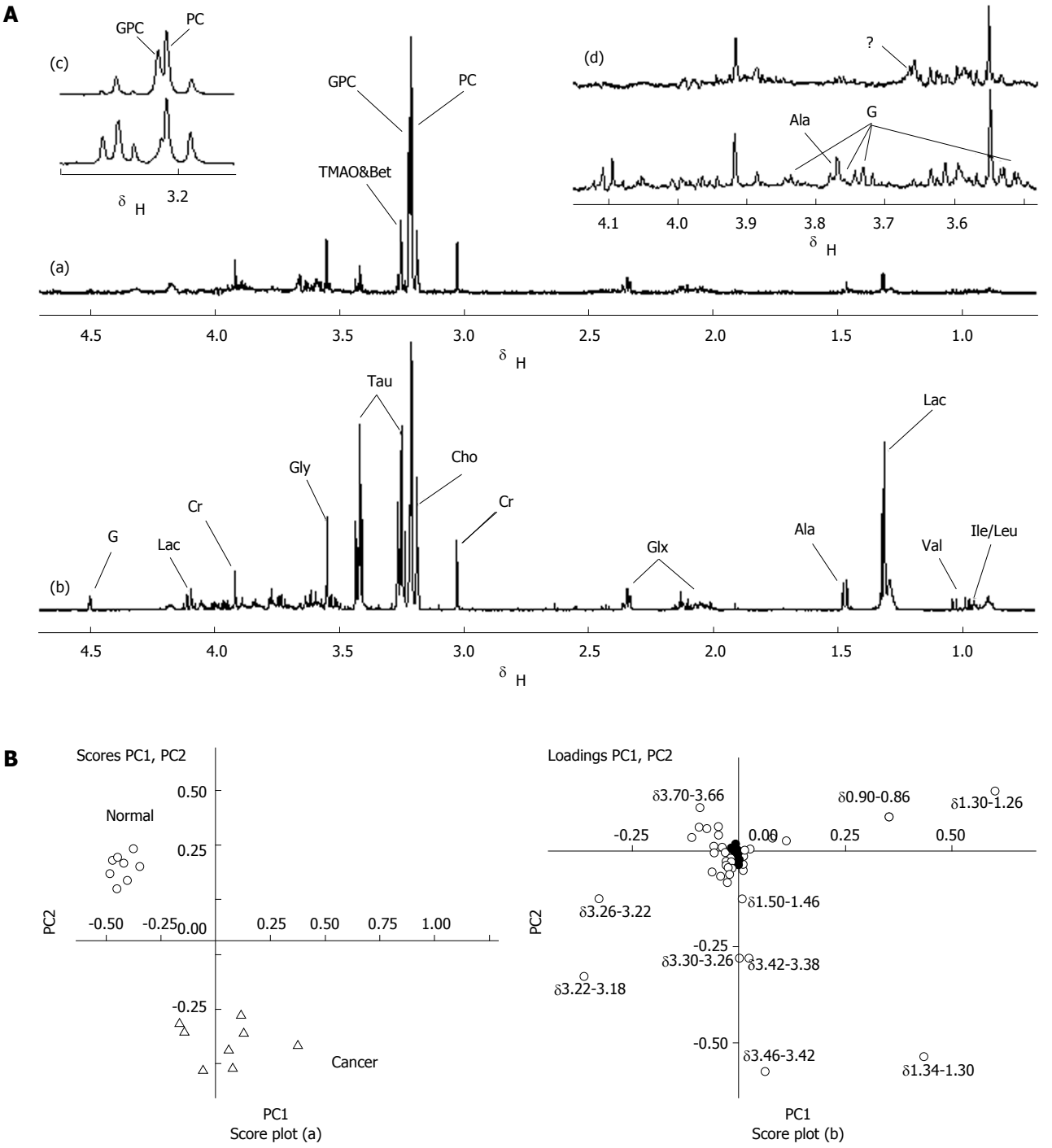

Figure 2 High-resolution magic angle spinning proton magnetic resonance spectroscopy spectra of normal pancreas and transplanted pancreatic tumor (500 mHz). A: Normal pancreas (a); Transplanted pancreatic tumor (b); Amplified data from spectra region δ3.30-3.15 (c); Amplified data from spectra region δ3.15-3.48 (d). For peak assignments, see list of abbreviations used; B: Principal Component Analysis to compare the metabolic profiles between normal pancreas and pancreatic cancer based on the high-resolution magic angle spinning proton magnetic resonance spectroscopy spectra. Panels (a) and (b) are score and loading plots. ❍: Normal pancreas; △: Pancreatic cancer.

sistent with what was observed in Figure 2A, corresponding to the residual lipid, Lac, Ala, Cho compound, Tau, and unknown chemicals.

As is well-known, absolute concentration quantification for metabolites is difficult in HRMAS spectroscopy, and the metabolite ratios are commonly used for statistical analysis. Table 1 shows the relative signal integrals and signal ratios for some metabolites that contributed to the classification of normal pancreas and pancreatic tumor tissues discussed in the above sections. Compared to the normal pancreas, concentrations of Ileu, Leu, Val, Lac, Ala, Glu, Tau, Cho, and some carbohydrates (G, contained galactose β-H possibly due to characteristic twin peak at δ 4.52) increased relatively in the pancreatic tumor samples, while GPC + PC, Bet, GPC/Cre, and unknown chemicals at δ 3.66 decreased relatively. The level

Table 1 Relative integrals and their ratios from some selected metabolites contributing to the classification of normal pancreas and pancreatic tumor tissues

		Normal pancreas	Pancreatic tumor	*P*-value
Metabolites	Choline	2.75 ± 1.37	3.99 ± 0.35	0.0376
	Taurine	1.99 ± 0.55	13.63 ± 2.92	0.0001
	Betaine	2.91 ± 0.57	1.58 ± 0.47	0.0002
	Glutamic acid	0.29 ± 0.11	0.46 ± 0.13	0.0260
	Alanine	0.60 ± 0.14	1.93 ± 0.16	0.0001
	Lactate	1.93 ± 0.86	8.30 ± 1.02	0.0001
	Acetic acid	0.06 ± 0.10	0.06 ± 0.03	0.7942
	Glycerophosphocholine+ phosphocholine	19.47 ± 1.36	16.61 ± 1.31	0.0007
Metabolites ratio	Glycerophosphocholine/ Creatine	3.51 ± 0.76	2.35 ± 0.58	0.0042
	Phosphocholine/ Creatine	5.19 ± 0.96	6.22 ± 1.52	0.1284

of Ace and PC/Cre showed no significant change.

Metabolic profiles of pancreatic tumor tissues after radiotherapy

The metabolic profiles of tumor tissues after radiotherapy were also detected by ^{1}H NMR. As shown in Figure 3A, no significant metabolic changes were observed in the ^{1}H NMR spectrum. PCA analysis was further conducted on samples in each group, with the spectrum integration region δ = 0.70-4.70, and the minimal region δ = 0.04 (Figure 3B). In score plots, most of samples in the control group concentrate in the upper left, but overlap partly with samples in the 10 Gy radiation dose group. A partial overlap is shown between the 10, 20, and 30 Gy radiation dose groups, but overall it seems that the three groups have a left, upper, and lower distribution trend in terms of scores. Loading plots showed the changes of Cho-containing compounds, along with Ace and Bet content among the three dose groups.

Table 2 shows the relative signal integrals and signal ratios for some metabolites that contributed to the evaluation of pancreatic tumor tissues response radiotherapy. Cho content showed a significant difference between the control and 30 Gy dose groups, as well as the 10 and 30 Gy dose groups. The Cho content decreased with an increase of radiation dosage. Bet content also decreased with an increase of radiation dosage. In contrast, Ace content showed a positive relationship with the radiation dosage.

DISCUSSION

Although HRMAS ^{1}H NMR combined with PCA has been demonstrated as an efficient method for studying a wide variety of animal and human cancers[12-17], this combined method has not been reported to analyze the metabolic features of cancer response to therapy. Here, for the first time to our knowledge, our findings demonstrate that applying this combined method has the potential for clinical assessment of the pancreatic cancer radiotherapeutic response.

Kaplan *et al*[22] conducted ^{1}H NMR analysis on perchlorate extract (water-soluble) of heterotopic transplanted pancreatic cancer tissue in nude mice. Compared with the normal pancreas of nude mice, Tau and Lac content in transplanted tumors increased, GPC content decreased, and there was little change in Cho and PC. However, in previous studies, some important information may be missed, and human factors introduced as a destructive process in extraction will lead to a negative impact on the results, along with poor experimental repeatability results from different pH values. Therefore, in this study, ^{1}H NMR combined with PCA was applied to the metabolic study on transplanted tumor tissues in a human pancreatic tumor-bearing nude mouse model. This has avoided the error factor involved in complex processes such as tissue extraction. Moreover, due to the application of the 500 mHz high-field strength NMR instrument, the spectrum resolution obtained is significantly higher than that reported in the literature, with more metabolites being found and variation characteristics of metabolites embodied more clearly. Consequently, not only did the accuracy of spectrum peak identification improve, but statistical analysis errors were also reduced. In this study using ^{1}H NMR combined with PCA, pancreatic cancer was shown to have higher Tau, Ileu, Leu, Val, Lac, Ala, Glu, and Cho levels relative to normal pancreas, while GPC + PC, and Bet and GPC/Cre levels decreased relatively. Compared to the other metabolites, Tau, Lac, and Ala had the most noticeable differences between normal pancreas and pancreatic cancer. Ace and PC/Cre showed no significant difference between normal and pathological conditions. The results suggest that these changes in the metabolite profile might be used as metabolic markers for the early diagnosis of pancreatic cancer.

Radiotherapy is a local treatment, and its ultimate goal is to eradicate tumor cells thoroughly, while protecting normal tissues and vital organs as much as possible[23]. The application of computer tomography simulations and the three-dimensional conformal technique in radiotherapy has boosted the pancreatic target dosage and offered better protection for the gastrointestinal tract. Currently, therapeutic evaluations of radiotherapy are mainly: remission from the symptoms of pain and jaundice, solid tumor size and its survival time, and the lack of a specific targeted method[23]. By imaging examination, tumor size and contrast agent enhancement were observed to determine tumor activity, and indirectly determine therapy efficacy, although lacking strong direct evidence[24-27]. In this study, we use ^{1}H NMR and PCA to compare pancreatic cancer metabolic variation characteristics before and after radiotherapy. Although no significant metabolic changes were observed in the ^{1}H NMR spectra, PCA results showed a trend of certain changes among different dosage groups. We found that the Ace level was increased, which positively correlated with the radiation dose. In contrast, Cho and Bet levels were decreased, which in-

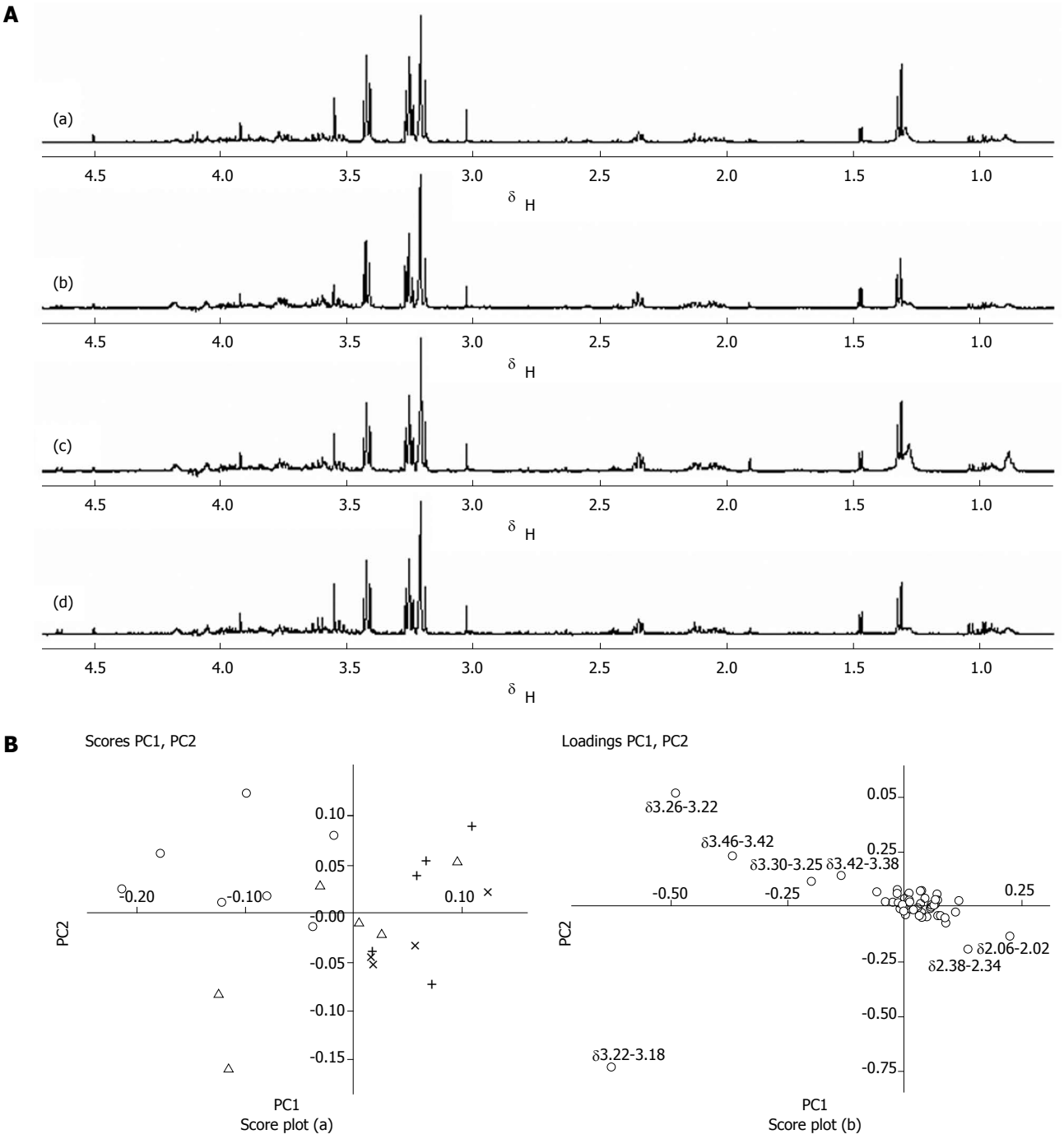

Figure 3 High-resolution magic angle spinning proton magnetic resonance spectroscopy spectra of transplanted pancreatic tumor after radiotherapy (500 mHz). A: Untreated group(a); 10 Gy treatment group (b); 20 Gy treatment group (c); 30 Gy treatment group (d); B: Principal component analysis to compare the metabolic profiles of the pancreatic tumor after radiotherapy based on the high-resolution magic angle spinning proton magnetic resonance spectroscopy spectra. Panels (a) and (b) are scores and loadings plots. ❍: Untreated group; Δ: 10 Gy treatment group; ×: 20 Gy treatment group; +: 30 Gy treatment group.

versely correlated with the radiation dose. Additionally, other metabolites, including Tau, Ileu, Leu, Val, Lac, Ala, Glu, and GPC + PC showed no significant change after radiotherapy. Thus, these data suggest that the changes in these metabolite profiles might provide a reference guide on therapeutic evaluation by NMR on pancreatic cancer *in vivo*.

Choline-containing metabolites (CCM) have already been chosen as biomarkers in various carcinoma studies[28,29]; however, they have not been mentioned in cancer treatment so far. CCM levels were shown to increase in most cancer tissues, which were explained as a result of high membrane concentration during the proliferation of cancer cells. We found that Cho level was reduced in pancreatic cancer after radiotherapy, suggesting that proliferation of cancer cells was inhibited in response to radiotherapy. However, PC and GPC levels showed no significant change in tumor tissue after radiotherapy. This

Table 2 Relative integrals and their ratios from some selected metabolites contributing to the evaluation of pancreatic tumor tissues response to radiotherapy

		Untreated	10 Gy	20 Gy	30 Gy	*P*-value
Metabolites	Choline	3.99 ± 0.35	3.97 ± 0.43	3.77 ± 0.36	3.44 ± 0.36	0.0075[1]
						0.3740[2]
						0.9012[3]
	Taurine	13.63 ± 2.92	13.43 ± 3.25	11.45 ± 2.20	12.41 ± 3.03	0.4262[1]
						0.1141[2]
						0.9005[3]
	Betaine	1.58 ± 0.47	1.69 ± 0.38	1.23 ± 0.45	0.79 ± 0.30	0.0013[1]
						0.1466[2]
						0.6275[3]
	Glutamic acid	0.46 ± 0.13	0.38 ± 0.07	0.43 ± 0.10	0.48 ± 0.17	0.8408[1]
						0.6480[2]
						0.1366[3]
	Alanine	1.93 ± 0.16	2.10 ± 0.40	2.01 ± 0.27	1.96 ± 0.42	0.8818[1]
						0.4821[2]
						0.2890[3]
	Lactate	8.30 ± 1.02	7.79 ± 1.43	7.51 ± 1.33	7.55 ± 0.85	0.1316[1]
						0.2031[2]
						0.4259[3]
	Acetic acid	0.06 ± 0.03	0.15 ± 0.06	0.25 ± 0.07	0.27 ± 0.13	0.0025[1]
						0.0001[2]
						0.0013[3]
	Glycerophosphocholine + phosphocholine	16.61 ± 1.31	19.95 ± 5.87	20.59 ± 5.79	20.80 ± 5.44	0.0522[1]
						0.0783[2]
						0.1383[3]
Metabolites ratio	Glycerophosphocholine/Creatine	2.35 ± 0.58	2.19 ± 0.15	2.49 ± 0.83	2.11 ± 0.36	0.3312[1]
						0.7087[2]
						0.4487[3]
	Phosphocholine/Creatine	6.22 ± 1.52	5.92 ± 0.44	5.87 ± 1.09	6.51 ± 1.28	0.6805[1]
						0.6065[2]
						0.6096[3]

[1]Untreated *vs* 30 Gy; [2]Untreatment *vs* 20 Gy; [3]Untreatment *vs* 10 Gy.

might be explained by a blockage of Cho-kinase and PC transferase, or by the consumption of PC through the CDP-Cho pathway[30,31]. Thus, we may deduce that increasing Cho and unchanged PC and GPC could be used as a unique profile of pancreatic cancer response to radiotherapy. Bet donates methyl groups for the remethylation of homocysteine to methionine and dimethylglycine, which supports proper liver and pancreatic function, cellular replication, and detoxification reactions. Because Cho is the precursor of Bet, the decrease of both Bet and Cho levels in pancreatic cancer after radiation treatment must be interrelated. Interestingly, the Ace level showed no significant difference between the normal pancreas and pancreatic cancer. However, Ace level dramatically increased with the radiation dose. The underlying significance of this needs to be further investigated.

In summary, although the number of samples in our study was limited, the potential of HRMAS NMR for the *in vitro* investigation of pancreatic disease response to radiotherapy should not be ignored. The above results clearly demonstrate that the metabolic profile changes of pancreatic cancer after radiotherapy were closely correlated with therapeutic effect through HRMAS ^{1}H NMR and the PCA combined method. Because metabolite changes observed by HRMAS NMR always occur before morphological changes investigated by MRIS, HRMAS NMR will certainly be beneficial to pathological research, early diagnosis, and therapy evaluation of pancreatic diseases.

COMMENTS

Background

Therapeutic evaluations of radiotherapy are mainly: remission from the symptoms of pain and jaundice, solid tumor size and its survival time, and the lack of a specific targeted method. During the last three decades, there has been ongoing magnetic resonance spectroscopy research in malignant diseases. These studies provided valuable data on the biochemistry and metabolism of tumors, along with the effects on nutrients, hormones, and growth factors.

Research frontiers

High-resolution magic angle spinning proton magnetic resonance spectroscopy (HRMAS ^{1}H NMR) is a well-recognized technique in metabonomics studies *in vitro*, by which biopsy or postmortem samples of intact tissues are spun at the magic angle, resulting in a significant improvement in the resolution of the spectrum obtained for some of line-broadening factors such as dipole–dipole interactions and chemical shift anisotropy. Magnetic field inhomogeneities are also averaged out. This approach requires minimal sample preparation and, unlike convenient ^{1}H NMR spectroscopy of tissue extracts, both aqueous and lipid-soluble metabolites can be observed simultaneously *in situ*.

Innovations and breakthroughs

Although HRMAS ^{1}H NMR combined with principal components analysis (PCA) has demonstrated to be an efficient method for studying a wide variety of animal and human cancers, this combined method has not been reported to analyze the metabolic features of cancer response to therapy. Here, HRMAS ^{1}H NMR and PCA were combined to highlight metabolite profiles of pancreatic cancer after radiotherapy, and by which the correlation between radiotherapy effect and metabolic change was analyzed, and the therapeutic scheme optimized.

Applications

The study has important implication for a reference guide on therapeutic evaluation by nuclear magnetic resonance spectroscopy on pancreatic cancer *in vivo*.

Peer review

The authors investigated whether metabolic profile changes of pancreatic cancer after radiotherapy were closely correlated with therapeutic effect through the HRMAS ^{1}H NMR and PCA combined method. The outcome of the study is interesting and beneficial to pathological research, early diagnosis, and therapeutic evaluation of pancreatic diseases.

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BRIEF ARTICLE

Laparoendoscopic single-site cholecystectomy *vs* three-port laparoscopic cholecystectomy: A large-scale retrospective study

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Abstract

AIM: To perform a large-scale retrospective comparison of laparoendoscopic single-site cholecystectomy (LESSC) and three-port laparoscopic cholecystectomy (TPLC) in a single institution.

METHODS: Data were collected from 366 patients undergoing LESSC between January 2005 and July 2008 and were compared with the data from 355 patients undergoing TPLC between August 2008 and November 2011 in our department. Patients with body mass index greater than 35 kg/m^2, a history of major upper abdominal surgery, signs of acute cholecystitis, such as fever, right upper quadrant tenderness with or without Murphy's sign, elevated white blood cell count, imaging findings suggestive of pericholecystic fluid, gallbladder wall thickening > 4 mm, and gallstones > 3 cm, were excluded to avoid bias.

RESULTS: Altogether, 298 LESSC and 315 TPLC patients met the inclusion criteria. The groups were well matched with regard to demographic data. There were no significant differences in terms of postoperative complications (contusion: 19 *vs* 25 and hematoma at incision: 11 *vs* 19), hospital stay (mean ± SD, 1.4 ± 0.2 d *vs* 1.4 ± 0.7 d) and visual analogue pain score (mean ± SD, 8 h after surgery: 2.3 ± 1.4 *vs* 2.3 ± 1.3 and at day 1: 1.2 ± 0.4 *vs* 1.3 ± 1.2) between the LESSC and TPLC patients. Four patients required the addition of extra ports and 2 patients were converted to open surgery in the LESSC group, which was not significantly different when compared with TPLC patients converted to laparotomy (2 *vs* 2). LESSC resulted in a longer operating time (mean ± SD, 54.8 ± 11.0 min *vs* 33.5 ± 9.0 min), a higher incidence of intraoperative gallbladder perforation (56 *vs* 6) and higher operating cost (mean ± SD, 1933.7 ± 64.4 USD *vs* 1874.7 ± 46.2 USD) than TPLC. No significant differences in operating time (mean ± SD, 34.3 ± 6.0 min *vs* 32.7 ± 8.7 min) and total cost (mean ± SD, 1881.3 ± 32.8 USD *vs* 1876.2 ± 33.4 USD) were found when the last 100 cases in the two groups were compared. A correlation was observed between reduced operating time of LESSC and increased experience (Spearman rank correlation coefficient, -0.28). More patients in the LESSC group expressed satisfaction with the cosmetic result (98% *vs* 85%).

CONCLUSION: LESSC is a safe and feasible procedure in selected patients with benign gallbladder diseases, with the significant advantage of cosmesis.

Key words: Cholecystectomy; Laparoscopy; Single site;

Retrospective studies

Core tip: This is a large-scale retrospective randomized study aimed to explore the safety and feasibility of laparoendoscopic single-site cholecystectomy (LESSC) for the treatment of benign gallbladder diseases, compared with three-port laparoscopic cholecystectomy in clinical outcomes. It was found that LESSC is a safe and feasible procedure in selected patients, with the significant advantage of cosmesis.

Cheng Y, Jiang ZS, Xu XP, Zhang Z, Xu TC, Zhou CJ, Qin JS, He GL, Gao Y, Pan MX. Laparoendoscopic single-site cholecystectomy *vs* three-port laparoscopic cholecystectomy: A large-scale retrospective study. *World J Gastroenterol* 2013; 19(26): 4209-4213 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i26/4209.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i26.4209

INTRODUCTION

Laparoendoscopic single-site cholecystectomy (LESSC) has increased in popularity due to its potential cosmetic benefits and faster recovery. It is predicted that this technique may become a standard approach to cholecystectomy[1-3]. The aim of this study was to report our experience in the LESSC with the aid of suture suspension by performing a retrospective comparison with conventional three-port laparoscopic cholecystectomy (TPLC).

MATERIALS AND METHODS

Between January 2005 and November 2011, 366 patients underwent LESSC and 355 underwent TPLC in the Department of Hepatobiliary Surgery, Zhujiang Hospital, Guangzhou, China. Retrospective data were collected from both case notes and the operating theater database.

As the LESSC procedure is a new technique and we have performed TPLC for nearly 15 years at this hospital, to avoid bias, the exclusion criteria for both the LESSC and TPLC groups included patients with a body mass index greater than 35 kg/m^2, history of major upper abdominal surgery, signs of acute cholecystitis, such as fever, right upper quadrant tenderness with or without Murphy's sign, elevated white blood cell count, imaging findings suggestive of pericholecystic fluid, gallbladder wall thickening > 4 mm, and gallstones > 3 cm. This study protocol was approved by the Institutional Review Board of the Second Affiliated Hospital of Southern Medical University, Guangzhou, China in November 2009 (No. ZJYY-2012-GDEK-001). Written informed consent for the procedure was obtained from all patients.

Eligible patients were assigned to the LESSC group (n = 298) and the TPLC group (n = 315). Collected data included patient demographics, intra-operative data about estimated blood loss, intra-operative complications, conversion to multi-port laparoscopic cholecystectomy (LC) or open surgery, and operating time (in all patients and in the last 100 patients in both groups), and postoperative data about length of hospital stay, visual analogue pain score, post-operative complications (contusion: an injury around the port site and bruised skin; hematoma: a localized collection of blood in the port site), total cost (for all patients and for the last 100 patients in both groups) and cosmetic results. The total costs for all procedures in the study were calculated using hospital financial records, which consisted of the cost of operating room usage and hospital ward stay during the perioperative period. Postoperative pain was assessed using a standard visual analogue scale [range, 0 (no pain) to 10 (maximum pain)] at 8 h after surgery and on postoperative day 1. The cosmetic effect was evaluated at the 2-wk follow-up visit, where patients were asked to assess the cosmetic results (satisfied or not very satisfied) by identifying the number and site of scars[4]. All operations were performed by two experienced surgeons who had performed more than 200 LC procedures before this study.

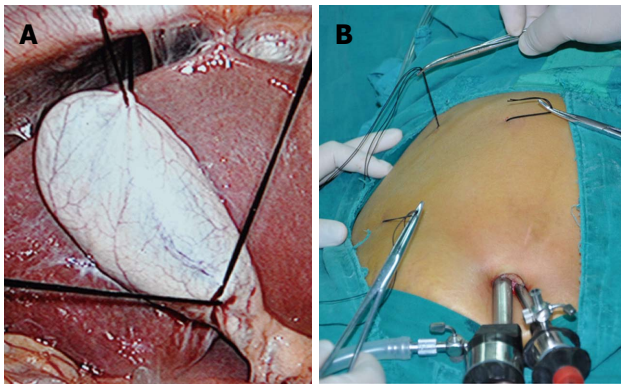

Figure 1 Suture suspension. A: The fundus and Hartmann's pouch were punctured and retracted by two sutures to expose Calot's triangle; B: Puncture spot at the superior chest wall along the costal margin in order to draw the liver up a bit more.

Surgical procedure

LESSC was performed with the help of 2 slings of sutures, and included the following steps.

Under general anesthesia, a single curved intra-umbilical 20-mm incision was made. One 10-mm trocar (Tonglu Kanger Medical Instrument Co., Ltd., Hangzhou, China) was placed to allow the insertion of a 30-degree laparoscope (Olympus, Tokyo, Japan) through the abdomen at the left side of the incision and a 5-mm trocar (Tonglu Kanger Medical Instrument Co., Ltd., Hangzhou, China) was inserted at the right side for the harmonic scalpel (Ethicon Endosurgery, 5 mm, Cincinnati, OH, United States). Tissues between the trocars were preserved to prevent air leakage. The first suture using a straight needle was inserted through the right 7th inter-costal space in the anterior axillary line, and the seromuscular layer of the gallbladder fundus was punctured and retracted toward the anterior abdominal wall. Hartmann's pouch was punctured and retracted using the second suture to expose Calot's triangle (Figure 1). A harmonic scalpel was used to dissect Calot's triangle. Once the cystic artery and duct were exposed, the cystic artery was cut using the

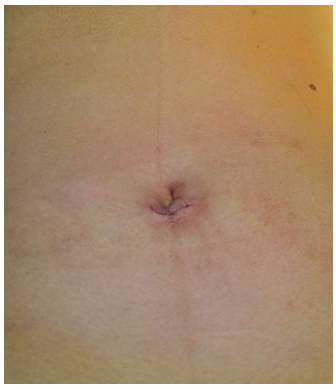

Figure 2 Umbilical incision was closed.

harmonic scalpel, and the cystic duct was ligated by three 5-mm titanium clips (Tonglu Kanger Medical Instrument Co., Ltd., Hangzhou, China) and divided. The harmonic scalpel was used to dissect the gallbladder from the gallbladder fossa. The specimen was placed into a specimen bag (TK Medical, Guangzhou, China), and removed through the umbilical incision. The umbilical incision was closed without a drainage tube in place (Figure 2)

In the TPLC procedure, the same instruments were used as in the LESSC procedure. A sub-umbilical incision, ultimisternal incision and right sub-costal incision were made. A 10-mm trocar was inserted into the sub-umbilical incision to allow introduction of the laparoscope, and another two trocars, a 10-mm and a 5-mm, respectively, were inserted for the grasp and harmonic scalpel. The operation was performed following the routine three-port cholecystectomy procedure[5], however, the cystic artery was divided and cut using the harmonic scalpel instead of being clipped and divided.

Statistical analysis

Statistical analysis was accomplished using the SPSS program for Windows 12.0 (SPSS, Chicago, IL, United States). The χ^2 test or t test was used as indicated. The Spearman rank correlation was used to investigate the relationship between operating time and experience. All data were presented as mean ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

There were no significant differences regarding demographic variables between the two groups (Table 1). In the LESSC group, four patients required additional ports (one or two) to adequately expose Calot's triangle. There were two conversions to open surgery in each group due to abnormal anatomy. There were no major intra- or post-operative complications such as bleeding, infection and bile leakage, however, LESSC resulted in a higher incidence of intraoperative gallbladder perforation than TPLC (56 cases *vs* 6 cases, $P < 0.001$). Overall, there were no significant differences in terms of surgical complications such as contusion (19 cases *vs* 25 cases, $P = 0.4540$) and hematoma at incision (11 cases *vs* 19 cases, $P = 0.1790$), hospital stay (1.4 ± 0.2 d *vs* 1.4 ± 0.7 d, $P = 1.0000$), and visual analogue pain score (8 h after surgery: 2.3 ± 1.4 *vs* 2.3 ± 1.3, $P = 1.0000$ and at day 1: 1.2 ± 0.4 *vs* 1.3 ± 1.2, $P = 0.2042$) between the LESSC and TPLC groups. LESSC resulted in a longer operating time (54.8 ± 11.0 min *vs* 33.5 ± 9.0 min, $P < 0.0010$). However, the operating time in the last 100 cases in the two groups was the same (34.3 ± 6.0 min *vs* 32.7 ± 8.7 min, $P = 0.1589$). A correlation was observed between reduced operating time and increased experience, with a Spearman rank correlation coefficient of -0.28.

Table 1 Demographic data

	LESSC (n = 298)	TPLC (n = 315)	P value
Age (yr)	41.5 ± 14.0	42.3 ± 11.0	0.3997
Female/male	170/128	191/124	0.3670
BMI (kg/m^2)	23.1 ± 4.0	23.5 ± 3.0	0.1279
ASA	1.4 ± 0.1	1.4 ± 0.2	1.0000
Clinical diagnosis			0.4530
Cholecystolithiasis	192	212	
Cystic polyps	106	103	

ASA: American Society of Anesthesiology; LESSC: Laparoendoscopic single site cholecystectomy; TPLC: Three-port laparoscopic cholecystectomy; BMI: Body mass index.

The total cost for LESSC per patient was 1933.7 USD compared with 1874.1 USD for the TPLC procedure (1933.7 ± 64.4 USD *vs* 1874.7 ± 46.2 USD, $P < 0.001$), and the overall cost of LESSC was approximately 57.8 USD more than the TPLC technique. However, no significant difference was found when the last 100 cases in the two groups were compared (1881.3 ± 32.8 USD *vs* 1876.2 ± 33.4 USD, $P = 0.0571$), suggesting that the cost difference was mainly due to the increased operating time.

Most patients were surprised by the reduced number of sites, and more patients who underwent LESSC satisfied with the cosmetic result than those who underwent TPLC (98% *vs* 85%, $P = 0.0010$) (Table 2).

DISCUSSION

Laparoendoscopic single-site surgery has attracted wide attention due to the decreased number of incisions needed and potentially good cosmetic results[6-13]. Recently, more studies have focused on comparing LESSC with multi-port LC and have reached an agreement that LESSC may become the gold standard treatment[14,15]. However, there is still a long way to go before this approach becomes the gold standard treatment as the standardization, safety, and other outcomes of LESSC require further validation[16-19].

Standardization is a prerequisite for clinical popularization of a surgical approach. Approaches to LESSC are technically immature. For example, to expose Calot' s triangle, trials on the use of sutures, Kirschner wires and loop retractors have been reported. The devices used in surgery vary from one surgeon to another: some use common trocars[20,21], some tend to use LESSC multiports[22] and others favor self-designed devices such as sterile gloves[23], in addition, there are differences in ma-

Table 2 Patient outcomes

	LESSC (n = 298)	TPLC (n = 315)	P value
Conversions to open surgery	2	2	1.0000
EBL (mL)	14 ± 6.0	15 ± 4.0	0.2643
Gallbladder perforation during surgery	56	6	< 0.001
Operating time (min)	54.8 ± 11.0	33.5 ± 9.0	< 0.001
Operating time of the last 100 cases (min)	34.3 ± 6.0	32.7 ± 8.7	0.1589
VAS (1–10)			
8 h after surgery	2.3 ± 1.4	2.3 ± 1.3	1.0000
Day 1	1.2 ± 0.4	1.3 ± 1.2	0.2042
Complications			
Contusion at incision	19	25	0.4540
Hematoma at incision	11	19	0.1790
Hospital stay (d)	1.4 ± 0.2	1.4 ± 0.7	1.0000
Cosmetic result	98%	85%	0.0010
Total cost (USD)	1933.7 ± 64.4	1874.7 ± 46.2	< 0.0010
Total cost of the last 100 cases (USD)	1881.3 ± 32.8	1876.2 ± 33.4	0.0571

EBL: Estimated blood loss; LESSC: Laparoendoscopic single site cholecystectomy; TPLC: Three-port laparoscopic cholecystectomy; VAS: Visual analogue score.

nipulative instruments such as routine instruments and reticulating instruments[22]. For example, to prevent air leakage, we have tried tri-ports and gel-ports at our center, but discontinued these due to high cost and longer trans-umbilical incision. We have used routine trocars because they are effective in preventing air leakage and are more cost-effective. With regard to surgical instruments, we have tried flexible forceps and laparoscopes, but have finally resorted to suture suspension assisted technology in LESSC, for which only one 30-degree laparoscope and one manipulative instrument are needed, eliminating the clashing of more instruments intra-operatively.

An appropriate method to place the sutures is essential for the operation. To achieve an ideal exposure of surgical site, we choose a puncture site at the superior chest wall along the costal margin so that the suture can draw the liver up a bit more, which is different from view of Piskun *et al*[23] that the puncture spot should be at the inferior costal margin. In addition, the use of harmonic scalpel is effective in occluding 3-mm blood vessels and dissecting tissues[24]. At our center, the cystic arteries were all cut using the harmonic scalpel, indicating the safety of this scalpel.

In this study, the groups were not randomized or operated on at the same time periods, thus inevitably increasing the risk of bias[25]. For example, TPLC was performed earlier than LESSC at our institution, suggesting a difference in operating experience between LESSC and TPLC. Many patients with signs of acute cholecystitis and other complications successfully underwent TPLC in our institution, but few patients with these complications successfully underwent LESSC during the study period. Therefore, exclusion criteria were applied, where patients with a history of major upper abdominal surgery, signs of acute cholecystitis, and gallstones > 3 cm, were excluded to minimize bias. However, despite the use of selection criteria, this study remains retrospective and was affected by the well-known bias due to this design.

Our results showed that the LESSC technique was more expensive and time-consuming than the TPLC technique. However, the comparisons in the last 100 patients between the two groups demonstrated that these differences were minimized through improvement of surgical skills. Analyses of operating time and total cost demonstrated a relationship between reduced operating time and increased experience, and a relationship between reduced total cost and increased experience. It is concluded that LESSC with the aid of suture suspension will not add a financial burden to the patient if the operator is skilled in this technique.

In conclusion, this large-scale retrospective trial demonstrated that LESSC with the aid of suture suspension is a safe and feasible procedure in selected patients. However, the limitations of the retrospective nature in this study preclude us from drawing a firm conclusion that LESSC is as safe as TPLC in terms of major complications, such as the bile duct injury, and from demonstrating its potential advantages, such as improved result, reduced postoperative pain and patient satisfaction. Therefore, more large-scale and multi-center randomized studies comparing LESSC with multi-port LC are needed to investigate the safety, potential benefits and clinical application of LESSC.

COMMENTS

Background

Recently, surgeons have begun performing laparoscopic cholecystectomy through a single umbilical incision, which is known as laparoendoscopic single-site cholecystectomy (LESSC). The potential benefits of this approach include reduced postoperative pain, improved cosmetic result and earlier return to normal life. Some investigators have predicted that LESSC may become an alternative standard approach for benign gallbladder diseases. However, there are still controversies with regard to its safety and efficiency, although increasing literatures demonstrate that single-incision laparoscopic surgery is a feasible and safe approach. This retrospective study explored the safety and efficiency of LESSC for the treatment of benign gallbladder diseases in selected patients compared with the three-port laparoscopic cholecystectomy (TPLC) in clinical outcomes.

Research frontiers

LESSC has attracted wide attention because of its potential advantages in cosmetic result and faster rehabilitation. However, whether LESSC could be an alternative to multi-port laparoscopic cholecystectomy remains unknown, and therefore it is necessary to compare the clinical outcome of LESSC and multiple-port laparoscopic cholecystectomy in a large cohort.

Innovations and breakthroughs

This is a large-scale retrospective study to explore the safety and efficiency of LESSC for the management of benign gallbladder diseases compared with the TPLC in selected patients.

Applications

LESSC is a safe and effective approach in selected patients with benign gallbladder diseases. LESSC has a better cosmetic benefit than TPLC.

Terminology

LESSC is a complementary approach to laparoscopic cholecystectomy, in which all operating procedures are completed through a single 15-25 mm incision around the navel. However, unlike the traditional multi-port laparoscopic approach, LESSC leaves only a single small scar.

Peer review

The authors have presented for an interesting manuscript in which they retrospectively compare a single incision laparoscopic cholecystectomy *vs* conventional 3 port cholecystectomy. The main strength of this study is the large simple size of considered groups of patients. The authors have compared the outcomes

of interest in a total of 613 eligible patients, 298 in the single incision group (LESSC) *vs* 315 in the three port group (TPLC). The procedures have been performed by two high experienced surgeons on laparoscopic cholecystectomy who have performed more than 200 laparoscopic cholecystectomy before this study. The authors have evaluated all necessary outcomes and they have accurately described the details of the performed surgical procedures. The study has concluded that LESSC is more expensive than TPLC, it requires longer operating time and it is a safe and feasible procedure in selected patients and in expert hands. Overall, the manuscript is well structured, clear and concise.

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BRIEF ARTICLE

Effect of amitriptyline on gastrointestinal function and brain-gut peptides: A double-blind trial

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Abstract

AIM: To study the effects of low-dose amitriptyline (AMT) on gastrointestinal function and brain-gut peptides in healthy Chinese volunteers.

METHODS: This was a double-blind, randomised, placebo-controlled, two-period cross-over trial. Twenty-eight healthy volunteers were randomised and administered 1-wk treatments of AMT (12.5 mg *tid*) or placebo. Before and during the final two days of treatment, gastric emptying, proximal gastric accommodation and visceral sensitivity were measured by drinking-ultrasonography test; the orocecal transit time (OCTT) was measured by lactulose hydrogen breath test, and fasting blood was collected. Plasma levels of ghrelin, motilin and neuropeptide Y (NPY) were measured by enzyme-linked immunosorbent assay kits.

RESULTS: AMT slowed the OCTT (109.2 ± 29.68 min *vs* 96.61 ± 23.9 min, $P = 0.004$) but did not affect liquid gastric emptying and had no effect on proximal gastric accommodation. AMT resulted in decreases in the visual analogue scale (VAS) for difficulty in drinking 600 and 800 mL of water (3.57 ± 0.94 *vs* 2.98 ± 0.85, 5.57 ± 0.82 *vs* 4.57 ± 0.98, $P < 0.01$ for both), although it had no significant effect on the VAS for difficulty in drinking 200 mL and 400 mL of water. AMT significantly increased the plasma ghrelin level (442.87 ± 176.79 pg/mL *vs* 526.87 ± 158.44 pg/mL, $P = 0.04$) and the neuropeptide-Y level (890.15 ± 131.46 pg/mL *vs* 965.64 ± 165.63 pg/mL, $P = 0.03$), whereas it had no effect on the MTL level.

CONCLUSION: Low-dose AMT could slow OCTT, make the stomach less sensitive and increase the plasma levels of ghrelin and NPY. Thus, we recommend the use of low-dose AMT for functional gastrointestinal disorders.

Key words: Amitriptyline; Orocecal transit time; Visceral hypersensitivity; Gastric emptying; Brain-gut peptides

Core tip: Low-dose amitriptyline has been used to treat functional gastrointestinal disorders for many years, but the precise mechanism is still not clear. Brain-gut peptides, such as motilin, ghrelin and neuropeptide Y, may regulate gastrointestinal functions. However, evidence indicating the possible effects of amitriptyline on the levels of brain-gut peptides in healthy Chinese volunteers is limited. In this study, we conclude that low-dose amitriptyline can slow orocecal transit time, make the stomach less sensitive and increase the plasma levels of ghrelin and neuropeptide Y. Thus, we recommend the use of low-dose amitriptyline for functional gastrointestinal disorders.

Huang W, Jiang SM, Jia L, You LQ, Huang YX, Gong YM,

Wang GQ. Effect of amitriptyline on gastrointestinal function and brain-gut peptides: A double-blind trial. *World J Gastroenterol* 2013; 19(26): 4214-4220 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i26/4214.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i26.4214

INTRODUCTION

Functional dyspepsia (FD) and irritable bowel syndrome (IBS) are the most common functional gastrointestinal disorders (FGIDs). The aetiology of FGIDs is unclear, and treatment options are limited[1,2]. Low-dose amitriptyline (AMT) is a tricyclic antidepressant that has been used to treat FGIDs for many years[3]; however, the exact mechanism of action is not clear.

Brain-gut peptides, including motilin (MTL), ghrelin, neuropeptide Y (NPY) and so on, also known as peptide hormones, can be found in the cerebral nervous system, enteric nervous system and endocrine cells in the gastrointestinal tract. Brain-gut peptides, can be neuropeptides and neuroendocrine and paracrine substances, regulate the secretory and motor functions of the gastrointestinal tract. MTL can reportedly accelerate gastric emptying and reduce the proximal gastric volume in patients with FD[4,5]. Ghrelin, the closest family member of MTL, was reported to be abnormal in FD[6]. NPY is a 36 amino-acid peptide in the central and peripheral nervous systems that can inhibit gastric emptying and stimulate colonic transit[7]. However, as far as we know, evidence indicating the possible effects of AMT on the levels of brain-gut peptides in healthy Chinese volunteers is limited.

We hypothesised that low-dose AMT is beneficial for FGIDs because of the changes in the gastrointestinal sensor, motor function and plasma levels of brain-gut peptides. Therefore, we aimed to explore the effects of low-dose AMT on liquid gastric emptying, proximal gastric accommodation, proximal gastric sensitivity, orocecal transit time (OCTT) and the plasma levels of MTL, ghrelin and NPY in healthy Chinese volunteers.

MATERIALS AND METHODS

Methods and drugs

This study was a randomised, double-blind, placebo-controlled, two-period cross-over trial in healthy Chinese volunteers (Clinical trial number: ChiCTR-TTRCC-12001967), which was approved by the ethics committee of the hospital. Written informed consent was obtained from healthy volunteers, which conformed to the Declaration of Helsinki.

Twenty-eight healthy volunteers were randomised to the two therapies: group A was treated for 1 wk with 12.5 mg AMT *tid* and then with placebo, while group B was treated with the opposite sequence. There was a 2-wk washout phase, followed by a crossover to the alternate treatment (Figure 1). AMT hydrochloride tablets were purchased from HuNan DongTing Pharmaceutical Co. Ltd. of China (batch number: B110824). The placebo

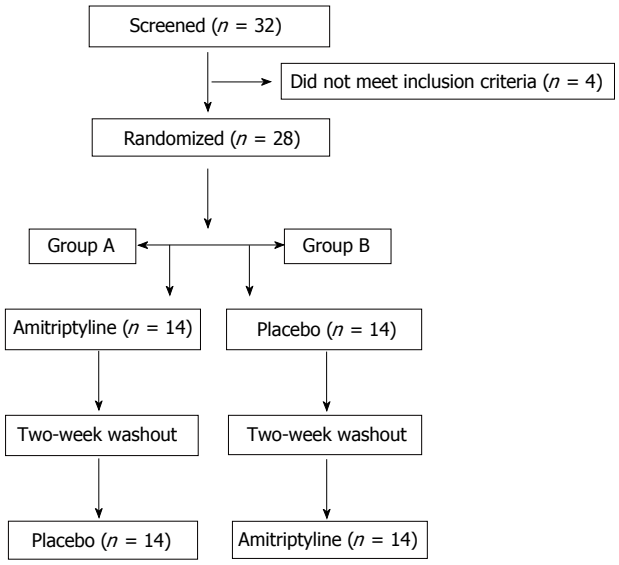

Figure 1 Consort diagram.

was supplied by ShenZhen WanHe Pharmaceutical Co. Ltd. of China. AMT and placebo tablets were similar, and the strength of each tablet was 25 mg. The investigators and patients were blinded to the treatment. The results were analysed by the investigators, and the original randomisation scheme was released after all of the analyses were performed.

Healthy volunteers

The exclusion criteria of healthy volunteers included: (1) history of FGIDs (in line with the definition of the Rome Ⅲ criteria) that may affect gastrointestinal motility; hypersensitivity or allergy to any tricyclic drug; (2) history of gastrointestinal surgery and psychiatric illness; (3) pregnancy or breast feeding; (4) use of medications that may affect gastrointestinal motor function (*e.g.*, prokinetics and anti-spasmotic agents) or the effect of AMT; (5) concomitant therapy with a monoamine oxidase inhibitor, history of urinary retention, known glaucoma, history of seizures and thyroid or liver dysfunction; and (6) participation in another clinical trial during the last two weeks.

Endpoints of the study

All healthy volunteers completed the Hamilton Anxiety and Depression Rating Scale before the treatment; a score less than 7 was defined as no anxiety or depression[8,9]. During the two days before therapy and the final two days of treatment, we assessed the following endpoints: (1) liquid gastric emptying, proximal gastric relaxation and visceral hypersensitivity by drinking- ultrasonography test; (2) OCTT by lactulose hydrogen breath test; and (3) plasma MTL, ghrelin and NPY levels by ELISA.

Drinking-ultrasonography test

The drinking-ultrasonography test was performed according to the method of Kato *et al*[10]. After an overnight

Table 1 Demographic and baseline characteristics of study in healthy volunteers

Variable	Group A (n = 14)	Group B (n = 14)	P value
Age (yr)	27.71 ± 8.56	32.5 ± 13.36	0.26
Sex (male)	7	7	1
BMI (kg/m^2)	20.09 ± 1.41	20.21 ± 1.42	0.82
HAMD	2.36 ± 1.28	1.71 ± 1.20	0.29
HAMA	2.71 ± 1.73	3.36 ± 1.45	0.18
Cross-sectional area of the proximal stomach (cm^2)			
200 mL	17.13 ± 4.53	18.75 ± 2.30	0.08
400 mL	29.19 ± 6.24	30.76 ± 6.59	0.95
600 mL	40.92 ± 11.5	40.66 ± 7.28	0.35
800 mL	46.21 ± 12.16	46.46 ± 6.81	0.24
Difficulty in drinking water VAS			
200 mL	0.79 ± 0.58	1.07 ± 0.62	0.23
400 mL	1.79 ± 0.58	1.86 ± 0.54	0.71
600 mL	3.21 ± 0.80	3.50 ± 0.65	0.31
800 mL	5.50 ± 0.76	5.43 ± 0.76	0.82
Gastric emptying			
5 min	77.65% ± 6.5%	81.46% ± 5.81%	0.67
10 min	62.61% ± 9.85%	65.18% ± 6.77%	0.55
OCTT (min)	88.93% ± 19.03%	81.43% ± 20.14%	0.46
Plasma levels (pg/mL)			
MTL	502.66 ± 127.52	440.85 ± 123.25	0.20
Ghrelin	460.06 ± 146.25	444.94 ± 202.43	0.82
NPY	888.88 ± 154.52	913.46 ± 139.32	0.66

Values are represented as mean ± SD. Symptom scores on 10-cm visual analogue scale. HAMD: Hamilton depression rating scale; HAMA: Hamilton anxiety rating scale; BMI: Body mass index; OCTT: Orocecal transit time; NPY: Neuropeptide Y; MTL: Motilin; VAS: Visual analogue scale.

fast, healthy volunteers ingested 200 mL of water (approximately 28 ℃) in 2 min for a total of four times with 2-min intervals. The subjects were in the supine position and ingested water through a straw. The emptying periods were calculated to be 5 and 10 min (by measuring the time) after drinking the total 800 mL of water. All of these examinations were performed by a single ultrasonography technician using a Philips IU22 ultrasound scanner (Philips Medical Systems, Bothell, Washington) and a Convex-type 5-20 MHz probe. The spleen served as an echo window, and the cross-section of the proximal stomach was measured *via* the 10th inter-costal space. The mucosal surface of the gastric lumen was traced from images acquired before the test at each 2 min interval after water consumption and 5 and 10 min after the end of the water consumption. The cross-sectional area was also calculated. Frozen images were saved on a hard disk. Before the test and every time after ingestion of water, abdominal symptoms were self-evaluated and recorded on a questionnaire using a visual analogue scale from 0 to 10 to investigate the difficulty (such as abdominal fullness) in drinking water.

Lactulose hydrogen breath test for OCTT

Subjects were placed on a low fibre diet 3 d before the test. After a 12-h overnight fast, two end-expiratory breath H_2 samples were collected as base values using a HHBT-01 breath hydrogen detector (Hydeway, China). After the subjects ingested 15 mL of lactulose syrup containing 10 g of lactulose, exhaled H_2 was recorded every 15 min for a total of three hours. OCTT was defined as the duration from the moment of lactulose administration to the moment when exhaled H_2 was increased over 12 ppm from the baseline[11].

Plasma ghrelin, MTL and NPY levels

After twelve-hours of fasting, the blood samples were collected and centrifuged at 3000 g for 10 min. Plasma samples were collected and stored at -70 ℃ until the procedure. We measured plasma levels of ghrelin, MTL and NPY using commercial ELISA kits (Shanghai Bluegene Biotech Co., Ltd., China).

Statistical analysis

Data analysis was performed using SPSS 13.0 software (SPSS Inc., Chicago IL, United States), and the measurement data are reported as the mean ± SD; baseline parameters and differences between the two treatments were compared using Student's t test. Differences between the baseline and AMT or placebo treatment were compared by paired t test. $P < 0.05$ was considered statistically significant.

RESULTS

Study participants

Thirty-two healthy volunteers were selected initially by public advertisement. After a screening visit, four subjects were not appropriate for the study by the exclusion criteria (two subjects had a history of gastrointestinal surgery and two subjects experienced abdominal pain during the last three months). Twenty-eight subjects completed the study. There were no statistically significant differences in age, gender, body mass index, Hamilton depression scale, Hamilton anxiety scale scores, proximal gastric accommodation, liquid gastric emptying, proximal gastric sensitivity, OCTT or the levels of MTL, ghrelin and NPY between group A and group B ($P > 0.05$) (Table 1).

Proximal accommodation, visceral hypersensitivity and gastric emptying using the drinking-ultrasonography test

There was no statistically significant difference in the proximal gastric accommodation between the AMT and placebo groups after consumption of 200, 400, 600 or 800 mL water ($P > 0.05$). Similarly, no differences were found in the gastric emptying rate (%) at 5 and 10 min after the completion of the drinking test (all $P > 0.05$). Moreover, there were no statistically significant differences between the two groups for the VAS test for difficulty in drinking 200 or 400 mL water (all $P > 0.05$). However, there were significant differences in VAS results for difficulty in drinking 600 and 800 mL between the two groups (all $P = 0.001$) (Table 2).

There were no significant differences between the baseline and placebo treatment in proximal accommoda-

Table 2 Effects of amitriptyline on gastrointestinal function and brain-gut peptides

Variable	Amitriptyline (n = 28)	Placebo (n = 28)	P value
Cross-sectional area of the proximal stomach (cm^2)			
200 mL	16.51 ± 3.78	16.56 ± 3.98	0.97
400 mL	27.14 ± 5.71	27.84 ± 5.95	0.49
600 mL	34.11 ± 6.11	34.85 ± 6.61	0.39
800 mL	39.58 ± 7.35	40.86 ± 8.45	0.34
Difficulty in drinking water VAS			
200 mL	0.93 ± 0.65	0.96 ± 0.56	0.58
400 mL	1.93 ± 0.46	1.82 ± 0.54	0.29
600 mL	2.98 ± 0.85	3.57 ± 0.94	0.001
800 mL	4.57 ± 0.98	5.57 ± 0.82	0.001
Gastric emptying			
5 min	78.40 ± 11.71	78.84 ± 7.47	0.87
10 min	66.72 ± 11.63	64.54 ± 10.29	0.47
OCTT (min)	109.29 ± 29.68	96.61 ± 23.9	0.004
Plasma levels (pg/mL)			
MTL	461.88 ± 129.66	473.40 ± 122.75	0.61
Ghrelin	526.87 ± 158.44	442.87 ± 176.79	0.04
NPY	965.64 ± 165.63	890.15 ± 131.46	0.03

Values are represented as mean ± SD. Symptom scores on 10-cm visual analogue scale. OCTT: Orocecal transit time; MTL: Motilin; NPY: Neuropeptide Y; VAS: Visual analogue scale.

Table 3 Baseline and after treatment with placebo

Variable	Baseline (n = 28)	Placebo (n = 28)	P value
Cross-sectional area of the proximal stomach (cm^2)			
200 mL	17.95 ± 3.62	16.56 ± 3.98	0.19
400 mL	29.97 ± 6.35	27.84 ± 5.95	0.09
600 mL	40.78 ± 9.54	34.85 ± 6.61	0.06
800 mL	46.34 ± 9.67	40.86 ± 8.45	0.06
Difficulty in drinking water VAS			
200 mL	0.93 ± 0.59	0.96 ± 0.56	0.70
400 mL	1.82 ± 0.54	1.82 ± 0.54	1.00
600 mL	3.36 ± 0.72	3.57 ± 0.94	0.34
800 mL	5.46 ± 0.73	5.57 ± 0.82	0.56
Gastric emptying			
5 min	79.55 ± 6.35	78.84 ± 7.47	0.47
10 min	63.89 ± 8.39	64.54 ± 10.29	0.79
OCTT (min)	85.18 ± 19.60	96.61 ± 23.90	0.07
Plasma levels (pg/mL)			
MTL	471.75 ± 127.02	473.40 ± 122.75	0.75
Ghrelin	452.50 ± 173.46	442.87 ± 176.79	0.35
NPY	901.17 ± 144.91	890.15 ± 131.46	0.12

Values are represented as mean ± SD. Symptom scores on 10-cm visual analogue scale. OCTT: Orocecal transit time; MTL: Motilin; NPY: Neuropeptide Y; VAS: Visual analogue scale.

tion, gastric emptying and VAS results for difficulty in drinking 200, 400, 600 or 800 mL of water ($P > 0.05$) (Table 3).

There were no significant differences between the baseline and AMT treatment in the cross-sectional area of the proximal stomach (cm^2) after drinking 200, 400, 600 or 800 mL of water ($P > 0.05$) (Table 4). Similarly, no significant differences were found between the baseline and AMT treatment in the VAS after drinking 200 or 400 mL of water ($P > 0.05$). However, the VAS results significantly dropped from baseline in response to AMT treatment after drinking 600 and 800 mL water ($P = 0.001$) (Table 4). No differences in gastric emptying were observed between the baseline and AMT treatment ($P > 0.05$; Table 4).

OCTT with lactulose hydrogen breath test

AMT slowed the OCTT, and there was a significant difference between the AMT and placebo groups ($P = 0.004$; Table 2). OCTT was not different between the baseline and placebo treatment (Table 3), although there was a significant difference between the baseline and treatment with AMT ($P = 0.001$; Table 4).

Plasma levels of MTL, ghrelin and NPY using ELISA

The fasting plasma concentration of MTL was similar in the AMT and placebo groups ($P = 0.61$; Table 2). There were no significant differences in the MTL levels between the baseline and treatment with placebo ($P = 0.75$; Table 3) or between the baseline and treatment with AMT ($P = 0.11$; Table 4). However, in the AMT group, the fasting plasma ghrelin concentration was significantly greater than the placebo group ($P = 0.04$; Table 2). There was no difference in the ghrelin level between the baseline and treatment with placebo ($P = 0.35$; Table 3), but the ghrelin concentration was elevated following AMT treatment compared to the baseline values ($P = 0.001$; Table 4). Compared with the placebo group, the AMT group had higher fasting plasma NPY levels ($P = 0.03$; Table 2). There was no significant difference between the baseline and treatment with placebo ($P = 0.12$; Table 3), but the NPY level was significantly elevated after AMT treatment ($P = 0.001$; Table 4).

Adverse effects and safety

Table 5 shows the adverse effects that occurred during the treatment. There were no adverse effects which required emergency evaluation or hospitalisation. No subjects dropped out of the study.

DISCUSSION

In previous studies, low-dose AMT was useful for FD and IBS, especially for the improvement of abdominal pain[12-15], possibly because AMT reduces the visceral sensitivity and increases the pain threshold in FGID patients, although this is still controversial. Mertz *et al*[12] suggested that after AMT (50 mg/d) treatment for 4 wk in FD, the perception of gastric distension using the barostat test was not different from the placebo treatment. Conversely, Thoua *et al*[13] demonstrated that after 3 mo of treatment with AMT (25-50 mg/d) in IBS, the rectal hypersensitivity to electrical current stress was decreased, however the study was uncontrolled. Obviously, it is not due to the antidepressant effect of AMT as the doses were below the effective doses of the antidepressant; the benefits are in patients who are not depressive, with responses occurring before the antidepressant effect[16].

Here, we measured visceral sensitivity using the non-invasive drinking-ultrasonography test in healthy volun-

Table 4 Baseline and after treatment with amitriptyline

Variable	Baseline (n = 28)	Amitriptyline (n = 28)	P value
Cross-sectional area of the proximal stomach (cm^2)			
200 mL	17.95 ± 3.62	16.51 ± 3.78	0.22
400 mL	29.97 ± 6.35	27.14 ± 5.71	0.09
600 mL	40.78 ± 9.54	34.11 ± 6.11	0.06
800 mL	46.34 ± 9.67	39.58 ± 7.35	0.06
Difficulty in drinking water VAS			
200 mL	0.93 ± 0.59	0.93 ± 0.65	0.99
400 mL	1.82 ± 0.54	1.93 ± 0.46	0.36
600 mL	3.36 ± 0.72	2.98 ± 0.85	0.01
800 mL	5.46 ± 0.73	4.57 ± 0.98	0.001
Gastric emptying			
5 min	79.55 ± 6.35	78.40 ± 11.71	0.96
10 min	63.89 ± 8.39	66.72 ± 11.63	0.17
OCTT (min)	85.18 ± 19.60	109.29 ± 29.68	0.001
Plasma levels (pg/mL)			
MTL	471.75 ± 127.02	461.88 ± 129.66	0.11
Ghrelin	452.50 ± 173.46	526.87 ± 158.44	0.001
NPY	901.17 ± 144.91	965.64 ± 165.63	0.001

Values are represented as mean ± SD. Symptom scores on 10-cm visual analogue scale. OCTT: Orocecal transit time; MTL: Motilin; NPY: Neuropeptide Y; VAS: Visual analogue scale.

teers, which is different from previous studies. We found that low-dose AMT reduced gastric sensitivity immediately after the volunteers ingested 600 and 800 mL water, which was consistent with the result of Thoua *et al*[13]. This might contribute to the potential centrally mediated visceral analgesic properties of AMT. As Morgan *et al*[16] suggested, low-dose AMT has a central effect on pain-related cerebral activation in the anterior cingulate cortex and left posterior parietal complex in IBS patients during mental stress.

A variety of gastrointestinal motility disturbances have been implicated in FGIDs[17]. Bouras *et al*[18] demonstrated that low-dose AMT could slow solid gastric emptying in healthy individuals. Vahedi *et al*[14] observed that low-dose AMT was effective for the treatment of diarrhoea-predominant IBS; the reason might be the anti-cholinergic effect of the drug. In our research, AMT did not affect liquid gastric emptying but did significantly prolong the OCTT (which is also reflects the small bowel transit time[19]). This is consistent with previous investigations in which imipramine delayed OCTT in controls and IBS patients[20]. The reason for the differences in the current results compared to previous studies might be that liquid emptying is related to the proximal portion or fundus relaxation, but solid emptying is associated with the distal stomach[21]. In the current study, there were no effects on proximal gastric accommodation with low-dose AMT. Similar conclusions have been previously reported and showed that AMT had no effect on drinking capacity in healthy volunteers[18]. The gold standard for the measurement of proximal gastric accommodation is gastric barostat[22], although this procedure is invasive. In this study, we used the new drinking-ultrasonography test, which is non-invasiveness, safe, reproducible, better accepted by volunteers and relatively simple to administer.

Table 5 Adverse effects of amitriptyline and placebo

Adverse effect	Amitriptyline (n = 28)	Placebo (n = 28)
Sleepiness	10	2
Bitter taste	7	2
Dry mouth	6	3
Tired in early morning	2	1
Dizziness	2	0
Constipation	1	1

MTL levels were not significantly different between the placebo and AMT groups. However MTL levels have been reported to be significantly elevated in patients with constipation who are receiving tricyclic antidepressant drugs[23]. It is possible that we evaluated healthy volunteers rather than patients in this study. In healthy individuals, AMT might not have any effect on the normal levels of MTL because of intact reflex mechanisms. Ghrelin plays a role in regulating appetite[24]. Lee *et al*[6] reported that unusually low preprandial ghrelin levels occur in FD patients due to dysmotility. It is possible that FD patients with dysmotility may respond to AMT effectively. Caproni *et al*[25] found that the plasma levels of NPY were markedly increased in migraine patients receiving AMT treatment (25 mg/d) for 3 mo. The present study extends the previous finding by showing that the plasma level of NPY was significantly increased with low-dose AMT treatment. A previous study showed that NPY may help patients with stress on the gut-brain axis[26], so the increase in NPY levels might be a reason for the treatment of FD and IBS patients who are often hypersensitive to stress[27].

This study included a small sample size of healthy volunteers. Further studies consisting of larger sample sizes that are powered to find smaller differences may be required. As the duration of AMT administration in the clinic is typically 4-12 wk[12-15], it is possible that the course of medication in our study was too short. A longer trial might have different effects on gastrointestinal function and brain-gut peptides.

In summary, low-dose AMT slows OCTT, decreases gastric sensitivity and increases the plasma levels of ghrelin and neuropeptide Y in healthy Chinese individuals, which may be the cause of the beneficial effects of low-dose AMT in FGID patients.

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COMMENTS

Background

Low-dose amitriptyline (AMT) has been used to study functional gastrointestinal disorders for many years, although the precise mechanism of the action is still not clear. Evidence indicating the possible effects of AMT on gastrointestinal

function and brain-gut peptides in healthy Chinese volunteers is limited.

Research frontiers

Therapeutic options for functional gastrointestinal disorders are limited. Antidepressant agents such as low dose AMT are effective in functional gastrointestinal disorders.

Innovations and breakthroughs

Based on previous data, this study first explored the possible effects of low dose AMT on gastrointestinal function and brain-gut peptides in healthy Chinese volunteers. The results of the present study revealed that low-dose AMT could slow orocecal transit time (OCTT), decrease gastric sensitivity and increase the plasma levels of ghrelin and neuropeptide Y in healthy Chinese individuals, which may be the cause of the beneficial effects of low-dose AMT in functional gastrointestinal disorders (FGID) patients.

Applications

Low dose AMT plays a role in regulating gastrointestinal function, supporting its clinical applicability for gastrointestinal disorders in China.

Terminology

The drinking-ultrasonography test is a novel method to measure proximal accommodation, visceral hypersensitivity and gastric emptying. The test is non-invasive, safe, better accepted by volunteers, reproducible and relatively simple to administer.

Peer review

This manuscript has originality so far. Low-dose AMT slows OCTT, decreases gastric sensitivity and increases the plasma levels of ghrelin and neuropeptide Y in healthy Chinese individuals, which may be the cause of the beneficial effects of low-dose AMT in FGID patients.

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BRIEF ARTICLE

Magnified and enhanced computed virtual chromoendoscopy in gastric neoplasia: A feasibility study

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Abstract

AIM: To evaluate the feasibility of a new computed virtual chromoendoscopy (CVC) device (M i-scan) in the diagnosis of gastric neoplasia.

METHODS: Patients with superficial lesions no larger than 1.0 cm found during high definition endoscopy were included. Those with advanced or obviously protruded or depressed lesions, lesions larger than 1.0 cm and/or lesions which were not amenable to observation by zoom function were excluded. The endoscopist was required to give the real-time descriptions of surface pit patterns of the lesions, based on surface pattern classification of enhanced magnification endoscopy. According to previous reports, types Ⅰ-Ⅲ represent non-neoplastic lesions, and types Ⅳ-Ⅴ represent neoplastic lesions. Diagnosis with M i-scan and biopsy was performed before histopathological diagnosis. Magnified images of gastric lesions with and without enhancement were collected for further analysis. The diagnostic yield of real-time M i-scan and effects on magnification image quality by tone enhancement (TE), surface enhancement (SE) and color enhancement (CE) were calculated. The selected images were sent to another endoscopist. The endoscopist rated the image quality of each lesion at 3 levels. Ratings of image quality were based on visualization of pit pattern, vessel and demarcation line.

RESULTS: One hundred and eighty-three patients were recruited. Five patients were excluded for advanced gastric lesions, 1 patient was excluded for poor preparation and 2 patients were excluded for superficial lesions larger than 1.0 cm; 132 patients were excluded for no lesions found by high definition endoscopy. In the end, 43 patients with 43 lesions were included. Histopathology revealed 10 inflammation, 14 atrophy, 10 metaplasia, 1 low grade dysplasia (LGD), 5 high grade dysplasia (HGD) and 3 cancers. For 7 lesions classified into type Ⅰ, histopathology revealed 6 atrophy and 1 metaplasia; for 10 lesions classified into type Ⅱ, histopathology revealed 2 inflammation, 7 atrophy and 1 metaplasia; for 10 lesions classified into type Ⅲ, histopathology revealed 1 inflammation, 8 metaplasia and 1 LGD; for 9 lesions classified into type Ⅳ, histopathology revealed 4 inflammation, 1 atrophy and 4 HGD; for 7 lesions classified into type Ⅴ, histopathology revealed 3 inflammation, 1 HGD and 3 cancers. A total of 172 still images, including 43 images by white light (MWL) and 129 images by M i-scan (43 with TE, 43 with SE and 43 with CE), were selected and sent to the endoscopist who did the analysis. General image quality of M i-scan with TE and SE was significantly better than that

of MWL (TE, 4.55 ± 1.07; SE, 4.30 ± 1.02; MWL, 3.25 ± 0.99; $P < 0.001$). Visualization of pit pattern was significantly improved by M i-scan with SE (1.93 ± 0.25 *vs* 1.50 ± 0.50, $P < 0.001$). Microvessel visualization was significantly improved by M i-scan with TE (1.23 ± 0.78 *vs* 0.76 ± 0.73, $P < 0.001$). Demarcation line visualization was improved by M i-scan with both TE and SE (TE, 1.75 ± 0.52; SE, 1.56 ± 0.59; MWL, 0.98 ± 0.44; $P < 0.001$). M i-scan with CE did not show any significant improvements of image quality in general or in the 3 key parameters. Although M i-scan with TE and SE slightly increased the diagnostic yield of MWL, there was no significant difference ($P > 0.1$).

CONCLUSION: Although digital enhancement improves the image quality of magnification endoscopy, its value in improving the diagnostic yield seems to be limited.

Key words: Computed virtual chromoendoscopy; i-scan; Gastric neoplasia; Diagnosis

Core tip: In this study, the authors applied a new endoscopic device combining magnification endoscopy and virtual chromoendoscopy, equipped with surface enhancement, tone enhancement and color enhancement (M i-scan), in the diagnosis of 43 patients with small superficial gastric lesions. The results showed that real-time diagnosis of the gastric cancerous lesions by using M i-scan corresponded well with their histopathology. In comparisons between different enhancement capabilities using offline images, images with surface enhancement and tone enhancement were found to be slightly superior to those with color enhancement.

Li CQ, Li Y, Zuo XL, Ji R, Li Z, Gu XM, Yu T, Qi QQ, Zhou CJ, Li YQ. Magnified and enhanced computed virtual chromoendoscopy in gastric neoplasia: A feasibility study. *World J Gastroenterol* 2013; 19(26): 4221-4227 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i26/4221.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i26.4221

INTRODUCTION

Since conventional endoscopy has limited definition and magnification, detection and characterization of early gastric cancer are still challenging in daily practice. Recently, advanced endoscopy techniques have been introduced to improve the diagnosis of early gastric cancer, such as chromoendoscopy with dyes[1], acetic acid-enhanced endoscopy[2,3], magnification endoscopy[4] and dyeless virtual chromoendoscopy[5]. Incorporation of magnification endoscopy and chromoendoscopy[6] or enhanced endoscopy[7] into one instrument is perfect, because chromoendoscopy and enhanced endoscopy serve as the red flag in detection, while magnification endoscopy serves in characterization. Magnified virtual chromoendoscopy is more preferable than dye spraying magnification chromoendoscopy for efficiency and safety[5]. One example is magnified narrow band imaging (M-NBI)[8-16]. Clinical trials suggest that M-NBI is helpful in the diagnosis of Barrett's esophagus[17-21], small colorectal lesions[22] and early gastric cancer[8,23]. Along with NBI, multi-band imaging virtual chromoendoscopy, such as Fuji Intelligence Chromoendoscopy (FICE)[24-30] and Pentax i-scan, are also available in clinical practice.

Unlike NBI, FICE and i-scan use reflection band filtering to achieve color enhancement of the mucosa. The instrument in this study not only incorporates color enhancement but also surface enhancement and magnification (M i-scan). The principle of surface enhancement is to adjust the dark-to-light contrast of the nearby pixels in order to show sharper surface details.

The aim of this study is to assess the accuracy of a real-time M i-scan in the diagnosis of gastric neoplasia (primary outcome). A comparison between magnified virtual chromoscopy and non-magnified virtual chromoscopy was made by using post-endoscopy still images (secondary outcome).

MATERIALS AND METHODS

Patients

From January 1st to March 31st 2012, consecutive patients who underwent high definition gastroscopy in Shandong University Qilu Hospital were recruited into this study. Patients aged 18-80 years, having superficial lesions with diameter less than 1 cm were included. Those with advanced or obviously protruded or depressed lesions, lesions larger than 1.0 cm and/or lesions which were not amenable to observation by zoom function (poor preparation, difficult positions, and non-cooperation of patients) were excluded. This study was approved by the local ethics committee (Ethics Committee of Shandong University Qilu Hospital) and adhered to the Declaration of Helsinki for Medical Research involving Human Subjects-Ethical Principles for Medical Research Involving Human Subjects. All the patients who participated in this study have provided their written informed consents.

Endoscopic procedure

The instruments applied in this study were an EG-2990Zi endoscope (Pentax, Tokyo, Japan) and an EPK-i endoscopic system (Pentax, Tokyo, Japan). This high definition endoscope incorporated surface enhancement (at +2, +4 and +6 levels), color enhancement (+4, +5 and +6 levels) and tone enhancement functions. It is also equipped with an adjustable image magnification in a continuous range up to 100-fold. The diameter and the length of the insertion tube of this instrument are the same as those of a standard upper endoscope. To achieve the maximum magnification, a transparent hood was attached to the distal tip of the endoscope to fix the distance between endoscope and gastric mucosa at 2 mm.

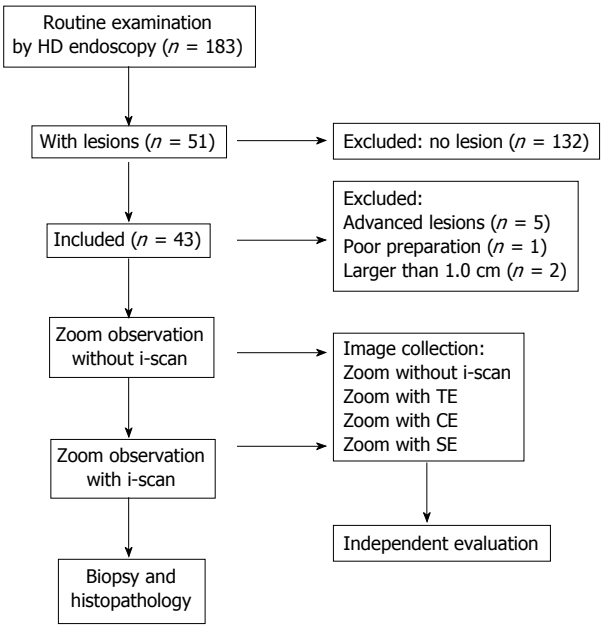

Figure 1 Study flow diagram. TE: Tone enhancement; SE: Surface enhancement; CE: Color enhancement.

All the patients underwent routine preparation before the procedure. The detected lesions were observed with magnification endoscopy in white light (MWL) mode and in enhancement (M i-scan) mode consecutively. The endoscopic procedures were performed by an experienced endoscopist who was familiar with magnification endoscopy diagnosis of early gastric cancer. The endoscopist was required to give the real-time descriptions of surface pit patterns of the lesions, based on surface pattern classification of enhanced magnification endoscopy. The surface pattern classification includes 5 types: type Ⅰ, small round pits of uniform size and shape; type Ⅱ, slit-like pits; type Ⅲ, gyrus and villous patterns; type Ⅳ, irregular arrangement and size; and type Ⅴ, destructive pattern. According to previous reports, types Ⅰ-Ⅲ represent non-neoplastic lesions, and types Ⅳ-Ⅴ represent neoplastic lesions[7]. Real-time diagnoses to determine neoplasia or non-neoplasia were not required from the endoscopist. Instead, the diagnoses were made by another investigator according to the diagnostic strategy and real-time description above. Images of MWL [without tone enhancement (TE), surface enhancement (SE) and color enhancement (CE)] and i-scan (with "g" TE, +2 SE or +4 CE) were collected and stored on USB devices during the procedures. Four best quality images per lesion were selected and sorted randomly by the investigator.

Post-endoscopy still image analysis

The selected images were sent to another endoscopist who did not participate in any of the endoscopic procedures. The endoscopist was kept blind to the clinical and endoscopic information of the patients. The endoscopist rated the image quality of each lesion at 3 levels. Ratings of image quality were based on visualization of pit pattern, vessel, and demarcation line[22], which are key parameters to detect and characterize the gastric neoplasia. Rating scales of image quality were: pit pattern, 0 for unassessable, 1 for fine, 2 for excellent; vessel, 0 for invisible, 1 for visible, 2 for clearly visible; demarcation line, 0 for unassessable, 1 for fine, 2 for clear. The endoscopist then recorded the descriptions of the still images according to the same standards as applied in the real time observation[7].

Biopsy and histopathology

The lesions were routinely biopsied, and the specimens were placed in 10% formalin solution and processed in the routine manner. The slices were examined by an experienced pathologist who had specific training in gastrointestinal pathology. The pathologist was kept blind to the clinical and endoscopic information of the patients. The histology report was based on the WHO (World Health Organization) classification of gastrointestinal tumors. The study flow diagram is illustrated in Figure 1.

Statistical analysis

Diagnostic accuracy of gastric neoplasia by using real-time M i-scan was presented with sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and likelihood ratio (LR). The agreement between real time M i-scan and histopathology was presented with kappa values (0.1-0.2 were considered slight agreement, 0.21-0.4 fair agreement, 0.41-0.6 moderate agreement, 0.61-0.8 substantial agreement and 0.81-0.99 almost perfect agreement). Parameters of still image quality were presented as mean ± SD, and differences of magnification image quality between MWL and i-scan were determined by one-way ANOVA test. A P value < 0.05 is considered to be significant. All data were analyzed by SPSS 13.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

Patients

One hundred and eighty-three patients were recruited. Five patients were excluded for advanced gastric lesions, 1 patient was excluded for poor preparation and 2 patients were excluded for superficial lesions larger than 1.0 cm; 132 patients were excluded for no lesions found by high definition endoscopy. In the end, 43 patients with 43 lesions were included. The average age of the patients was 47.5 (18-74) years, of which 32 were males. Locations of the lesions were: 5 in cardia and fundus, 2 in body, 4 on angle and 32 in antrum. All the lesions could be easily identified and zoomed. Histopathology revealed 10 inflammation, 14 atrophy, 10 metaplasia, 1 low grade dysplasia (LGD), 5 high grade dysplasia (HGD) and 3 cancers.

Real-time diagnosis by M i-scan

For 7 lesions classified into type Ⅰ, histopathology revealed 6 atrophy and 1 metaplasia; for 10 lesions classified into type Ⅱ, histopathology revealed 2 inflammation, 7 atrophy and 1 metaplasia; for 10 lesions classified into type Ⅲ, histopathology revealed 1 inflammation, 8 meta-

Table 1 Histopathology and pit patterns of the lesions classified by M i-scan

Pit	Histology						Total
	Inflammation	Atrophy	Metaplasia	LGD	HGD	Cancer	
Type Ⅰ	0	6	1	0	0	0	7
Type Ⅱ	2	7	1	0	0	0	10
Type Ⅲ	1	0	8	1	0	0	10
Type Ⅳ	4	1	0	0	4	0	9
Type Ⅴ	3	0	0	0	1	3	7
Total	10	14	10	1	5	3	43

HGD: High grade dysplasia; LGD: Low grade dysplasia.

plasia and 1 LGD; for 9 lesions classified into type Ⅳ, histopathology revealed 4 inflammation, 1 atrophy and 4 HGD; for 7 lesions classified into type Ⅴ, histopathology revealed 3 inflammation, 1 HGD and 3 cancers. The real-time descriptions of pit patterns and the corresponding histopathology are shown in Table 1. Typical images representing pit patterns of types Ⅰ-Ⅴ are illustrated in Figure 2.

When the histopathology was re-classified into 2 categories (as non-cancerous lesions including inflammation, atrophy, metaplasia and LGD, or cancerous lesions including HGD and cancer) and the pit patterns re-classified into 2 categories as described above, sensitivity, specificity, PPV, NPV and likelihood ratio of M i-scan regarding gastric neoplasia were 100%, 77.1%, 50%, 100% and 4.37% respectively. Kappa value calculated from agreement between M i-scan and histopathology was 0.557 (moderate agreement). The diagnostic yield after re-classification is shown in Table 2.

Post-endoscopy still image analysis

A total of 172 still images, including 43 images by MWL and 129 images by M i-scan (43 with TE, 43 with SE and 43 with CE), were selected and sent to the endoscopist who did the analysis. General image quality of M i-scan with TE and SE was significantly better than that of MWL (TE, 4.55 ± 1.07; SE, 4.30 ± 1.02; MWL, 3.25 ± 0.99; $P < 0.001$). Regarding the 3 key parameters, visualization of pit pattern was significantly improved by M i-scan with SE (1.93 ± 0.25 *vs* 1.50 ± 0.50, $P < 0.001$). Microvessel visualization was significantly improved by M i-scan with TE (1.23 ± 0.78 *vs* 0.76 ± 0.73, $P < 0.001$). Demarcation line visualization was improved by both M i-scan with TE and SE (TE, 1.75 ± 0.52; SE, 1.56 ± 0.59; MWL, 0.98 ± 0.44; $P < 0.001$). M i-scan with CE did not show any significant improvements of image quality in general or in the 3 key parameters.

Descriptions of the still images based on lesions demonstrated that diagnosis by MWL revealed a sensitivity, specificity, PPV, NPV and LR of 87.5%, 71.4%, 41.2%, 96.2% and 3.06%, respectively. Although M i-scan with TE and SE slightly increased the diagnostic yield, there was no significant difference ($P > 0.1$). M i-scan with CE did not change the diagnostic yield by MWL. M i-scan with SE perfectly matched the results of real-time

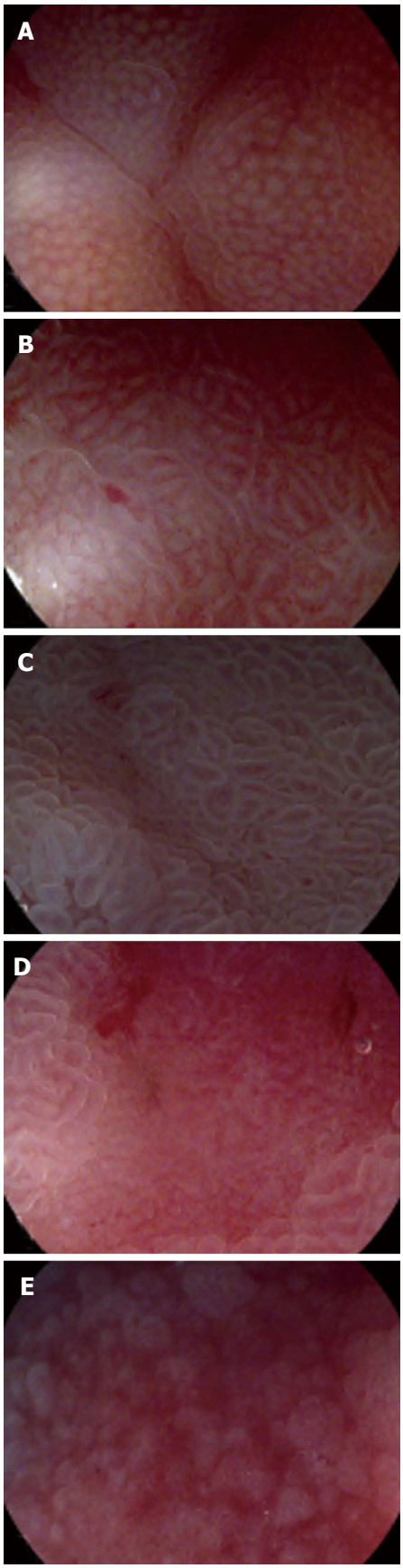

Figure 2 Images representing typical pit pattern classification by M i-scan. A: Type Ⅰ, small round pits of uniform size and shape; B: Type Ⅱ, slit-like pits; C: Type Ⅲ, gyrus and villous patterns; D: Type Ⅳ, irregular arrangement and size; E: Type Ⅴ, destructive pattern.

Table 2 Diagnostic yield of gastric neoplasia by real time M i-scan

M i-scan	Histopathology		Total
	Cancerous	Non-cancerous	
Neoplasia	8	8	
Non-neoplasia	0	27	
Total	8	35	43

Sensitivity: 100%; Specificity: 77.1%; Positive predictive value: 50%; Negative predictive value: 100%; Likelihood ratio: 4.37.

descriptions. Representative images showing image quality differences among different modes are illustrated in Figure 3.

DISCUSSION

Detection and characterization of early gastric cancer by dyeless virtual chromoendoscopy, such as NBI and computed virtual chromoendoscopy (CVC), are preferable for the endoscopist, because of time, labor and potential risks reduction[31,32]. Virtual chromoendoscopy with magnification is thought to be the dream team, since the former provides the detection "red flag" followed by real-time characterization by the latter. It was reported that real-time characterization of Barrett's esophagus[33,34], gastric cancer[35,36] and colorectal adenoma[37,38] can be improved by dyeless virtual chromoendoscopy, such as NBI or FICE[25-29,39]. In this pilot feasibility study, we aimed to evaluate application of M i-scan in the diagnosis of small superficial gastric lesions, both in real-time investigation and post-endoscopy still image analysis. The preliminary results showed that M i-scan is helpful for the *in vivo* prediction of small gastric superficial lesions with excellent sensitivity and NPV, acceptable specificity and LR, and poor PPV. The post-endoscopy still image analysis showed that M i-scan with TE and SE can slightly increase the image quality.

One feature of M i-scan is to mimic the surface enhancement of EME by acetic acid spraying. In this study, the still image analysis showed that SE significantly improves visualization of surface pit pattern and demarcation line compared to MWL. Although there were excellent sensitivity and NPV, and acceptable specificity results, the PPV was poor, just as the results of enhanced magnification endoscopy[7]. This is partly due to the low percentage of neoplastic lesions in the sample (18.6%, 8/43). On the other hand, erosion is sometimes difficult to be differentiated from neoplasia by surface pit pattern evaluation, as in both lesions surface pits could be lost. In these cases, evaluation of microvessel pattern in addition to surface pit pattern may be helpful. However, observation of microvessels is not satisfactory by M i-scan. Although still image analysis shows that TE significantly improves the visualization of microvessels, which only happens in cases with visible microvessels (visible to clearly visible), visualization of those cases with invisible microvessels (41.2%) remains unchanged.

This study has several limitations. Firstly, this is a feasibility study with small sample size and no sample size calculation. Secondly, the detection rate of small superficial gastric lesions was not evaluated. There has not been any report on the detection rate of small gastric lesions by CVC yet. In our own practice, CVC is not suitable for screen gastroscopy with insufficient luminous intensity. Thirdly, only one endoscopist performed the real-time and still image analysis, so there was no interobserver agreement analysis. However, the perfect match between surface classification of real-time and still image with

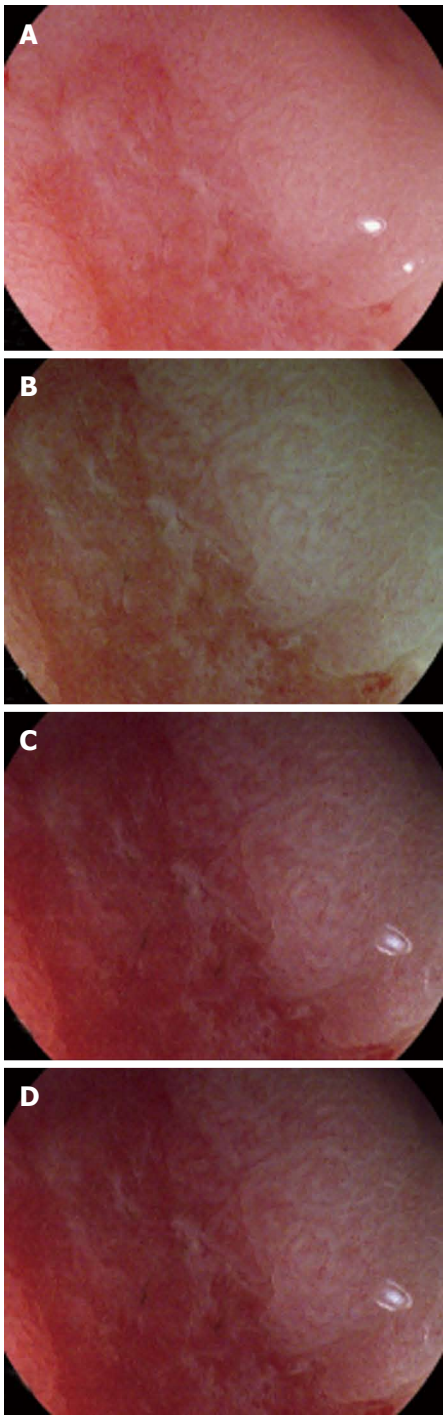

Figure 3 Representative images showing white light (A), M i-scan with tone enhancement (B), surface enhancement (C) and color enhancement (D), respectively.

SE suggests an excellent consistency, which should be validated in future studies. Fourthly, there was no comparison between M i-scan and magnification chromoendoscopy with indigo carmine or other contrast agents. And finally (the last may not be the least), gold standard histopathology was only performed by biopsy. Although we only included lesions smaller than 1.0 cm to minimize the heterogeneity, a discrepancy between biopsy and autopsy still remains.

In conclusion, real-time prediction of the histopathology of small superficial gastric lesions by M i-scan is feasible. Although digital enhancement increases image quality, its value in the diagnosis of gastric neoplasia seems to be limited.

COMMENTS

Background

Magnified chromoendoscopy is a promising tool in the surveillance and diagnosis of gastric neoplasia. Enhanced magnification endoscopy is superior to conventional endoscopy with detailed surface characterization.

Research frontiers

Dyeless virtual chromoendoscopy with magnification might be preferable for reduction of labor and health risks. The endoscope used in this study is a magnification endoscope with both color and surface enhancement.

Innovations and breakthroughs

To date, this is the first endoscopic device with surface enhancement mimicking acetic acid spraying enhanced magnification endoscopy. With the surface enhancement, the gastric pit patterns can be classified into 5 categories according to the classification from enhanced magnification endoscopy, which enables the detailed characterization of the gastric mucosa. With classification of gastric pits, different common gastric pathologies such as atrophy, intestinal metaplasia and neoplasia can be identified in real-time procedures or by still image analysis. The margin of gastric lesions can be more easily identified although the differences were not significant.

Terminology

Although digital enhancement improves the image quality of magnification endoscopy, its value in improving the diagnostic yield seems to be limited.

Peer review

This is a quite interesting study on virtual chromoscopy on gastric neoplasia. However, data are limited.

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BRIEF ARTICLE

Effects of propranolol or propranolol plus isosorbide-5-mononitrate on variceal pressure in schistosomiasis

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Abstract

AIM: To compare the effects of propranolol (PR) to that of PR plus isosorbide-5-mononitrate (ISMN) on variceal pressure in patients with schistosomiasis.

METHODS: Forty-eight patients with schistosomiasis who had no previous variceal bleeding were treated with PR alone or PR plus ISMN. Seven patients refused variceal pressure manometry (3 receiving PR and 4 receiving PR plus ISMN). One patient withdrew from the trial due to headache after taking ISMN. At the time of termination, twenty patients were randomly assigned to treatment with PR plus ISMN or PR alone. The dose of PR was adjusted until the resting heart rate had been reduced by 25% or was less than 55 bpm. In the PR plus ISMN group, after PR was titrated to the same target, the dose of ISMN was increased up to 20 mg orally twice a day. Variceal pressure was measured using a noninvasive endoscopic balloon technique at the end of the 6-mo treatment period.

RESULTS: In 40 patients (20 in the PR group and 20 in the PR plus ISMN group), variceal pressure was measured before treatment and at the end of the 6-mo treatment period. PR or PR plus ISMN treatment caused a significant reduction in variceal pressure (PR group: from 24.15 ± 6.05 mmHg to 22.68 ± 5.70 mmHg, P = 0.001; PR plus ISMN group: from 25.69 ± 5.26 mmHg to 20.48 ± 5.43 mmHg; $P < 0.001$). The percentage decrease in variceal pressure was significant after PR plus ISMN compared with that after PR alone (15.93% ± 8.37% *vs* 6.05% ± 3.67%, P = 0.01). One patient in the PR plus ISMN group and two patients in the PR group had variceal bleeding during follow-up. There were no significant differences between the two groups regarding the incidence of variceal bleeding. In the PR plus ISMN group, three patients had headache and hypotension. The headache was mild and transient and promptly disappeared after continuation of the relevant drug in two patients. Only one patient withdrew from the trial due to severe and lasting headache after taking ISMN. No side effects occurred in the PR group.

CONCLUSION: PR plus ISMN therapy may be an alternative treatment for patients with schistosomiasis who have a high risk of bleeding.

Key words: Esophageal varices; Schistosomiasis; Portal hypertension; Bleeding; Propranolol; Variceal pressure; Isosorbide-5-mononitrate

Core tip: The results of the present study suggested that the combination of propranolol and isosorbide-5-mononitrate was more effective than propranolol alone in decreasing variceal pressure. This drug combination will reduce the rate of bleeding in patients with schistosomiasis, high-risk esophageal varices and no previous history of variceal bleeding.

Kong DR, Ma C, Wang M, Wang JG, Chen C, Zhang L, Hao JH, Li P, Xu JM. Effects of propranolol or propranolol plus isosorbide-5-mononitrate on variceal pressure in schistosomiasis. *World J Gastroenterol* 2013; 19(26): 4228-4233 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i26/4228.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i26.4228

INTRODUCTION

Variceal bleeding is the most frequent and severe complication of portal hypertension in patients with cirrhosis. Identification of those who have a high risk of variceal hemorrhage is effective for preventive therapy in patients with a high disease predisposition[1]. Variceal size and the red color sign are considered to be the most important endoscopic parameters in predicting variceal bleeding[2]. However, endoscopic findings alone can not be used to reliably predict the risk of variceal bleeding. The formation of esophageal varices depends on an elevation in portal pressure; a hepatic venous pressure gradient (HVPG) greater than 10 mmHg is necessary for the development of and bleeding from esophageal varices[3-6]. On the other hand, a more rational approach would be to guide pharmacologic therapy based on hemodynamic response, defined as a decrease in HVPG to < 12 mmHg or a decrease of > 20% from baseline levels[7]. However, limitations to the generalized use of HVPG measurement are the lack of local expertise and poor adherence to guidelines that will ensure reliable and reproducible measurements, and its invasive nature[5]. In the majority of published studies, the dose of nonselective β-blockers was titrated to decrease the heart rate by 25% from baseline or maximal tolerated doses[5,7].

Propranolol (PR) or isosorbide-5-mononitrate (ISMN) is effective in preventing the first variceal bleeding in patients with cirrhosis[1,5]. ISMN enhances the reductive effect of PR on variceal pressure in cirrhotic patients[1,5]. In contrast to liver cirrhosis, published data regarding the effect of PR on schistosomiasis-related portal hypertension are scarce and contradictory, and the effect of ISMN plus PR treatment is unknown in these patients[8,9]. A short-term study in patients with schistosomiasis and previous variceal bleeding after PR treatment found that the portal pressure was not decreased[8]. Moreover, the required mean dose to achieve a 20%-25% reduction in heart rate from baseline was up to 400 mg/d[8]. Cohort studies indicated that PR treatment achieved a reduction in rebleeding rates and increased the survival of patients with no serious side effects[9]. Recently, a study from Brazil found that PR significantly reduced variceal pressure in schistosomiasis patients who had never bled[10]. However, it is not clear whether ISMN plus PR is better than PR alone in the treatment of schistosomiasis patients who had never bled. In this study, we will ascertain whether the combination of PR and ISMN is more effective than PR alone in decreasing variceal pressure.

MATERIALS AND METHODS

Selection of patients

From September 2007 to October 2010, patients admitted to our hospital due to schistosomiasis-related portal hypertension were assessed for inclusion in the trial. The diagnosis of schistosomiasis was established in accordance with the World Health Organization criteria[11]. The eligibility criteria were age between 18 and 65, schistosome eggs in stool specimens, the characteristic ultrasound criteria, and endoscopic evidence of esophageal varices. The exclusion criteria were previous treatment for portal hypertension (*e.g.*, beta-blockers, sclerotherapy, or endoscopic band ligation), severe hepatic disease (*e.g.*, Child-Pugh score higher than 12 points or hepatorenal syndrome), previous variceal bleeding, presence of any neoplastic disease, portal vein thrombosis, inability to attend follow-up, contraindications to beta-blockers (severe chronic pulmonary obstructive disease, asthma, severe insulin-dependent diabetes mellitus, heart failure, grade II atrioventricular block, sinus bradycardia < 50 bpm, aortic stenosis, peripheral arterial disease, arterial hypotension with systolic pressure < 85 mmHg), or long-acting nitrates (glaucoma). The study was approved by the Ethics Committee of Anhui Medical University, and all patients gave written informed consent to participate in the study. Patients were assigned to one of two treatment groups according to the sequential method of randomization.

Treatment

Patients who fulfilled the inclusion and exclusion criteria were immediately randomized into the two treatment groups using consecutively numbered envelopes that contained the treatment assignments, which were generated by a system using computer-allocated random digit numbers. PR was given orally at an initial dose of 20 mg 3 times daily. The dose was subsequently adjusted over a period of 5 d until the resting heart rate had been reduced by 25% or was less than 55 bpm. In the PR plus ISMN group, after PR was titrated to the same target in resting heart rate, the dose of ISMN was increased up to an oral dose of 20 mg twice a day.

Methods

Measurement of variceal pressure was performed after

Table 1 Demographic profile of the study population

	PR group (n = 20)	PR + ISMN group (n = 20)	P value
Sex			0.619
Male	12	11	
Female	8	9	
Age (yr)	47.87 ± 15.16	44.14 ± 9.51	0.585
Child-Pugh grade			1.000
A	9	8	
B	11	12	
Child-Pugh score	8.87 ± 1.88	8.00 ± 1.63	0.358
Albumin (g/L)	30.63 ± 3.82	33.34 ± 5.30	0.271
Total bilirubin (μmol/L)	29.45 ± 17.02	25.11 ± 11.26	0.577
Prothrombin time (s)	16.80 ± 1.82	16.65 ± 1.59	0.875
VP (mmHg)	24.15 ± 6.05	25.69 ± 5.26	0.248
Varix grade			0.608
F2	10	9	
F3	10	11	
Red color signs	12	14	1.000

VP: Variceal pressure; PR: Propranolol; ISMN: Isosorbide-5-mononitrate.

an overnight fast during upper gastrointestinal endoscopy. Variceal pressure was assessed with a previously described noninvasive technique using an esophageal variceal manometer (EVM; Esophageal Varix Manometer; Treier Endoscopie AG, Beromünster, Switzerland) and recorded by the workstation which was developed by our group[12,13]. To minimize esophageal tonus and peristalsis, all patients received premedication with 5 mg diazepam and 20 mg *n*-butylscopolamine intravenously. The reliability of the endoscopic measurement of variceal pressure was determined in a previous study which found a good correlation with needle puncture measurement[13-15]. In the current study, endoscopic measurement of variceal pressure was used because of the unique hemodynamic pattern of pre-sinusoidal portal hypertension. The largest varix situated above the cardia was chosen for measurement of variceal pressure. The pressure in each patient was measured five times. Variceal pressure was calculated as the mean of five satisfactory measurement periods recorded.

After variceal pressure measurement, the size of the varix was estimated in the absence of peristaltic waves, by comparing the varix with the scales in the balloon variceal markers (5-mm intervals). The maximal size of the varices and the red color signs were recorded as proposed by the Japanese Research Society for portal hypertension[16].

Follow-up and endpoints

All patients were followed in the outpatient clinics at 3-month intervals and assessed for adverse events, compliance (direct questioning, prescription renewal, and reinforcement), variceal bleeding, and progression of liver disease. Variceal pressures in all patients were measured before and after 6 mo of continuous PR or PR plus ISMN therapy. The primary end point was variceal bleeding and secondary end points were treatment-related complications and mortality. Variceal bleeding was defined as hemetemesis or melena, with an associated drop in hematocrit by 10%, in the absence of any other source of gastrointestinal bleeding on endoscopy. In the case of variceal bleeding, physicians were free to choose endoscopic treatment to prevent rebleeding.

Statistical analysis

Statistical analyses were performed with SPSS (version 10; SPSS, Inc., Chicago, IL, United States). All quantitative data were tested for normal distribution. Quantitative data were expressed as mean ± SD if the data were normally distributed. Each continuous parameter was analyzed with the independent-samples t-test. The paired-samples t-test was used to examine change from baseline to follow-up. Categorical data were examined using Fisher's exact test. P-values < 0.05 were considered statistically significant.

RESULTS

Baseline data

Twenty-five patients received PR plus ISMN and 23 patients received PR alone (dosage of PR: 60 to 160 mg/d, median: 80 mg; dosage of ISMN: 20 mg/d). Seven patients refused to variceal pressure manometry (3 receiving PR and 4 receiving PR plus ISMN). One patient withdrew from the trial due to headache after taking ISMN. Therefore, there were 20 patients in each treatment group. Clinical and endoscopic data of the patients in the subsets are shown in Table 1. There were no significant differences between the two groups at baseline with regard to clinical and demographic characteristics or baseline variceal pressure (Table 1, PR group = 24.15 ± 6.05 mmHg; PR plus ISMN = 25.69 ± 5.26 mmHg).

Changes in variceal pressure

In 40 patients (20 in the PR group and 20 in the PR plus ISMN group), variceal pressure was measured again the end of a 6-mo continuous treatment period. PR or PR plus ISMN caused a significant reduction in variceal pressure (PR group: from 24.15 ± 6.05 mmHg to 22.68 ± 5.70 mmHg, P = 0.001; PR plus ISMN group: from 25.69 ± 5.26 mmHg to 20.48 ± 5.43 mmHg; P < 0.001). The percentage decrease in variceal pressure after PR plus ISMN was more significant than that after PR alone (Table 2, 15.93% ± 8.37% *vs* 6.05% ± 3.67%, P = 0.01).

Bleeding

One patient in the PR plus ISMN group and two patients in the PR alone group had variceal bleeding during the 6-mo follow-up period. There were no significant differences between the two groups regarding the incidence of variceal bleeding.

Adverse effects

In the PR plus ISMN group, three patients had headache and hypotension. The headache was mild and transient and promptly disappeared after continuation of the rel-

Table 2 Effects of propranolol and propranolol plus isosorbide-5-mononitrate on variceal pressure, liver function and systemic hemodynamics in patients with 6 mo of follow-up

	PR		PR + ISMN	
	Baseline	6 mo	Baseline	6 mo
(ΔVP)%	0	15.93 ± 8.37	0	6.05 ± 3.67[a]
ALB (g/L)	30.63 ± 3.82	31.14 ± 3.08	33.34 ± 5.30	34.30 ± 5.09
TB (umol/L)	29.45 ± 17.02	27.26 ± 12.27	25.11 ± 11.26	26.74 ± 12.96
SBP (mmHg)	132 ± 20	124 ± 21[d]	130 ± 19	125 ± 19[d]
DBP (mmHg)	77 ± 10	72 ± 11[d]	74 ± 10	70 ± 13[d]

[a]$P < 0.05$ *vs* PR group, [d]$P < 0.01$ *vs* baseline. (ΔVP)%: Percentage difference in variceal pressure from baseline; PR: Propranolol; ISMN: Isosorbide-5-mononitrate; ALB: Albumin; TB: Total bilirubin; SBP: Systolic blood pressure; DBP: Diastolic blood pressure.

evant drug in two patients. One patient withdrew from the trial due to severe and lasting headache after taking ISMN. No side effects occurred in the PR group. There was no worsening of liver function or impairment of renal function in the 2 groups within the 6-mo treatment period (Table 2).

DISCUSSION

Nonselective β-blockers are the most commonly used drugs to prevent variceal bleeding in patients with cirrhosis and esophageal varices[6,14]. Although many trials have shown that variceal hemorrhage risk was reduced with β-blockers, these drugs do not protect all treated patients, probably due to an inadequate decrease in the HVPG[5,17]. Most published studies have shown that PR and ISMN have a synergistic effect on reducing portal pressure and a combination of the two could be more effective than PR alone[17,18]. Recently, PR was found to significantly reduce variceal pressure and wall tension in patients with schistosomiasis[10]. However, it is uncertain whether the combination of PR and ISMN is more effective than PR alone in decreasing variceal pressure in schistosomiasis patients who have never bled.

This study investigated the efficacy of PR compared with PR plus ISMN in schistosomiasis patients that had never bled. Our approach was to assess variceal pressure in patients with high-risk varices, using the same methodology reported for cirrhotic patients[12]. Variceal bleeding is believed to occur when the tension exerted over the thin wall of the varices increases beyond a critical value determined by the elastic limit of the vessel[5]. Variceal pressure and size are key factors determining variceal wall tension. Not only is variceal pressure the best parameter for predicting rupture of varices and consequent complications, but it is also a useful guide for studying the effect of the pharmacotherapy of portal hypertension and a measure of the effects of transjugular intrahepatic portosystemic shunting[6,19-21]. As confirmed by one study, the measurement of variceal pressure can efficiently monitor the direct effect of the prophylaxis of variceal bleeding compared with the rate of bleeding in cirrhotic patients[17].

In the present study, we observed that PR and PR plus ISMN administration caused a significant reduction in variceal pressure in patients with schistosomiasis. After a 6-mo continuous treatment period, the percentage decrease in variceal pressure was more obvious in patients receiving PR plus ISMN than PR alone (15.93% ± 8.37% *vs* 6.05% ± 3.67%, $P = 0.01$). Thus, the results of our study suggest that PR plus ISMN is superior to PR alone in reducing variceal pressure in patients with schistosomiasis. These results are consistent with the data from different randomized clinical trials which show that the effect in patients treated with combined pharmacological therapy was greater than that obtained with PR alone[17,18]. Therefore, the pharmacological therapy of choice in the prevention of variceal bleeding is probably the combination of PR and ISMN.

The mean dosage of PR used in our study was lower than that in other studies for cirrhotic patients and schistosomiasis patients[1,5]. However, the low dosage of PR in the current study was expected because it is well know that the metabolism of this drug is different between Asian and European patients[22]. In a previous study, Lay *et al*[23] found that the mean daily dosage of PR was 68.2 ± 32.8 mg, which was sufficient to reduce the heart rate by 25%. Therefore, it is possible that a lower dosage of PR to reach a target heart rate reduction of 25% would have enough power to result in a lower bleeding rate in a Chinese population.

Three patients treated with PR plus ISMN experienced side effects. Most reported side effects caused by β-blockers (hypotension, tiredness, breathlessness, poor memory, insomnia) can be easily managed by adjusting the dose of the medication, which does not affect the treatment effect. ISMN may increase vasodilatation leading to more side effects such as headache and hypotension[1,5]. In a trial performed in patients with cirrhosis and ascites which compared β-blockers with ISMN, the latter medication was associated with more side effects[24]. Furthermore, other studies also found a trend toward more side effects requiring withdrawal of the combination therapy compared with PR alone[17,18,25]. In our study, two patients experienced mild and transient headache on the first administration of ISMN, which disappeared after continuation of the relevant medication. One patient withdrew from the trial due to severe and lasting headache after taking PR plus ISMN. When the resting heart rate was reduced by 25% or was less than 55 bpm, most patients showed a significant reduction in variceal pressure (15.93% ± 8.37%) after receiving PR plus ISMN.

We are aware of the limitations of the current study. First, we found that the measurement of variceal pressure is technically difficult and time consuming in patients with small varices, which may reduce the applicability of measurements in clinical practice. However, because very large varices and red color signs indicate imminent bleeding, these patients are at high risk of bleeding and require prophylactic measures even though their variceal pressure

is not high[26,27]. On the other hand, the measurement of variceal pressure is probably not very important in patients with very small varices due to rare bleeding[28-31]. Second, patients not suitable for PR plus ISMN therapy need to be investigated in future studies. Third, future randomized controlled studies with a larger number of patients are warranted to confirm these findings and to demonstrate the long-term decrease in the frequency of bleeding episodes and mortality.

In conclusion, we found that combination treatment with PR plus ISMN compared with PR alone, more effectively decreased variceal pressure in schistosomiasis patients. Future randomized controlled studies with a larger number of patients are warranted to demonstrate the long-term decrease in variceal pressure, to determine when side effects will outweigh the benefits, and monitor the frequency of bleeding episodes and mortality.

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COMMENTS

Background

Non-cirrhotic portal hypertension and gastrointestinal bleeding are complications of the infection caused by the intravascular parasitic trematode *Schistosoma mansoni*. The prophylactic treatment of variceal bleeding is therefore crucial in the management of these patients.

Research frontiers

Treatment with propranolol plus isosorbide-5-mononitrate resulted in a synergistic decrease in variceal pressure compared with propranolol alone in cirrhotic patients. However, this has not been demonstrated in non-cirrhotic portal hypertension caused by *Schistosoma mansoni* infection.

Innovations and breakthroughs

In this study, the authors found that the combination of propranolol and isosorbide-5-mononitrate was more effective than propranolol alone in decreasing variceal pressure, which is important in reducing the rate of bleeding in patients with schistosomiasis, high-risk esophageal varices and no previous history of variceal bleeding.

Applications

The results suggest that the combination of propranolol plus isosorbide-5-mononitrate should be recommended as the first prophylaxis of variceal bleeding in non-cirrhotic portal hypertension caused by *Schistosoma mansoni* infection. Additional studies with long-term follow-up are needed to confirm the results concerning mortality.

Peer review

The authors have compared the effect of propranolol alone with the combination of propranolol and isosorbide-5-mononitrate on variceal pressure in patients with portal hypertension due to schistosomiasis. The results suggested that the combination led to a more pronounced decrease of variceal pressure than propranolol did.

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META-ANALYSIS

Hepatitis B or C viral infection and risk of pancreatic cancer: A meta-analysis of observational studies

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Abstract

AIM: To investigate if there is an association between hepatitis B virus (HBV) or hepatitis C virus (HCV) infection and the risk of pancreatic cancer.

METHODS: All relevant studies published before 11 October, 2012 were identified by a systematic search of MEDLINE, EMBASE, BIOSIS Previews and the Cochrane Library databases and with cross-referencing. The observational studies that reported RR or OR estimates with 95%CIs for the association between HBV or HCV and pancreatic cancer were included. A random-effects model was used to summarize meta-analytic estimates. The Newcastle-Ottawa quality assessment scale was applied to assess the quality of the methodology in the included studies.

RESULTS: A total of 8 eligible studies were selected for meta-analysis. Overall, chronic hepatitis B and inactive hepatitis B surface antigen (HBsAg) carrier state (HBsAg positive) had a significantly increased risk of pancreatic cancer with OR of 1.20 (95%CI: 1.01-1.39), especially in the Chinese population (OR = 1.30, 95%CI: 1.05-1.56). Past exposure to HBV (possible occult HBV infection) had an increased OR of pancreatic cancer risk (OR = 1.24, 95%CI: 1.05-1.42), especially among those patients without natural immunity [anti hepatitis B core (HBc) positive/hepatitis B surface antibody (anti HBs) negative], with OR of 1.67 (95%CI: 1.13-2.22). However, past exposure to HBV with natural immunity (anti-HBc positive/anti-HBs positive) had no association with pancreatic cancer development, with OR 0.98 (95%CI: 0.80-1.16), nor did the HBV active replication (hepatitis B e antigen positive status), with OR 0.98 (95%CI: 0.27-1.68). The risk of pancreatic cancer among anti-HBs positive patients was significantly lower than among anti-HBs negative patients (OR = 0.54, 95%CI: 0.46-0.62). Past exposure to HCV also resulted in an increased risk of pancreatic cancer (OR = 1.26, 95%CI: 1.03-1.50). Significant between-study heterogeneity was observed. Evidence of publication bias for HBV/HCV infection-pancreatic cancer association was not found.

CONCLUSION: Chronic HBV and HCV infection increases pancreatic cancer risk. Our findings underscore the need for more studies to confirm this potential relationship.

Key words: Hepatitis B; Hepatitis C; Pancreatic cancer; Observational studies; Meta-analysis

Core tip: Based on the meta-analysis, we identified that chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection is associated with pancreatic cancer, especially among Chinese population. Patients with past exposure to HBV/HCV should be screened for hepatocellular carcinoma and other malignancies, especially pancreatic cancer.

Xu JH, Fu JJ, Wang XL, Zhu JY, Ye XH, Chen SD. Hepatitis B or C viral infection and risk of pancreatic cancer: A meta-analysis of observational studies. *World J Gastroenterol* 2013; 19(26): 4234-4241 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i26/4234.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i26.4234

INTRODUCTION

Pancreatic cancer is one of the most lethal and devastating human malignancies and the fourth leading cause of cancer-related fatality worldwide. Because of absence of early symptoms, lack of sensitive and specific tests to screen the cancer in the initial phases, limited therapeutic options and rapid progression, nearly all patients die of the disease within one year of diagnosis with the overall 5-year survival rate being less than 5%[1]. Therefore, it is crucial to identify the intrinsic genes as well as other risk factors, that may influence the progression of the cancer, and to develop more accurate screening programs for early monitoring and intervention strategies. Although several risk factors associated with pancreatic cancer have been explored, the causative factors for pancreatic cancer are far from being understood, one of which is chronic hepatitis infection[2,3].

The prevalence of hepatitis B virus (HBV) and hepatitis C virus (HCV) infection varies worldwide, ranging from less than 0.5% in Western countries to 7% and 25% in East Asian and African countries[4,5]. Infection with HBV is a huge global public health concern, especially in China. According to the World Health Organization, there are 2 billion people infected with HBV globally, with China accounting for 65% of the HBV infective public health burden of the world[6]. HBV/HCV have been detected not only in hepatotropic tissue but also in extrahepatic sites such as the pancreas[7,8]. Several studies reported conflicting results regarding the association between HBV infection and the risk of subsequent pancreatic cancer. Studies by Berrington de Gonzalez *et al*[9] and Hong *et al*[10] found no relationship between chronic infection with HBV and the development of pancreatic cancer, while several published studies found an association between presence of HBV infection and incidence of pancreatic cancer, not only in countries with a lower prevalence of HBV infection such as the United States but also in countries with a higher number of HBV infections such as China[11,12]. Meanwhile, little is known about the association between the presence of HCV infection and the risk of pancreatic cancer[3].

The purpose of the present study is to summarize all available evidence of observational studies in order to better define the impact of HBV and HCV infection on the risk of pancreatic cancer in patients following the meta-analysis of observational studies in epidemiological guidelines.

MATERIALS AND METHODS

Data sources and searches

A comprehensive literature search was carried out on observational studies and trials, and no language or time restriction was applied. All literature from January 1, 1980 to October 18, 2012 was searched using the following databases: Pubmed, ISI web of Science, Embase and Cochrane library. The following main keywords or corresponding MeSH terms were used: hepatitis, virus, viral, pancreas, cancer OR adenocarcinoma OR neoplasm OR tumor. A manual search was also performed for references cited in the selected articles.

Study selection

Studies were included in the meta-analysis if (1) they were the principal published reports of original data from case-control or cohort studies; (2) they were independent from other studies to avoid giving double weight to estimate the same study; (3) the exposure of interest was a history of HBV/HCV infection; (4) the outcome of interest was pancreatic cancer incidence or mortality; and (5) they had sufficient information to allow adequate estimation of OR or RR and 95%CI to estimate cancer risk under HBV/HCV exposure. Two authors (Fu JJ and Xu JH) independently evaluated all of the studies retrieved from database, then compared their results. Any disagreements were resolved by consensus.

Data extraction

The following data were extracted from each study: authors, publication year, study design, country of origin, sample size, measure of outcome, duration of follow-up, marker of hepatitis serostatus, covariates adjusted for in the analysis, and the effect estimates with corresponding 95%CIs.

Quality evaluation

The Newcastle-Ottawa quality assessment scale (NOS)[13] was applied to assess the quality of the methodology in the included studies. A star system was used to judge the data according to the selection populations, comparability of groups and exposure/outcome of interest. The NOS scale consists of 8 questions with 9 possible points. The assessment score ranged from 0 to 9. Studies with a total score of 6 or lower indicated low quality while study

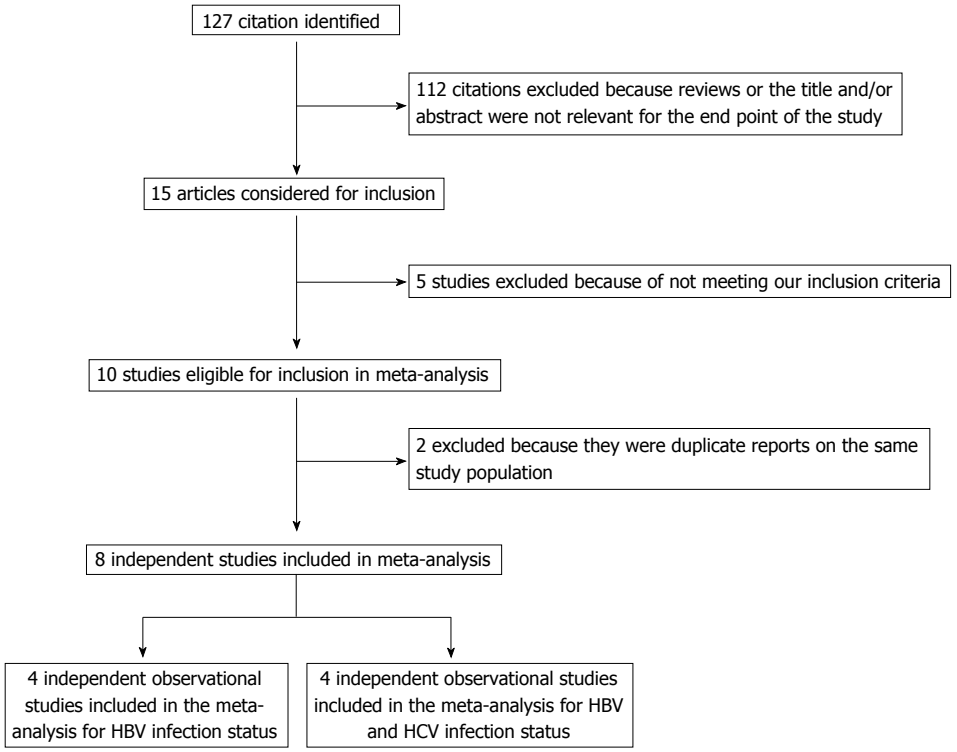

Figure 1 Flowchart of selection of studies for inclusion in meta-analysis. HBV: Hepatitis B virus; HCV: Hepatitis C virus.

Table 1 Assessment of study quality

Ref.	Quality indicators from NOS									Score
	Selection				Comparability		Exposure/outcome			
	1	2	3	4	5	6	7	8	9	
Hong *et al*[10]	Yes	Yes	No	Yes	Yes	No	Yes	Yes	No	6
Hassan *et al*[11]	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	7
Wang *et al*[18]	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	7
Zhu *et al*[17]	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	7
Ben *et al*[12]	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	7
Berrington de Gonalez *et al*[9]	Yes	Yes	Yes	Yes	Yes	No	Yes	No	No	6
El-Serag *et al*[3]	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	8
Iloeje *et al*[2]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	9

NOS: Newcastle-Ottawa quality assessment Scale. For case-control studies: (1) represents cases with independent validation; (2) cases are consecutive or representative; (3) controls are community; (4) controls have no history of pancreatic cancer; (5) study controls are comparable for age and sex; (6) study controls for any additional factor(s); (7) cases and controls have the same method of ascertainment; (8) was follow-up long enough for outcomes to occur; and (9) cases and controls have complete follow-up. For cohort studies: (1) indicates the exposed cohort study representative of the population; (2) the non exposed cohort drawn from the same population; (3) the exposure ascertainment are from secure record or structured interview; (4) the pancreatic cancer was not present at start of study; (5) cohorts are comparable for age and sex; (6) cohorts are comparable for any additional factor(s); (7) assessment of pancreatic cancer is from secure record; (8) follow-up long enough for pancreatic cancer to occur; and (9) complete follow-up.

scores of 7 or higher were considered to be of high quality. Two reviewers (Xu JH and Fu JJ) independently evaluated and cross-checked the qualities of the included studies (Table 1).

Statistical analysis

Statistical analyses were completed with STATA version 10.0 (STATA, College Station, TX, United States). Summary odd ratio estimates with the corresponding 95%CIs were combined and weighted to produce pooled ORs using a random-effects model, which considers both within- and between-study variations[14]. Q and I^2 statistics were both examined to investigate the source of heterogeneity across studies. I^2 values of 25, 50 and 75% were assigned to low, moderate, and high heterogeneities, respectively[15]. The Begg's adjusted rank correlation test and the Egger's regression test (significant at $P < 0.1$) were performed to test for evidence of publication bias[16].

RESULTS

Description of the studies

The participant flow diagram for the study inclusion in the meta-analysis is shown in Figure 1. A total of 8 articles were retrieved and checked for relevance in terms of infectious status, population studied, and reporting of pancreatic cancer risk data[2,3,9-12,17,18]. Seven of other articles were not included in the meta-analysis for the following reasons: (1) two referred to the same cohort[19,20]; (2) two were editorials responding to originated studies[21,22]; (3) one reported HCV infection and pancreatic cancer incidence in the abstract but no OR (95%CI) information was found[23]; and (4) two studies were manual search cited in the selected articles, but did not meet our inclusion criteria after reading the text[24,25].

The main characteristics of the 8 studies pooled in

Table 2 Characteristics of studies included in the meta-analysis

Ref.	Population	Study design	Country	Ethnicity	Case (*n*)	No. of control	Confirmation of HBV/HCV	Confirmation of PC	Matching criteria
Berrington de Gonalez *et al*[9]	Population-based	Cohort	South Korea	Asian	2194	628978	HBsAg	clinic diagnosed	Age, sex
Hong *et al*[10]	Hospital-based	CC	South Korea	Asian	506	1008	Anti-HCV, Anti-HBs, HBsAg	histologically confirmed	Age, sex
Hassan *et al*[11]	Hospital-based	CC	United States	White	476	879	Anti-HCV,Anti-HBs, Anti-HBc	pathologically confirmed	Age, sex, race
El-Serag *et al*[3]	Population-based	Cohort	United States	Asian	140	477	Anti-HCV, HBV	ICD-9	Age, sex
Iloeje *et al*[2]	Population-based	Cohort	Taiwan	Asian	48	22471	HBsAg, HBV DNA	pathologically confirmed	Age, sex, smoking, alcohol
Wang *et al*[18]	Hospital-based	CC	China	Asian	645	711	HBsAg, Anti-HBs, Anti-HBc	pathologically confirmed	Age, sex
Zhu *et al*[17]	Hospital-based	CC	China	Asian	80	77	HBsAg, Anti-HCV, Anti-HBc	pathologically confirmed	Age, sex
Ben *et al*[12]	Hospital-based	CC	China	Asian	943	1128	HBsAg, Anti-HBs, Anti-HBc	pathologically confirmed	Age, sex, smoking, DM, BMI

CC: Case-control study; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HBsAg: Hepatitis B surface antigen; Anti-HBs: Anti-hepatitis B surface antigen; Anti-HBc: Anti-hepatitis B core antigen; Anti-HCV: Anti-hepatitis C virus; ICD-9: International Classification of Diseases, Ninth Revision; DM: Diabetes mellitus; BMI: Body mass index.

the present analysis are reported in Table 2. All studies except one prospective study were retrospective. Five studies were case-control, and three were cohort studies conducted between 1988 and 2010 and published between 2008 and 2012. These studies included a total of 744 120 investigated patients and 3758 cases of pancreatic cancer events. Four studies were conducted in China, two in South Korea and two in the United States.

Quantitative data synthesis

Hepatitis B surface antigen (HBsAg) was seropositive in 4492 patients across all of the groups. Meta-analysis of 6 studies in a random-effects model found that compared to individuals without a history of chronic hepatitis B, those with chronic hepatitis B and in inactive HBsAg carrier state (HBsAg positive) had a 20% greater risk of pancreatic cancer (OR = 1.20, 95%CI: 1.01-1.39), with moderate heterogeneity among studies (test for heterogeneity I^2 = 29.6%, P = 0.213). To further evaluate the HBsAg carrier state associated with pancreatic cancer in the Chinese population, a subgroup type was used to analyze the data. As shown in Figure 2, 4 studies conducted in the Chinese population revealed that the odds ratio of pancreatic cancer for HBsAg positivity was 1.30 (95%CI: 1.05-1.56). Moderate heterogeneity (I^2 = 40.2%, P = 0.171) was seen across studies.

Prior infection with hepatitis B, as determined by the presence of anti- hepatitis B core (HBc), resulted in a significantly increased risk of pancreatic cancer showing OR of 1.24 (95%CI: 1.05-1.42) (I^2 = 71.4%, $P_{heterogeneity}$ = 0.015) which is summarized in Figure 2. Furthermore, an increased risk of pancreatic cancer was observed for hepatitis B surface antibody (anti-HBs)-seronegative/anti-HBc-seropositive carriers who were previously exposed to HBV without natural immunity, with OR of 1.67 (95%CI: 1.13-2.22) (I^2 = 0.0%, $P_{heterogeneity}$ = 0.329), but not for past exposure to HBV carriers with natural immunity (anti-HBs-seropositive/anti-HBc-seropositive), with OR of 0.98 (95%CI: 0.80-1.16) (I^2 = 82.3%, $P_{heterogeneity}$ = 0.003).

We observed non-significant positive associations between markers of active viral replication and pancreatic cancer risk, as illustrated in Figure 2. The risk of developing pancreatic cancer was 0.98 (95%CI: 0.27-1.68) (I^2 = 0.0%, $P_{heterogeneity}$ = 0.399) for HBsAg-seropositive/hepatitis B e antigen (HBeAg)- seropositive subjects compared with that of HBsAg-seronegative subjects while a significant positive association between the protective markers of HBV, anti-HBs and pancreatic cancer risk was found for studies conducted in the pooled analysis with OR of 0.54 (95%CI: 0.46-0.62) (I^2 = 92.7%, $P_{heterogeneity}$ = 0.000).

As summarized in Figure 2, the incidence of pancreatic cancer risks were also significantly increased in previously HCV infected population, with OR of 1.26 (95%CI: 1.03-1.50) (I^2= 0.0%, $P_{heterogeneity}$ = 0.949).

We also carried out stratified analyses to assess the impact of confounding factors of the RRs on the chronic carriers of HBV subgroup. As shown in Table 3, when we restricted the meta-analysis to those studies adjusted for smoking, the association between chronic HBV infection and pancreatic cancer risk was positive, the pooled OR was 1.32 (95%CI: 1.08-1.56). No positive association between chronic HBV infection and pancreatic cancer risk was found in the studies that were not adjusted for smoking, the pooled OR was 0.99 (95%CI: 0.68-1.30). In the stratified analysis, the association between chronic HBV infection and pancreatic cancer risk was also similar between the studies that were adjusted for alcohol drinking (the pooled OR = 1.51, 95%CI: 1.17-1.85) and those that were not adjusted for alcohol drinking (the pooled OR = 1.05, 95%CI: 0.83-1.28). Meanwhile, there was no positive association between chronic HBV infection and pancreatic cancer risk in the studies adjusted for diabetes (the pooled OR = 1.25, 95%CI: 0.95-1.55), neither was

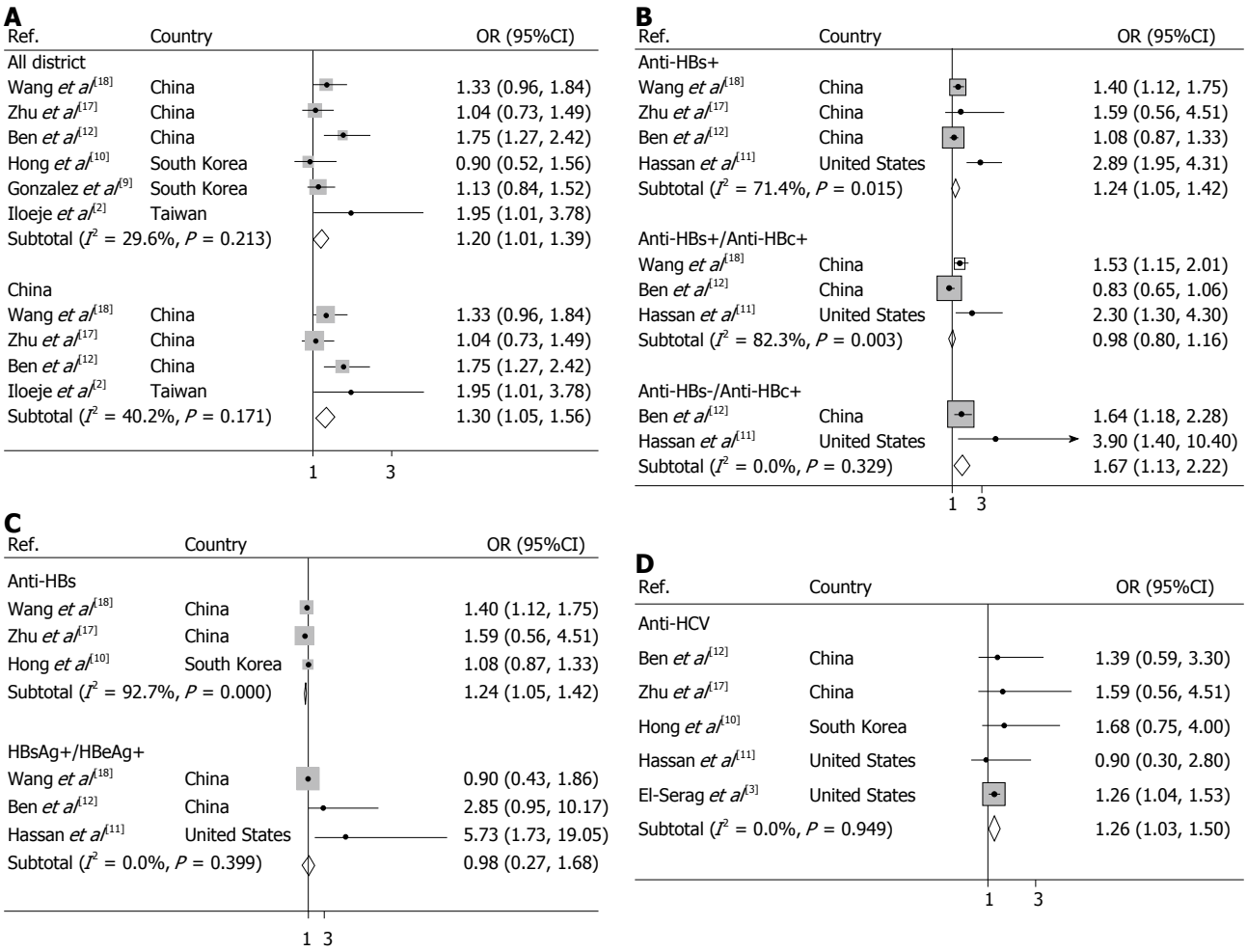

Figure 2 Forest plots of risk of pancreatic cancer. A: Associated with chronic hepatitis B (HBsAg carrier state) around the world and Chinese population; B: Associated with anti-HBc status; C: Associated with active hepatitis B virus viral replication and anti-HBs status; D: Associated with hepatitis C virus infection.

Table 3 Stratified analysis of pancreatic cancer risk by adjusted covariates

Stratifying variables	Studies (*n*)	OR (95%CI)	Tests for heterogeneity		
			χ^2	*P* value	I^2
Adjusted for smoking					
Yes	4	1.32 (1.08-1.56)	4.14	0.246	27.60%
No	2	0.99 (0.68-1.30)	0.18	0.670	0.00%
Adjusted for drinking					
Yes	3	1.51 (1.17-1.85)	1.70	0.428	0.00%
No	3	1.05 (0.83-1.28)	0.53	0.766	0.00%
Adjusted for diabetes					
Yes	4	1.25 (0.95-1.55)	5.22	0.156	42.50%
No	2	1.24 (0.50-1.98)	1.54	0.214	35.20%

in the studies that were adjusted for diabetes (the pooled OR, 1.24, 95%CI: 0.50-1.98).

Publication bias

No publication bias was apparent following an assessment by funnel plot (Figure 3, Begg's test $P = 0.711$, Egger's test $P = 0.868$).

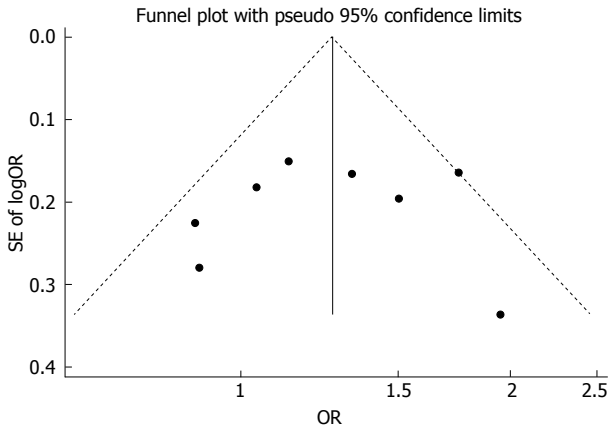

Figure 3 Begg's funnel plot with 95% confidence limits to detect publication bias. Each point represents a separate study for the indicated association.

DISCUSSION

This is the first comprehensive meta-analysis of observational studies on the association between chronic hepa-

titis viral infection and pancreatic cancer risk. We found that HBV and HCV infection is associated with 20% and 23% higher risk of pancreatic cancer, respectively. Our results reveal that prior infection with hepatitis B, especially in those without natural immunity would significantly increase the risk of pancreatic cancer. However, active hepatitis B viral replication does not increase the pancreatic cancer incidence. These observations provide evidence supporting the importance role of chronic HBV and HCV infection in the development of pancreatic cancer. In light of the fact that pancreatic cancer is a highly fatal tumor with a 5-year survival rate of less than 5% and that the number of people with HBV is 2 billion[6,26], our findings have substantial clinical and public significance on a global scale. It points to the need for further investigation on the etiological causes involved in human pancreatic carcinogenesis, the recognition of pancreatic damage mechanisms caused by chronic hepatic viral infection, and for long-term, large scale clinical studies to confirm this clinical association.

If the positive association between the chronic or inactive HBV or HCV carriers and the development of pancreatic cancer is a true, what mechanism could explain such a link? From the anatomical point of view, the proximity of the liver to the pancreas, as well as the sharing of the two organs blood vessels and ducts may make the pancreas a potential reservoir of hepatitis viruses. HBV or HCV may travel through the blood stream and be deposited in non-liver tissue[27,28]. In fact, by means of *situ* hybridization and immunohistochemical techniques, the serological markers of present or past HBV infection, HBsAg was detected in chronic inflammatory pancreatic acinar cells and in the pancreatic duct epithelia with pancreatic adenocarcinoma[29]. The same was true with HCV antigen, which was also found in pancreatic acinar cells[30]. These findings demonstrated the possibility of HBV infection and evidence of a chronic inflammatory reaction in non-hepatic tissues.

HBV and HCV replication intermediates in pancreatic cells support the assumption that the permissiveness of these extrahepatic cells for viral replication might also induce the chronic inflammatory response, thus eventually promoting tumor development. Anti-HBc-positive status, the sero biological marker of past exposure to HBV had an increased risk of developing pancreatic cancer. The observation provides some biological plausibility to the idea that long-lasting persistence of viral infection could indeed replicate in the pancreas. In fact, HBV and HCV are oncogenic viruses, and both are able to integrate the viral RNA or DNA into the genome of the infected cells[31,32]. DNA integration may play a key role in the regulation of the cell cycle, inducing carcinogenesis associated with HBV infection[33].

The third reason why our finding of a relationship between HBV infection and pancreatic cancer incidence may not be surprising is that the presence of HBV infection protection marker, seroconversion from HBsAg to anti-HBs, which is considered a sign of disease protection, leads to a significantly decreased risk of pancreatic cancer showing an OR of 0.54 in the pooled studies.

As with all meta-analyses of observational studies, our findings might have some limitations. First, because five of eight studies used a case-control design[10-12,17,18], the findings provided by this meta-analysis should be viewed with caution since more recall and selection bias might be seen in case-control studies. In addition, all the case-control studies were hospital-based and therefore may not fully represent the general population of pancreatic cancer patients, thereby introducing potential for selection bias into our meta-analysis.

Second, when investigating the association between hepatic virus infection and the risk of pancreatic cancer, the potential residual confounding and the allocation bias, with hepatic virus infection being at different stage and baseline risk of pancreatic cancer would affect the results. For example, it is difficult to understand the biological explanation for finding the cancer risk in subjects with chronic infection and not in those with current and active replication of the virus as shown by a positive HBeAg. The progression from active hepatitis virus infection to chronic inflammatory response targeted to pancreatic carcinogenesis is still unknown, for there is a lack of such data[34]. This incomplete information on HBV/HCV in the pathogenesis of progressive stages limits our knowledge on the true relationship of these oncogenic viruses with pancreatic cancer development. Although an increased risk of pancreatic cancer was observed for anti-HBs-seronegative/anti-HBc-seropositive carriers who were previously exposed to HBV without natural immunity, it is very difficult to interpret a pooled analysis of only 2 studies. Another issue is that none of the studies directly tested for the presence of markers of hepatitis virus infection in the pancreatic tissue. Therefore, a correlation between the level of the markers of HBV/HCV infection in peripheral blood and that in pancreatic tissue could not be established, which would throw some doubt into the reliability of the summary of RRs. More research is necessary to assess a dose-response association to examine the influence of viral load on the progression of pancreatic cancer to support biological plausibility.

Third, possible confounding factors and biases that may not have been fully adjusted for in this study exist. In fact, risk factors such as cigarette smoking, alcohol intake and diabetes, all of which could increase risk of pancreatic cancer associated with a history of chronic hepatitis virus infection. Only 4[2,10-12], 3[2,11], and 4[10-12,17] studies provided risk estimated adjusting for smoking, alcohol intake, and diabetes, respectively. The positive association between chronic HBV infection and pancreatic cancer risk was found after adjustment by smoking and alcohol drinking, but no positive correlation was maintained after adjustment by diabetes, suggesting that residual confounding by diabetes modified the association between chronic HBV infection and pancreatic cancer risk.

Finally, it is also important to realize that there is still a significant heterogeneity observed across studies, mostly

due to the diversity of the study designs and the varying incidence of pancreatic cancer and HBV/HCV infection rates may vary from continent to continent.

Even with these limitations, our meta-analysis supports the hypothesis that chronic HBV and HCV infection may significantly increase pancreatic cancer risk. The findings of this study raise the question of whether the early detection and provision of aggressive antiviral treatment for chronic hepatitis virus infection could prevent the development of pancreatic cancer, and whether patients with past exposure to HBV/HCV should be screened for malignancies other than HCC particularly in patients at high risk of HBV/HCV infection.

In conclusion, our meta-analysis favors the association between HBV/HCV infection and pancreatic cancer risk. However, observational studies were moderately heterogeneous and biased. Additional long-term prospective evidence for HBV/HCV infection among higher risk of pancreatic disease patients should be monitored and new evaluations on the effects of early intervention including HBV/HCV treatment, especially in occult HBV infection (anti-HBc-seropositive status), on the molecular carcinogenesis of pancreatic cancer are warranted.

COMMENTS

Background

Data from epidemiological studies related to the association of hepatitis B virus (HBV) and hepatitis C virus (HCV) and pancreatic cancer risk are inconsistent, with some studies supporting the excess pancreatic cancer with HBV/HCV infection compared to non-infected controls, and some studies showing differently. The aim of this meta-analysis was to clarify the association of chronic hepatitis viruses with the risk of pancreatic cancer.

Research frontiers

To date, several studies have assessed the association between the chronic HBV/HCV and pancreatic cancer risk in different ethics; however, the results are inconsistent and inconclusive. No quantitative summary of the evidence has ever been performed.

Innovations and breakthroughs

Based on the meta-analysis, the authors identified that chronic HBV and HCV infection is associated with pancreatic cancer, especially among Chinese population. Early intervention of HBV and HCV infection might decrease pancreatic cancer incidence.

Applications

The results support the hypothesis that chronic HBV/HCV infection significantly increases pancreatic cancer risk. Findings of this analysis are comparable with previous studies, and long-term prospective studies. Patients with past exposure to HBV/HCV should be screened for hepatocellular carcinoma and other malignancies, especially pancreatic cancer.

Terminology

Anti-hepatitis B core antigen (HBc)+/anti-hepatitis B surface antigen (HBs)± are serum biomarkers of possible occult HBV infection. Anti-HBc+/anti-HBs+ is the status of past exposure to HBV with evidence of HBV immunity or recovery, but possibly harboring persistent HBV infection. Anti-HBc+/ anti-HBs- is the status of past exposure to HBV without natural immunity.

Peer review

The meta-analysis was aimed at assessing the association between HBV/HCV chronic infection and risk of pancreatic cancer. This is an appealing issue, leading to interesting results.

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META-ANALYSIS

Association of *Helicobacter pylori babA2* with peptic ulcer disease and gastric cancer

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Abstract

AIM: To investigate the association between *babA2* gene and peptic ulcer disease (PUD) and gastric cancer (GC) in *Helicobacter pylori*-infected populations.

METHODS: We evaluated the relationship between *babA2* and clinical outcomes (PUD and GC) using a meta-analysis. A literature search was performed using the PubMed and Web of Science databases for relevant case-control studies that met the defined inclusion criteria. The ORs and 95%CIs were calculated to estimate the association between *babA2* genotype and clinical outcomes. A fixed-effect or random-effect model was performed depending on the absence or presence of significant heterogeneity.

RESULTS: A total of 25 articles with 38 studies met the inclusion criteria and were finally included in this meta-analysis. The results showed that the *babA2* genotype was significantly associated with an increased risk of PUD (OR = 2.069, 95%CI: 1.530-2.794, $P < 0.001$) and especially in the subgroup of duodenal ulcer (OR = 1.588, 95%CI: 1.141-2.209, $P = 0.006$). Moreover, a significant association between *babA2* gene and PUD and duodenal ulcer (OR = 2.739, 95%CI: 1.860-4.032, $P < 0.001$; OR = 2.239, 95%CI: 1.468-3.415, $P < 0.001$, respectively) was observed in western countries but not in Asian countries.

CONCLUSION: We demonstrated that the presence of *babA2* may be associated with increased risks for PUD, especially duodenal ulcer, in western countries.

Key words: *Helicobacter pylori*; *babA2*; Peptic ulcer; Gastric cancer; Risk

Core tip: BabA encoded by *babA2* gene is an outer member protein of *Helicobacter pylori* (*H. pylori*), which plays a key role in facilitating bacterial colonization in the stomach. The association between *babA2* and *H. pylori*-related gastroduodenal diseases is still controversial. We summarized a total of 25 case-control articles with 38 studies in this meta-analysis and evaluated the relationship between *babA2* and clinical outcomes. The presence of *babA2* may contribute to increased risk of peptic ulcer disease (PUD), especially duodenal ulcer, in western countries. In Asians, *babA2* genotype only showed a marginal association with PUD risk, which requires further investigation.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative spiral bacterium that may colonize the human gastric mucosa and establish a life-long infection[1]. Although *H. pylori* infects approximately half of the population worldwide, especially in developing countries, the majority of infected people remain asymptomatic. Only 15%-20% of those infected develop severe gastroduodenal diseases, such as peptic ulcer disease (PUD), gastric cancer (GC), and mucosa-associated lymphoid tissue lymphoma[2,3]. In addition to the host and environmental factors, another important reason for the diverse clinical outcomes is the differences in virulence factors among *H. pylori* strains[3]. For example, *H. pylori* strains harboring the vacuolating toxin A (*vacA*) and the cytotoxin-associated antigen (*cagA*) have been proposed as possible risk factors for PUD and GC[4].

Successful colonization in the stomach is the most important step for the pathogenicity of *H. pylori* infection. It is generally accepted that bacterial attachment to the gastric epithelium is the first critical stage of colonization by *H. pylori*[5]. The blood group antigen binding adhesin (BabA) is a well-described outer member protein of *H. pylori* that targets fucosylated Lewis^b blood group antigens presented on gastric epithelium[6,7]. Three *bab* allelic types have been identified, including *babA1*, *babA2* and *babB*; however, only the product of the *babA2* gene is necessary for endowing the bacteria with Lewis^b binding activity[6]. In 1999, Gerhard *et al*[8] first reported a positive association between a *babA2*-gene-positive strain and duodenal ulcer (DU) and GC. Subsequently, a series of studies of the association between *babA2* gene and PUD and GC have been done, but with inconsistent or conflicting conclusions[9-11].

We proposed a hypothesis that bacterial adherence factor BabA mediating close attachment to the epithelium may contribute to pathogenesis of PUD and/or GC. So far, it has not been possible to draw any causal conclusion about the relationship between the *babA2* gene and specific diseases, partly because of the small size of individual studies. Therefore, in the present study, we conducted a meta-analysis, combining available data from published case-control studies, to obtain a more precise estimate of the association between *babA2* gene and PUD and GC in *H. pylori*-infected populations.

MATERIALS AND METHODS

Literature search strategy

A literature search was performed using the PubMed and Web of Science databases for articles estimating the association between *babA2* gene and clinical outcomes in *H. pylori*-infected populations. All enrolled studies were published from January 1997 to October 2012 and retrieved using one of the keywords "*babA*" or "*babA2*" in combination with "*Helicobacter pylori*". The search was performed without restriction on language.

Inclusion criteria

The criteria used to select studies for this meta-analysis were as follows: (1) fully published case-control studies [case group included DU, gastric ulcer (GU), PUD or GC, and the control group included gastritis or nonulcer disease (NUD)]; (2) studies described the relationship between *babA2* gene status and clinical outcomes; (3) the presence of *babA2* was examined by polymerase chain reaction (PCR); and (4) the papers were written in English.

Exclusion criteria

The exclusion criteria were as follows: (1) the results came from review articles; (2) there was no integrated raw data; (3) *in vitro* studies or animal experiments; (4) studies with abstract only; and (5) studies with children.

Data extraction

Evaluation of all potentially relevant articles and extraction of raw data were independently performed by two investigators (Chen MY and He CY). Disagreements were resolved through discussion. We collected information on the following items from each study: first author's name, year of publication, countries and areas of the study population, *babA2* status and clinical outcomes (DU, GU, PUD and GC), and the total number of cases and controls.

Statistical analysis

All statistical analyses were performed using STATA version 11.0 (College Station, TX, United States). Two-sided *P* values were evaluated in this meta-analysis and $P < 0.05$ was considered statistically significant. The strength of the association between the *babA2* gene and clinical outcomes was estimated by OR and corresponding 95%CIs. The statistical heterogeneity among the included studies was assessed by χ^2-based Q and I^2 statistics. If the heterogeneity was considered not significant (with $P > 0.1$ for Q test) among studies, a fixed-effects model based on the Mantel-Haenszel method[12] was used to calculate the pooled OR. On the contrary, a random-effects model based on the DerSimonian and Laird method[13] was used to assess the pooled OR when the P value of the Q test was < 0.1. In addition, a sensitivity analysis was performed to estimate the effects of each included study on the overall risk of clinical outcomes. ORs and 95%CIs were recalculated when any single study was excluded in turn. Begg's test[14] and Egger's test[15] were performed to estimate the publication bias.

RESULTS

Characteristics of selected studies

According to the literature search strategy, a total of 220

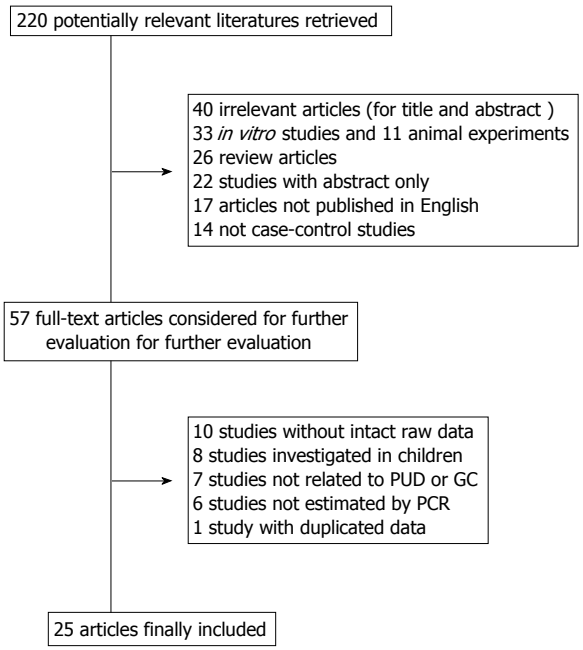


Figure 1 Flowchart of literature search and studies selection. PUD: Peptic ulcer disease; GC: Gastric cancer; PCR: Polymerase chain reaction.

possibly relevant studies were retrieved and 195 were excluded. The main reasons for exclusion were that the articles were reviews, *in vitro* studies, irrelevant to the theme of our research, or did not meet our inclusion criteria (Figure 1). Twenty-five case-control studies met the inclusion criteria[8-11,16-36]. Four of these studies[10,11,19,32] investigated the association between *babA2* gene and clinical outcomes in several different countries. Considering that these data partially evaluated the geographic variation of the influence of *babA2* gene status on the risk of *H. pylori*-related gastroduodenal diseases, data that came from different countries were treated as a separate study. Therefore, with respect to geographical location, 16 studies[11,17,19,22-26,29,33-36] were concerned with Asian populations, and 23[10,16,18,20,21,27,28,30-32] analyzed western populations. One of the latter group, which involved a study from Sweden[10], was excluded because of insufficient data. Finally, a total of 38 independent studies with 4556 patients were included in this meta-analysis (Table 1).

Association between babA2 gene and PUD

There were 36 studies[8-11,17-20,22-36] that investigated the distribution difference of *babA2* genotypes between patients with PUD and gastritis and/or NUD, which consisted of 1859 cases and 1909 controls. The overall prevalence of *babA2* gene was 73.96% (1375/1859) in PUD patients and 57.94% (1106/1909) in control subjects. Data from Oleastro *et al*[19] (Japan, South Korea, Brazil population), Sheu *et al*[26] and Lai *et al*[34] showed that the prevalence of *babA2* gene was 100% in both case and control groups, and the OR and standard error could not be estimated; thus, these studies were excluded. We found that the *babA2* gene significantly increased the risk of PUD in a random-effects model, with a pooled OR of 2.069 (95%CI: 1.532-2.794, $P < 0.001$), and moderate heterogeneity was observed ($I^2 = 62.8$, $P < 0.001$) (Figure 2).

To explore the source of heterogeneity, subgroup analysis was performed. PUD was classified into DU and GU. Among the total of 36 PUD-related studies, 19[8,9,11,17,22-24,26,27,29,31-35] could be used to evaluate risk for DU and eight[22,24,26,27,29,33-35] for GU. For DU analysis, the overall prevalence of *babA2* gene in DU and control subjects was 77.20% (813/1053) and 71.77% (811/1130), respectively. After removal of two studies with 100% prevalence of *babA2* genotype[26,27], the pooled OR based on the random-effects model was 1.588 (95%CI: 1.141-2.209, $P = 0.006$), and mild heterogeneity was observed ($I^2 = 45.8$, $P = 0.021$) (Figure 2). For GU analysis, the overall prevalence of *babA2* genotype seemed to be lower in GU (73.73%, 174/236) than in controls (80.89%, 402/497). Two studies with 100% prevalence of *babA2* genotype were also excluded because of statistical limitation[26,34]. No significant association was observed between *babA2* genotype and GU in a fixed-effects model (OR = 0.755, 95%CI: 0.496-1.150, $P = 0.191$), and there was no heterogeneity among the studies ($I^2 = 0.0\%$, $P = 0.845$) (Figure 2).

When geographical location was considered, data from different countries were subdivided into Asian and western groups. For PUD, the overall prevalence of *babA2* gene was 78.36% (822/1049) in Asian countries and 68.27% (553/810) in western countries. Furthermore, in western countries, the presence of *babA2* substantially increased PUD risk, with a pooled OR of 2.739 (95%CI: 1.860-4.032, $P < 0.001$), while in Asian countries, the *babA2* genotype was only borderline associated with PUD (OR = 1.370, 95%CI: 0.941-1.994, $P = 0.100$) (Figure 2). For DU, the *babA2* genotype significantly increased the risk of DU in western countries (OR = 2.239, 95%CI: 1.468-3.415, $P < 0.001$), but not in Asian countries (OR = 1.158, 95%CI: 0.802-1.672, $P = 0.433$) (Figure 2). The results suggested that differences in geographical distribution of *babA2* genotype may also confer heterogeneity to the studies. Only one study with a small sample size investigated the relationship of *babA2* gene and GU in a western country[27]; therefore, we did not perform subgroup analysis according to geographical area.

Sensitivity analysis was conducted to assess the influence of individual studies on the overall risk of PUD and DU by excluding any single study in turn and recalculating the pooled OR and 95%CI. A similar OR and 95%CI were generated, which indicated high stability of the results (Figure 3).

Association between babA2 and GC

A total of 16 studies[8,9,16,17,21-24,29,31-36] investigated the association between *babA2* gene and GC. The overall prevalence of *babA2* gene was 70.72% (384/534) in GC cases and 60.64% (607/1001) in gastritis or NUD controls. One study with both 100% prevalence of *babA2* in cases and controls was excluded from our meta-analysis[34]. In a random-effects model, the risk of GC increased 1.972-fold (95%CI: 1.103-3.525, $P = 0.022$) in the pres-

Table 1 Characteristics of studies included in the meta-analysis *n* (%)

Ref.	Population	Gastritis or NUD	PUD	GU	DU	GC
		babA2 +	*babA2* +	*babA2* +	*babA2* +	*babA2* +
Asian						
Saxena *et al*[36]	India	35 (26.32)	19 (52.78)			10 (28.57)
Talebi Bezmin Abadi *et al*[9]	Iran	17 (26.15)	10 (18.18)		10 (18.18)	38 (95.00)
Safaei *et al*[17]	Iran	30 (68.18)	20 (74.07)		20 (74.07)	8 (80.00)
Oleastro *et al*[19]	Japan	28 (100.00)	42 (100.00)			
Oleastro *et al*[19]	South Korea	37 (100.00)	28 (100.00)			
Chomvarin *et al*[24]	Thai	57 (91.94)	31 (91.18)	17 (85.00)	14 (100.00)	15 (93.75)
Erzin *et al*[23]	Turkey	7 (23.33)	14 (46.67)		14 (46.67)	29 (87.88)
Zhang *et al*[22]	China	89 (66.92)	89 (60.14)	28 (59.57)	61 (60.40)	54 (68.35)
Sheu *et al*[26]	Taiwan	85 (100.00)	60 (100.00)	30 (100.00)	30 (100.00)	
Zheng *et al*[25]	China	11 (37.93)	17 (39.53)			
Han *et al*[29]	China	28 (65.12)	50 (64.94)	15 (50.00)	35 (74.47)	12 (57.14)
Lai *et al*[34]	Taiwan	41 (100.00)	46 (100.00)	15 (100.00)	31 (100.00)	14 (100.00)
Maeda *et al*[33]	Japan	52 (96.30)	40 (95.24)	20 (100.00)	20 (90.91)	11 (100.00)
Yamaoka *et al*[32]	Korea	47 (88.68)	111 (96.52)		111 (96.52)	
Yamaoka *et al*[32]	Japan	112 (88.89)	172 (95.56)		112 (88.89)	
Mizushima *et al*[35]	Japan	34 (80.95)	73 (84.88)	38 (84.44)	35 (85.37)	36 (90.00)
Western						
Mattar *et al*[16]	Brazil	22 (64.71)				14 (41.18)
Oleastro *et al*[18]	Portugal	7 (11.67)	27 (47.37)			
Bartchewsky *et al*[21]	Brazil	102 (79.07)				40 (78.43)
Oleastro *et al*[19]	Portugal	16 (32.00)	25 (50.00)			
Oleastro *et al*[19]	France	3 (50.00)	22 (81.48)			
Oleastro *et al*[19]	Sweden	4 (40.00)	10 (83.33)			
Oleastro *et al*[19]	Germany	6 (60.00)	7 (77.78)			
Oleastro *et al*[19]	United States	12 (92.31)	10 (100.00)			
Oleastro *et al*[19]	Brazil	12 (100.00)	10 (100.00)			
Oleastro *et al*[20]	Portugal	18 (32.14)	25 (50.00)			
Gatti *et al*[27]	Brazil	16 (43.24)	20 (40.00)	11 (37.93)	9 (42.86)	
Gatti *et al*[28]	Brazil	37 (54.41)	3 (20.00)			
Olfat *et al*[10]	Finland	12 (46.15)	22 (70.97)			
Olfat *et al*[10]	Portugal	12 (19.67)	19 (63.33)			
Olfat *et al*[10]	Germany	19 (28.36)	22 (88.00)			
Oliveira *et al*[31]	Brazil	24 (31.58)	43 (53.75)		43 (53.75)	29 (55.77)
Zambon *et al*[30]	Italy	26 (27.96)	20 (48.78)			
Yamaoka *et al*[32]	Colombia	28 (70.00)	34 (85.00)		34 (85.00)	34 (82.93)
Yamaoka *et al*[32]	United States	28 (70.00)	35 (85.37)		35 (85.37)	19 (63.33)
Yamaoka *et al*[11]	United States	66 (71.74)	123 (84.83)		123 (84.83)	
Yamaoka *et al*[11]	Colombia	37 (71.15)	53 (82.81)		53 (82.81)	
Gerhard *et al*[8]	Munich	13 (37.14)	23 (100.00)		23 (100.00)	21 (77.78)

NUD: Nonulcer disease; PUD: Peptic ulcer disease; GU: Gastric ulcer; DU: Duodenal ulcer; GC: Gastric cancer.

ence of *babA2* compared with the controls; however, high heterogeneity among studies was observed (I^2 = 76.8%, $P < 0.001$). Meta-analyses were conducted repeatedly when each study was omitted. As showed in Figure 4, two studies[9,23] showed larger differences in the risk estimates compared with other studies in the sensitivity analysis. Sensitivity analysis excluding these studies generated an OR of 1.303 (95%CI: 0.881-1.927, $P = 0.185$) among homogeneous studies (I^2 = 45.0%, $P = 0.040$), which was different from the OR of 1.972 (95%CI: 1.103-3.525, $P = 0.022$) before the removal of those studies (Figure 4). In terms of geographical area, no statistically significant findings were found among the Asian or western subpopulations, with a pooled OR of 1.132 (95%CI: 0.763-1.680, $P = 0.539$) in the former and 1.303 (95%CI: 0.881-1.927, $P = 0.349$) in the latter (Figure 4).

Publication bias analysis

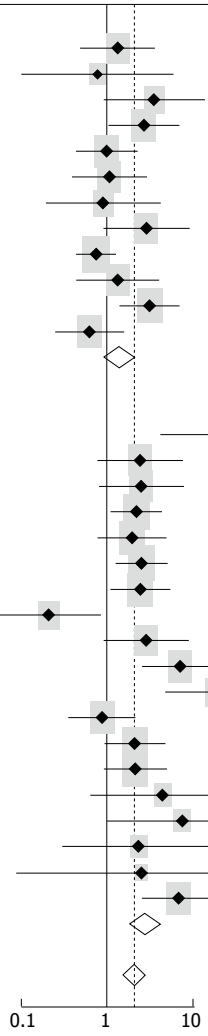
Publication bias was preliminarily estimated by Begg's and Egger's tests. No significant publication bias was observed in all the comparisons based on Begg's test (all $P > 0.1$), but P value was 0.08 in Egger's test, suggesting a slight publication bias.

DISCUSSION

The Gram-negative bacterium *H. pylori* is known to have a remarkably high level of genetic diversity, and is implicated in human diseases after decades of persistence in the stomach[37-39]. A crucial virulence factor BabA, encoded by the *babA2* gene, facilitates colonization by *H. pylori* in the stomach and may be involved in the pathogenesis of different *H. pylori*-related gastroduodenal diseases, such as PUD and gastric malignancy[8]. To date, there have been numerous relevant studies published but with divergent results on the relationship between the *babA2* gene and PUD and GC[9-11]; moreover, there is no comprehensive meta-analysis on the significance

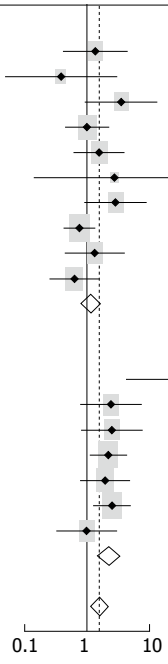
A

Ref.	Country	PUD *babA2*+/-	NUD/GS *babA2*+/-	OR (95%CI)	%Weight
Asian					
Mizushima *et al*[35]	Japan	73/13	34/8	1.32 (0.50, 3.49)	3.61
Maeda *et al*[33]	Japan	40/2	52/2	0.77 (0.10, 5.70)	1.62
Yamaoka *et al*[32]	South Korea	111/4	47/6	3.54 (0.96, 13.13)	2.75
Yamaoka *et al*[33]	Japan	172/8	112/4	2.69 (1.09, 6.61)	3.80
Han *et al*[29]	China	50/27	28/15	0.99 (0.45, 2.17)	4.15
Zheng *et al*[25]	China	17/26	11/18	1.07 (0.41, 2.82)	3.61
Chomvarin[24]	Thai	31/3	57/5	0.91 (0.20, 4.05)	2.37
Erzin *et al*[23]	Turkey	14/16	7/23	2.88 (0.95, 8.72)	3.23
Zhang *et al*[22]	China	89/59	89/44	0.75 (0.46, 1.22)	5.01
Safaei *et al*[17]	Iran	20/7	30/14	1.33 (0.46, 3.88)	3.34
Saxena *et al*[36]	India	19/17	35/98	3.13 (1.46, 6.69)	4.22
Talebi Bezmin Abadi *et al*[9]	Iran	10/45	17/48	0.63 (0.26, 1.51)	3.86
Subtotal (I^2 = 46.3%, P = 0.039)				1.37 (0.94, 1.99)	41.58
Western					
Gerhard *et al*[8]	Munich	23/0	13/22	78.33 (4.39, 1397.21)	0.92
Yamaoka *et al*[32]	Colombia	34/6	28/12	2.43 (0.81, 7.30)	3.26
Yamaoka *et al*[32]	United States	35/6	28/12	2.50 (0.83, 7.50)	3.26
Yamaoka *et al*[11]	United States	123/22	66/26	2.20 (1.16, 4.18)	4.57
Yamaoka *et al*[11]	Colombia	53/11	37/15	1.95 (0.81, 4.73)	3.85
Oliveira *et al*[31]	Brazil	43/37	24/52	2.52 (1.31, 4.84)	4.45
Zambon *et al*[30]	Italy	20/21	26/67	2.45 (1.15, 5.26)	4.21
Gatti *et al*[28]	Brazil	3/12	37/31	0.21 (0.05, 0.81)	2.66
Olfat *et al*[10]	Finland	22/9	12/14	2.85 (0.96, 8.51)	3.27
Olfat *et al*[10]	Portugal	19/11	12/49	7.05 (2.66, 18.69)	3.59
Olfat *et al*[10]	Germany	22/3	19/48	18.53 (4.96, 69.22)	2.74
Gatti *et al*[27]	Brazil	20/30	16/21	0.88 (0.37, 2.07)	3.92
Oleastro *et al*[20]	Portugal	25/25	18/38	2.11 (0.96, 4.64)	4.13
Oleastro *et al*[19]	Portugal	25/25	16/34	2.13 (0.94, 4.79)	4.06
Oleastro *et al*[19]	France	22/5	3/3	4.40 (0.68, 28.60)	1.78
Oleastro *et al*[19]	Sweden	10/2	4/6	7.50 (1.04, 54.12)	1.65
Oleastro *et al*[19]	Germany	7/2	6/4	2.33 (0.31, 17.54)	1.60
Oleastro *et al*[19]	United States	10/0	12/1	2.52 (0.09, 68.60)	0.72
Oleastro *et al*[18]	Portugal	27/30	7/53	6.81 (2.65, 17.52)	3.68
Subtotal (I^2 = 59.1%, P = 0.001)				2.74 (1.86, 4.03)	58.42
Overall (I^2 = 62.8%, P = 0.000)				2.07 (1.53, 2.79)	100.00
Note: Weights are from random effects analysis					



B

Ref.	Country	DU *babA2*+/-	NUD/GS *babA2*+/-	OR (95%CI)	%Weight
Asian					
Mizushima *et al*[35]	Japan	35/6	34/8	1.37 (0.43, 4.37)	5.18
Maeda *et al*[33]	Japan	20/2	52/2	0.38 (0.05, 2.92)	2.24
Yamaoka *et al*[32]	South Korea	111/4	47/6	3.54 (0.96, 13.13)	4.40
Yamaoka *et al*[32]	Japan	112/4	112/14	1.00 (0.46, 2.19)	7.92
Han *et al*[29]	China	35/12	28/15	1.56 (0.63, 3.87)	6.89
Chomvarin *et al*[24]	Thai	14/0	57/5	2.77 (0.14, 53.09)	1.15
Erzin *et al*[23]	Turkey	14/16	7/23	2.88 (0.95, 8.72)	5.48
Zhang *et al*[22]	China	61/40	89/44	0.75 (0.44, 1.29)	10.40
Safaei *et al*[17]	Iran	20/7	30/14	1.33 (0.46, 3.88)	5.73
Talebi Bezmin Abadi *et al*[9]	Iran	10/45	17/48	0.63 (0.26, 1.51)	7.10
Subtotal (I^2 = 23.1%, P = 0.231)				1.16 (0.80, 1.67)	56.49
Western					
Gerhard *et al*[8]	Munich	23/0	13/22	78.33 (4.39, 1397.21)	1.20
Yamaoka *et al*[32]	Colombia	34/6	28/12	2.43 (0.81, 7.30)	5.53
Yamaoka *et al*[32]	United States	35/6	28/12	2.50 (0.83, 7.50)	5.54
Yamaoka *et al*[11]	United States	123/22	66/26	2.20 (1.16, 4.18)	9.31
Yamaoka *et al*[11]	Colombia	53/11	37/15	1.95 (0.81, 4.73)	7.07
Oliveira *et al*[31]	Brazil	43/37	24/52	2.52 (1.31, 4.84)	9.19
Gatti *et al*[27]	Brazil	9/12	16/21	0.98 (0.33, 2.90)	5.65
Subtotal (I^2 = 28.1%, P = 0.214)				2.24 (1.47, 3.42)	43.51
Overall (I^2 = 45.8%, P = 0.021)				1.59 (1.14, 2.21)	100.00
Note: Weights are from random effects analysis					



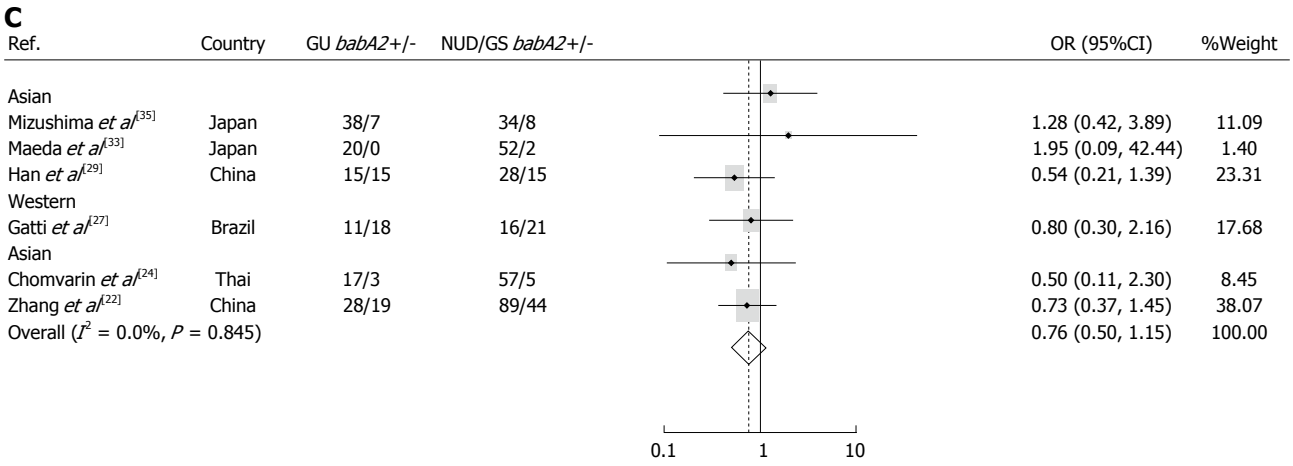


Figure 2 Results of the association between *babA2* gene and peptic ulcer disease, duodenal ulcer and gastric ulcer risk. A: Association between *babA2* and peptic ulcer disease (PUD); B: Association between *babA2* and duodenal ulcer (DU); C: Association between *babA2* and gastric ulcer (GU). ORs and 95%CIs were calculated by a random-effect (A, B) and fixed-effect (C) model. NUD: Nonulcer disease.

of *babA2*. Therefore, we performed the present meta-analysis of the available published literature to obtain a more precise conclusion. Our meta-analysis showed that *babA2* was significantly associated with increased risks of PUD, especially DU, with corresponding ORs of 2.069 and 1.588; moreover, statistically significant findings were more apparent in western populations with ORs of 2.739 for PUD and 2.239 for DU. The summary ORs for PUD and DU in Asians, however, were relatively small (1.370 and 1.158, respectively) and without statistical significance. No significant risk association was observed for GU and GC, but a decreased tendency was noted for GU with a pooled OR of 0.755.

Over the past 20 years, there has been marked progress in our understanding of the role of *H. pylori* infection in the etiology of gastroduodenal diseases. It is well known that *H. pylori* infection increases the risk of developing PUD, including both GU and DU subtypes[40]. Our meta-analysis confirmed a positive association of *H. pylori* with *babA2* genotype with PUD development. Among the major outer membrane proteins of *H. pylori*, BabA has significance not only in triggering bacterial colonization of the gastric epithelium, but also in regulating its functional interaction with host cells, which mainly acts through binding to Lewis^b and fucosylated ABO blood group antigens present in the stomach[41,42]. Gene inactivation experiments have demonstrated that only the product of *babA2* gene is essential for Lewis^b binding activity[7]. Rad *et al*[43] have reported a high density of *H. pylori* colonization in the stomach in the presence of *babA2* genotype, which increases interleukin-8 secretion and granulocytic infiltration, resulting in intense mucosal inflammation. In addition, Ishijima *et al*[41] have demonstrated that *babA2*-positive strains with Lewis^b binding activity are potentiators of the type IV secretion system (T4SS), implying a possible combined effect of *babA2* and other virulence factors related to T4SS. Although the detailed mechanism of the pathogenicity of *babA2* in PUD development has not been fully established, our meta-analysis suggests an important role of *babA2* genotype in distinguishing *H. pylori*-related PU and especially DU from NUD.

Intriguing findings in this study further suggested that individuals infected with *babA2*-positive pathogens have a unique pathogenicity in DU development; conversely, there was no significant association between *babA2* and GC. This difference may be partially due to the distinct etiologies of DU and GC development. Generally, *H. pylori*-related chronic severe gastritis could progress in two different directions[44]. One possibility is that *H. pylori*-related gastritis, predominating in the antrum as well as generating gastric acid, usually induces DU[45]. Patients with DU rarely develop atrophic gastritis of the corpus, and therefore GC risk may decrease in such cases[46]. Another possibility is that patients with extensive gastritis in the corpus and antrum, involving decreased acid output, tend to develop intestinal metaplasia, atrophic gastritis, and even GC[45]. It is speculated that *babA2* combined with other virulence factors may also lead to GC development. Studies conducted by Gerhard *et al*[8] and Erizin *et al*[47] have suggested that triple-positive *H. pylori* strains with *cagA, vacAs1* and *babA2* coexpression increase the risk of developing GC. Zambon *et al*[30] have also reported that infections with these triple-positive strains carry a higher risk of intestinal metaplasia, known as a gastric precancerous lesion. The different risk associations between GC and DU should be interpreted with caution, which should be further investigated in the future.

Our stratified analysis according to geographical areas demonstrated that *babA2* genotype is closely involved in the risk of PUD, especially DU in western populations, but not in Asian populations. This important information about geographical difference in the *babA2* gene suggests a potential biomarker distinguishing PUD, especially DU, from other NUDs in western populations, and reveals a phylogenetic difference between Asian and western *H. pylori* strains. Previous studies have also reported divergence in genes accounting for BabA and other virulence genes, such as *cagA* and *vacA*, between Asian and western strains[48-50]. The above-mentioned findings support the

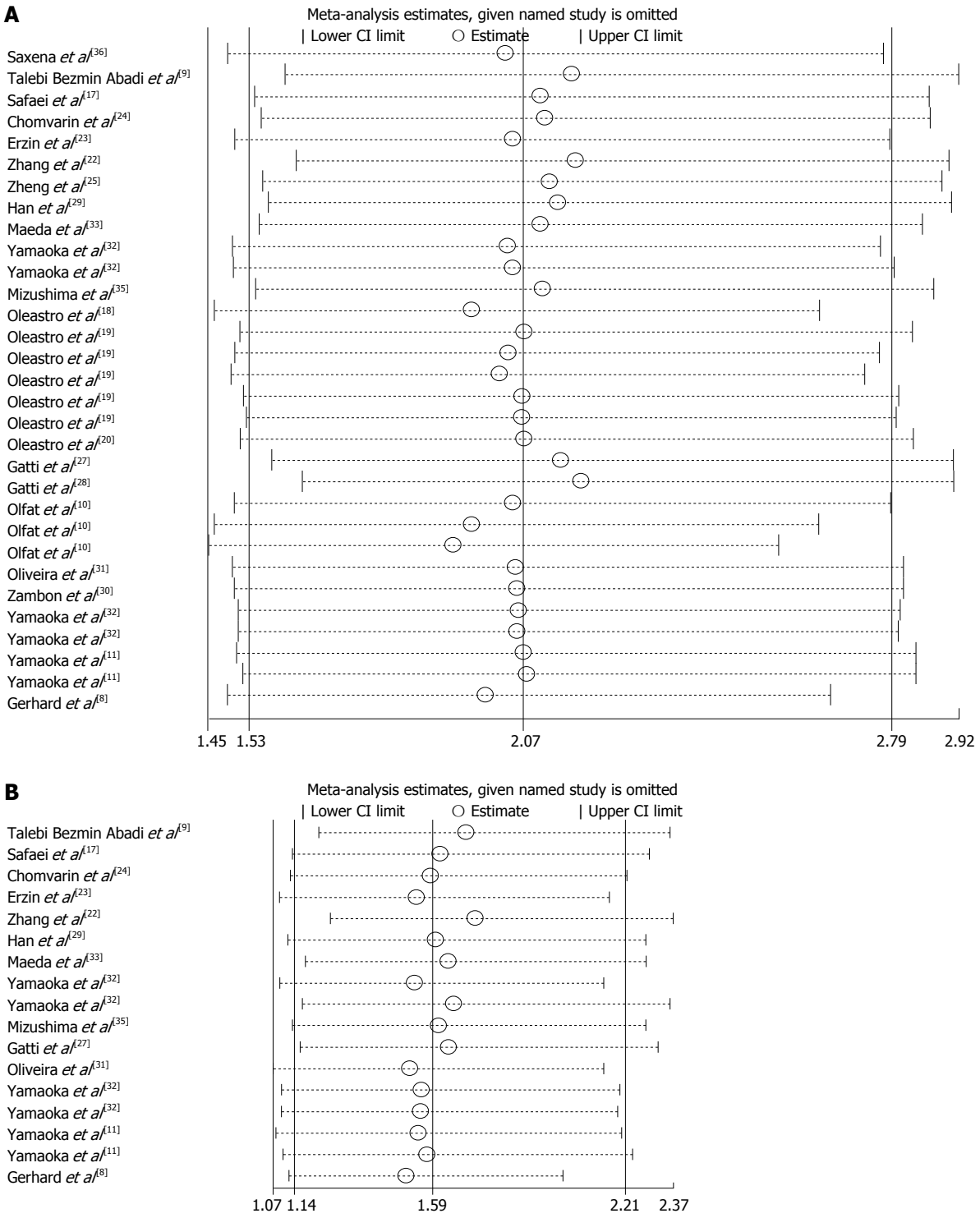
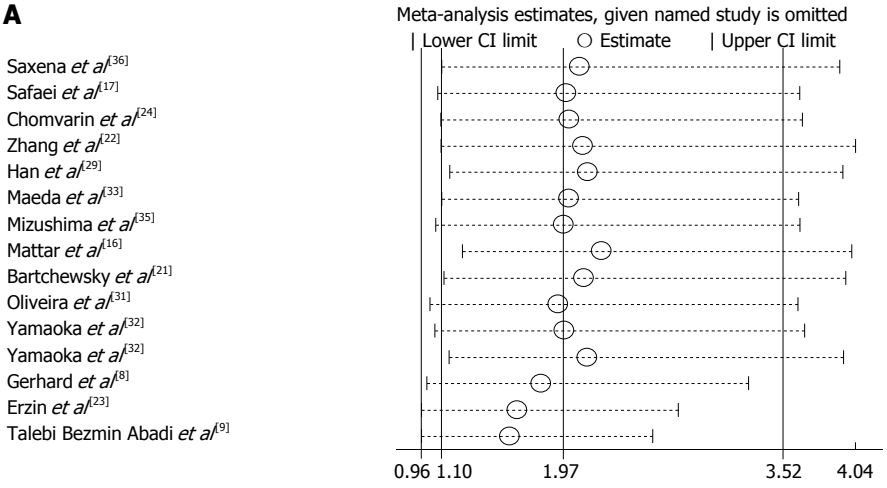


Figure 3 Influence of the summary OR coefficients on the association between *babA2* genotype and peptic ulcer disease and duodenal ulcer risk. A: *babA2* genotype and peptic ulcer disease (PUD) risk; B: *babA2* genotype and duodenal ulcer risk. Results were calculated by omitting each study (on the left) in turn. Bars, 95%CI. Meta-analysis random-effects estimates (exponential form) were used.

suggestion that genetic variability within the *H. pylori* genome, especially in probable host interaction genes, plays a critical role in its different adaptive ability and pathogenicity among different ethnicities[49].

There were several unavoidable limitations to our meta-analysis that should be considered. First, 17 studies[10,18-20,25,28,30,36] related to PUD lacked information about the distribution of DU and GU, which may have influenced the results of the stratified analysis. Second, a lack of original data on histopathological types of GC limited the subgroup analysis according to differences in these types. An unstable result was obtained according to the sensitivity analysis that assessed the relationship between *babA2* and GC, but there were insufficient data to explore

A



B

Ref.	Country	GC *babA2*+/-	NUD/GS *babA2*+/-	OR (95%CI)	%Weight
Asian					
Mizushima *et al*[35]	Japan	36/4	34/8	2.12 (0.58, 7.68)	6.19
Maeda *et al*[33]	Japan	11/0	52/5	1.10 (0.05, 24.38)	1.47
Han *et al*[29]	China	12/9	28/15	0.71 (0.25, 2.08)	7.84
Chomvarin *et al*[24]	Thai	15/1	57/5	1.32 (0.14, 12.13)	2.67
Zhang *et al*[22]	China	54/25	89/44	1.07 (0.59, 1.94)	13.11
Safaei *et al*[17]	Iran	8/2	30/14	1.87 (0.35, 9.96)	4.24
Saxena *et al*[36]	India	10/25	35/98	1.12 (0.49, 2.57)	10.21
Subtotal (I^2 = 0.0%, P = 0.918)				1.13 (0.76, 1.68)	45.73
Western					
Gerhard *et al*[8]	Munich	21/6	13/22	5.92 (1.90, 18.47)	7.27
Yamaoka *et al*[32]	Colombia	34/7	28/12	2.08 (0.72, 6.00)	7.93
Yamaoka *et al*[32]	United States	19/11	28/12	0.74 (0.27, 2.02)	8.41
Oliveira *et al*[31]	Brazil	29/23	24/52	2.73 (1.32, 5.67)	11.38
Bartchewsky *et al*[21]	Brazil	40/11	102/27	0.96 (0.44, 2.12)	10.65
Mattar *et al*[16]	Brazil	14/20	22/12	0.38 (0.14, 1.02)	8.63
Subtotal (I^2 =73.8%, P = 0.002)				1.43 (0.68, 3.00)	54.27
Overall (I^2 = 45.0%, P = 0.040)				1.30 (0.88, 1.93)	100.00

Note: Weights are from random effects analysis

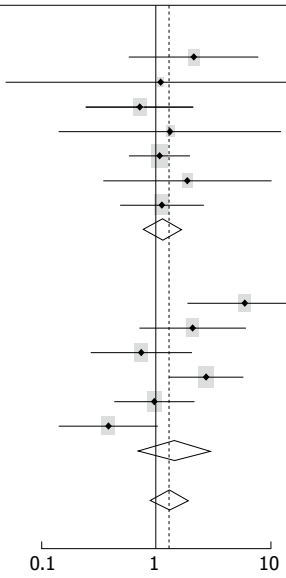


Figure 4 Influence of summary OR coefficients and results on the association between *babA2* genotype and gastric cancer risk. A: Influence analysis. Results were calculated by omitting each study (on the left) in turn. Bars, 95%CI. Meta-analysis random-effects estimates (exponential form) were used; B: Results. ORs and 95%CIs were calculated by a random-effect model.

the source of heterogeneity related to histopathological types. Third, most of the studies had a relatively small sample size.

In conclusion, our results suggest that the presence of *babA2* may contribute to increased risk of PUD, especially DU development, in western countries. In Asians, *babA2* genotype only showed a marginal association with PUD risk, which requires further investigation in the future.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) is a common bacterium with a high prevalence rate and severe pathogenicity, which has been identified as a major cause of severe gastroduodenal diseases, such as peptic ulcer disease (PUD) and gastric cancer (GC). The genome of various *H. pylori* strains demonstrates significant genetic diversity. Genetic variation in specific virulence genes of *H. pylori* may participate in the pathogenic process of *H. pylori* infection in the stomach, thereby contributing to the variable risk of diverse clinical outcomes.

Research frontiers

BabA encoded by the *babA2* gene is a crucial virulence factor of *H. pylori*, which may be involved in the pathogenesis of PUD and GC. Although a few studies have focused on the association between *babA2* gene and the risks of *H. pylori*-related gastroduodenal diseases, those studies showed discrepant results. Moreover, there is no comprehensive meta-analysis integrating the currently available data on the relationship between *babA2* gene and PUD and GC.

Innovations and breakthroughs

This meta-analysis investigated the association between *babA2* gene and PUD and GC. They observed that the presence of *babA2* may contribute to increased risk of PUD, especially duodenal ulcer (DU) development, in western countries. However, in Asians, the presence of *babA2* only showed a marginal association with PUD risk, which requires further investigation. This meta-analysis achieved a relatively comprehensive conclusion on the relationship between *babA2* and clinical outcomes.

Applications

The study suggested that individuals infected with *H. pylori* harboring *babA2* gene were associated with increased risk of PUD, especially DU, in western countries. Eradication of *H. pylori*, in particular *H. pylori* harbouring *babA2*, may contribute to a lower incidence of PUD.

Terminology

babA2: Three *bab* allelic types have been identified, including *babA1*, *babA2* and *babB*, and only the product of the *babA2* gene is necessary for endowing *H. pylori* with Lewis^b antigen binding activity. *babA2* encodes the blood group antigen binding adhesion that binds to fucosylated Lewis^b blood group antigens on gastric epithelial cells.

Peer review

This was a well-performed meta-analysis of currently available studies on the association between *babA2* gene and PUD and GC, and concluded that the presence of *babA2* may be associated with increased risk of PUD, with an emphasis on DU and in western countries. This study was well designed and performed, and the results are well discussed.

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CASE REPORT

Microscopic colitis: Is it a spectrum of inflammatory bowel disease?

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Abstract

Lymphocytic and collagenous colitis are forms of microscopic colitis which typically presents in elderly patients as chronic watery diarrhea. The association between microscopic colitis and inflammatory bowel disease is weak and unclear. Lymphocytic colitis progressing to ulcerative colitis has been previously reported; however there is limited data on ulcerative colitis evolving into microscopic (lymphocytic or collagenous) colitis. We report a series of six patients with documented ulcerative colitis who subsequently were diagnosed with collagenous colitis or lymphocytic colitis suggesting microscopic colitis could be a part of the spectrum of inflammatory bowel disease. The median duration of ulcerative colitis prior to being diagnosed with microscopic colitis was 15 years. We noted complete histological and/or symptomatic remission in three out of six cases while the other three patients reverted back into ulcerative colitis suggesting lymphocytic or collagenous colitis could present as a continuum of ulcerative colitis. The exact molecular mechanism of this histological transformation or the prognostic implications is still unclear. Till then it might be prudent to follow up these patients to assess for the relapse of inflammatory bowel disease as well as for dysplasia surveillance.

Key words: Ulcerative colitis; Lymphocytic colitis; Microscopic colitis; Collagenous colitis; Inflammatory bowel disease

Core tip: Lymphocytic colitis (LC), together with collagenous colitis (CC) is a part of the spectrum of "microscopic colitis" (MC) characterized by profuse non-bloody watery diarrhea, without endoscopic or radiological lesions, but with histological abnormalities. The association between LC and inflammatory bowel disease (IBD) is weak and unclear. The case reports of CC progressing to ulcerative colitis (UC) and vice versa has been previously reported but however to our knowledge we report the first case series of six patients with chronic UC subsequently developing into CC or LC suggesting MC could be a part of the spectrum of IBD.

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INTRODUCTION

Lymphocytic colitis (LC), together with collagenous

colitis (CC) is a part of the spectrum of "microscopic colitis" (MC) characterized by profuse non-bloody watery diarrhea, without endoscopic or radiological lesions, but with histological abnormalities. LC is characterized by increased lymphocytic infiltration of the colonic epithelium and lamina propria. CC in addition to the inflammatory infiltrate is characterized by a markedly thickened sub epithelial collagen band adjacent to the basal membrane. The association between LC and inflammatory bowel disease (IBD) is weak and unclear. The case reports of CC progressing to ulcerative colitis (UC) and vice versa has been previously reported but however to our knowledge we report the first case series of six patients with chronic UC subsequently developing into CC or LC suggesting MC could be a part of the spectrum of IBD[1-4].

We evaluated more than 1000 UC patients from a retrospectively collected UC colonoscopy database who had more than 3000 colonoscopies at our institution from 1998-2011. We identified a total of six patients with documented UC who subsequently were diagnosed with biopsy proven CC or LC from this database. All six patients were seen at our institution with the underlying UC for further management and the diagnosis of UC was reconfirmed by colonoscopic study in our institution. When these patients were followed up either for the change in symptoms or surveillance with the colonoscopic studies, colonic biopsies revealed CC or LC with no evidence of UC (Table 1).

Diagnostic criteria used by our pathologists to diagnose LC is increased intraepithelial lymphocytes (IELs > 20/100 colonic surface epithelial cells) in an architecturally normal colonic mucosa, accompanied by surface epithelial damage and a mixed mononuclear inflammatory infiltrate in the lamina propria[5]. These patients were treated for LC and on subsequent follow up three out of six patients reverted back to UC. All the slides were re-examined by a single pathologist and the diagnosis was confirmed (Liu X).

CASE REPORT

Patient 1

A 79-year-old female with a history of UC for 14 years presented with complaints of left lower quadrant abdominal pain for 1 month duration. She also had intermittent watery diarrhea and fecal urgency over the past 2 years. She denied bloody diarrhea or recent weight loss. She was on maintenance mesalamine, and UC was kept under complete remission. She was evaluated with colonoscopy for the present complaints which revealed very mild generalized redness throughout colon. Random colonic biopsies suggested marked surface epithelial lymphocytosis with collagen deposition in all areas of the colon consistent with the diagnosis of CC without any evidence of UC. She was treated with mesalamine 2400 mg/d and complete resolution of symptoms occurred within a month. She was asked to continue mesalamine and follow up if symptoms recur again. She has not had any recurrence of any symptoms.

Patient 2

A 75-year-old male with a history of UC for 25 years came for the surveillance colonoscopy to our institution. The surveillance colonoscopy with random colon biopsies revealed chronic quiescent UC involving the entire colon with no dysplasia. Subsequently, the patient developed intermittent watery diarrhea with fecal urgency. He denied nocturnal symptoms, bloody diarrhea and recent weight loss. He had a history of aspirin intake but there was no temporal relationship between aspirin intake and onset of diarrhea. Further work up with colonoscopy revealed mild erythema in left colon. The colon biopsies were consistent with LC throughout the colon with a significant increase in the number of intraepithelial lymphocytes without any evidence of UC. He was started on sulfasalazine and symptoms resolved within a month. Surveillance colonoscopy done 2 years later was macroscopically normal. Histopathological studies were negative both for UC and LC. He was asymptomatic to follow up till date.

Patient 3

A 61-year-old male with a long standing history of left-sided UC maintained on remission with sulfasalazine and 6-mercaptopurine had a surveillance colonoscopy performed for UC at our institution 20 years later and was macroscopically normal. Colonic biopsies revealed evidence of LC in the right colon. He was not treated for LC since he was asymptomatic on maintenance treatment. Four years later another surveillance colonoscopy revealed chronic quiescent UC. He was completely asymptomatic through these years.

Patient 4

A 59-year-old female was diagnosed with left-sided UC at the age of 46 and was maintained on mesalamine with complete symptomatic remission. She subsequently developed symptoms of intermittent watery diarrhea accompanied by abdominal cramps. She was treated with prednisone and mesalamine with no symptomatic improvement. She had a history of ibuprofen intake for back pain for a long time and there was no association between ibuprofen and the development of new symptoms. She was further evaluated with colonoscopy which revealed increased intraepithelial lymphocytes with collagen deposition throughout the colon consistent with CC. She was treated with bismuth and her symptoms resolved within 2 mo. The follow up colonoscopy a year later was negative for CC.

Patient 5

A 53-year-old female with a history of left-sided UC for 16 years had surveillance colonoscopy performed which revealed inactive UC in rectum and a tubular adenoma in the descending colon. She was referred to our institution for an opinion regarding UC and tubular adenoma. She

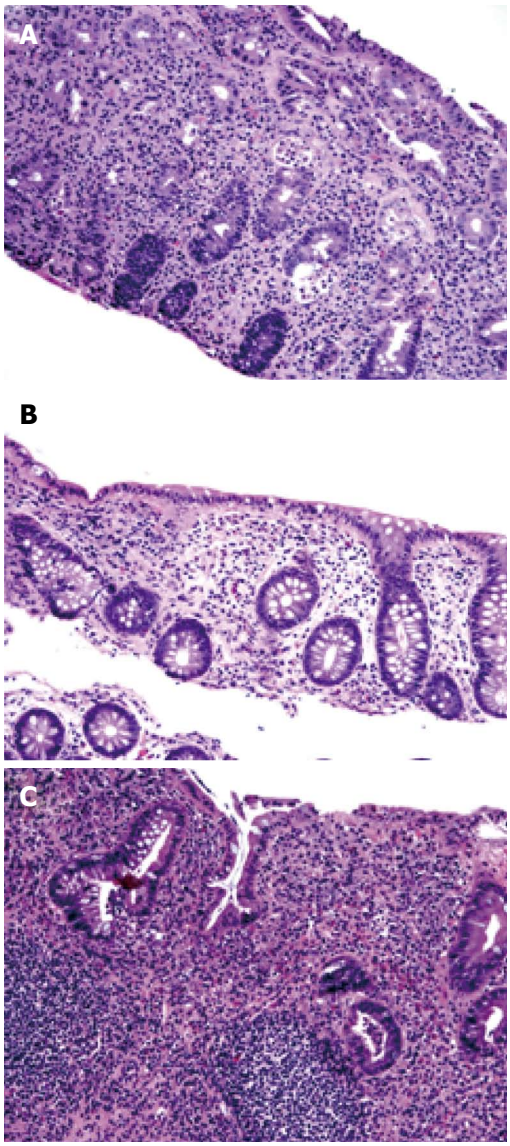

Figure 1 Histologic evolution of colitis (hematoxylin and eosin stain, × 200). A: Active colitis manifested by epithelial injury and cryptitis, in the context of clinical history of ulcerative colitis (UC) and lack of other etiologies for active colitis, this is consistent with early exacerbation of UC; B: Lymphocytic colitis (LC) (3 years after the exacerbation depicted in A). There is surface intraepithelial lymphocytosis and epithelial injury but without significant chronic information. The crypt architecture is normal; C: Reverting to active ulcerative colitis manifested by basal lympho plasmacytosis, architectural distortion, and cryptitis (3 years after an episode of LC-pattern of injury).

was treated with mesalamine for UC in the past and she was kept under complete remission. She was evaluated with a colonoscopy in our institution which suggested macroscopically normal colon with a histop anthology consistent with LC without any evidence of dysplasia. There was no change in treatment. Subsequently, surveillance colonoscopy done a year later revealed mild inactive UC in rectum and sigmoid colon without any evidence of LC and dysplasia. Two subsequent surveillance colonoscopies were positive for chronic UC in sigmoid colon and rectum without any dysplasia.

Patient 6

A 36-year-old female had a long standing history of extensive colitis for 18 years. She developed steroid dependent UC and required 6-mercaptopurine and mesalamine for symptom control (Figure 1A). She was maintained on complete remission on these medications. She had a surveillance colonoscopy performed which revealed mild erythema in distal rectum. The colonic biopsy studies were consistent with LC (Figure 1B) in all other areas of the colon. She had a follow up colonoscopy three years later which showed left-sided chronic active UC without any evidence of LC (Figure 1C). She has been asymptomatic at the time of last follow-up.

DISCUSSION

LC is characterized by chronic watery diarrhea and specific histopathological changes in a macroscopically normal colonic mucosa. The incidence has been reported to be 5.5 per 100000 and prevalence is 63.7 per 100000[6], but the incidence and prevalence appears to be increasing over time. The median age at diagnosis is 59 years with female: male ratio of 2.4:1. The most frequent symptoms at presentation are diarrhea, abdominal pain, weight loss and fecal urgency. Most patients are in remission with a limited disease duration of 6 mo; it can be chronic intermittent or chronic continuous in minority of patients. 7 However, relatively limited data has been published on the relationship between inflammatory bowel disease (IBD) and LC[7-11].

The etiology of LC is largely unknown and probably multifactorial. At present, it is thought to be caused by immunological reaction to different mucosal insults in predisposed individuals. The frequent association of LC with other autoimmune disorders (thyroid disease, diabetes mellitus, celiac disease, psoriasis, and rheumatoid arthritis), inflammation in the lamina propria with increased intraepithelial lymphocytes and the fair response to steroids support this theory. Infectious agents, drugs, or food antigen such as gluten may be precipitating factors. The importance of genetic factors in LC is still unclear but Olesen *et al*[7] suggested a family history of IBD in patients with LC. We describe six cases of UC that subsequently evolved into CC or LC. The median age at the time of UC diagnosis was 38 years in our series. The median duration of UC prior to being diagnosed with CC or LC was 15 years. The median age at the time of LC diagnosis was 51 years. We noted complete histological and/or symptomatic remission in three out of six cases while the other three patients reverted back into UC suggesting that LC could present as a continuum of UC. The triggering factor for this transformation is still unknown. The association between MC and IBD is found predominantly in patients with extensive colitis[12]. However, in our case series only 50% of patients had extensive colitis when LC or CC was diagnosed.

The relationship between LC and IBD is unclear. Sur-

Table 1 Clinical characteristic of patients

Variables	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
Age at the time of last follow-up (yr)	81	75	62	60	52	36
Gender	Female	Male	Male	Female	Female	Female
Age at diagnosis of UC	65	42	28	46	34	18
Age at diagnosis of LC/CC	79	72	48	54	45	33
Duration of UC when LC/CC was diagnosed	14	30	20	8	11	15
Extent of UC when LC/CC was diagnosed	Extensive colitis	Extensive colitis	Left-sided	Left-sided	Left-sided	Extensive colitis
Extent of LC/CC at diagnosis	Extensive colitis	Extensive colitis	Right-sided	Extensive colitis	Extensive colitis	Extensive colitis
Endoscopic findings at the time of LC/CC diagnosis	Mild generalized redness	Mild erythema at left colon	Normal	Normal	Normal	Mild erythema at distal rectum
Medication at the time of LC/CC diagnosis	Mesalamine	None	Sulfasalazine, 6-mercaptopurine	Mesalamine	Mesalamine	Mesalamine, 6-mercaptopurine
Medications added when LC/CC was diagnosed	None	Sulfasalazine	None	Bismuth	None	None
Outcome on subsequent follow up	Asymptomatic	Asymptomatic	Ulcerative colitis	Asymptomatic	Ulcerative colitis	Ulcerative colitis
Family history of IBD/LC/CC	UC	None	None	None	None	UC
Smoking History	Yes	Ex-smoker	No	Yes	Yes	Ex-smoker
Alcohol use history	Yes alcoholic	Yes	No	No	Yes	No
History of aspirin/NSAIDS intake	No	Aspirin	No	NSAIDS	No	No
Period of follow up after LC/CC was diagnosed	3 mo	3 yr	10 yr	3 yr	8 yr	3 yr

NSAIDS: Non steroidal anti-inflammatory drugs; CC: Collagenous colitis; LC: Lymphocytic colitis; UC: Ulcerative colitis; IBD: Inflammatory bowel disease.

veillance colonoscopic biopsies from IBD patients with inactive disease may show a collagenous colitis pattern in UC and a focal LC-like pattern with Crohn's disease[1,12,13]. In our patients, the histopathological diagnosis of LC when UC was in complete remission further raises the question whether the observed LC pattern is an expression of healing and inactive UC disease in reality. However, the presence of symptoms in some patients and lack of IBD flare immediately prior to LC diagnosis makes this "healing" theory unlikely. A previous study had demonstrated that the transcriptional factor nuclear factor κB activation occurs in both UC and CC patients. However in CC patients, nuclear factor κB activation occurs only in epithelial cells whereas it occurs both in epithelial cells and lamina propria macrophages in UC patients[14]. Hence it is possible that the site of nuclear factor κB activation determines the pathological manifestation of the disease. Either UC or MC may precede the onset of the other. Based on these data, it seems reasonable to assume that UC and MC could represent both ends of the spectrum of the same disorder.

With the aggregate of cases we have reported along with other few case reports it seems highly reasonable to assume that LC or CC could be a part of the spectrum of IBD. Whether it is a random coincidence of MC and IBD in the same patient remains to be answered. Since there was no specific cause of LC in our patients such as an infection or drugs, along with the absence of autoimmune conditions usually associated with LC further supports our current view. The prognostic implication of this histological transformation to LC in inactive UC patients is still unknown and has to be further evaluated with prospective studies. Until then it might be prudent to consider MC as a part of the natural history of IBD, at least in some cases, and follow up these patients to assess for the relapse of IBD as well as for dysplasia surveillance.

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CASE REPORT

Alveolar echinococcosis-spreading disease challenging clinicians: A case report and literature review

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Abstract

Human alveolar echinococcosis (AE) is a potentially deadly disease; recent studies have shown that the endemic area of *Echinococcus multilocularis*, its causative agent, is larger than previously known. This disease has low prevalence and remains underreported in Europe. Emerging clinical data show that diagnostic difficulties are still common. We report on a 76-year old patient suffering from AE lesions restricted to the left lobe of the liver who underwent a curative extended left hemihepatectomy. Prior to the resection a liver biopsy under the suspicion of an atypical malignancy was performed. After the intervention he developed a pseudoaneurysm of the hepatic artery that was successfully coiled. Surprisingly, during surgery, the macroscopic appearance of the tumour revealed a growth pattern that was rather typical for cystic echinococcosis (CE), *i.e.*, a gross tumour composed of multiple large vesicles with several centimeters in diameter. In addition, there were neither extensive adhesions nor infiltrations of the neighboring pancreas and diaphragm as was expected from previous imaging results. The unexpected diagnosis of AE was confirmed by definite histopathology, specific polymerase chain reaction and serology results. This is a rare case of unusual macroscopic presentation of AE that posed immense diagnostic challenges and had an eventful course. To our knowledge this is the first case of an autochthonous infection in this particular geographic area of Germany, the federal state of Saxony. This report may provide new hints for an expanding area of risk for AE and emphasizes the risk of complications in the scope of diagnostic procedures and the limitations of modern radiological imaging.

Key words: Alveolar echinococcosis; *Echinococcus multilocularis*; Autochthonous infection; Liver resection; Hemihepatectomy

Core tip: We describe a rare case of uncommon macroscopic presentation of autochthonous infection with *Echinococcus multilocularis* that posed immense diagnostic challenges and had an eventful course. To our knowledge this is the first case of an autochthonous infection in this geographic area. This report may deliver new hints for an expanding area of risk for alveolar

echinococcosis and emphasizes the risk of complications in the scope of diagnostic procedures and the limitations of modern imaging techniques.

Atanasov G, Benckert C, Thelen A, Tappe D, Frosch M, Teichmann D, Barth TFE, Wittekind C, Schubert S, Jonas S. Alveolar echinococcosis-spreading disease challenging clinicians: A case report and literature review. *World J Gastroenterol* 2013; 19(26): 4257-4261 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i26/4257.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i26.4257

INTRODUCTION

The fox-tapeworm *Echinococcus multilocularis* (*E. multilocularis*) is the causative agent of alveolar echinococcosis (AE), a potentially deadly parasitic disease. AE is prevalent in the northern hemisphere and central Europe is an endemic focus[1-3]. In the era prior to anthelmintic treatment the cumulative lethality for AE was about 90% ten years after a diagnosis has been established[4]. Imaging techniques of hepatic involvement by AE commonly reveal an ill-defined lesion of the liver parenchyma and contrast computer tomography (CT) and magnetic resonance imaging (MRI) are considered to clearly demonstrate infiltrative structure and extension of the parasitic tumour to adjacent structures[5-8]. In macroscopic sections of the human liver the larval parasite usually exhibits an alveolar (spongy) structure composed of numerous irregular vesicles with diameters between less than 1 and up to 20 mm[9]. Biologically, the lesions behave like a slow-growing liver cancer, without sharp boundaries between the parasitic tissue and the liver parenchyma.

Publications on surgical procedures and results are rare but essential, and prospective studies are not available because the incidence of the disease is low. According to current treatment guidelines, surgery should be the first choice if the parasitic mass is resectable *in toto*[10]. Complete resections of the parasitic lesion can cure the patient while available drugs are only parasitostatic[10-12].

Emerging clinical data indicate that the parasite's geographic range has widened in recent years[13,14]. Growing fox populations in Europe, especially in urban zones, have drawn attention to a potentially increased infection risk for humans with a phase lag of 10-20 years[15-17]. In addition, since AE is not adequately considered as a differential diagnosis, the disease remains thus underdiagnosed in Europe[18].

We report on a 76-year old patient with AE of the liver, who underwent a curative resection. Prior to resection the patient had an eventful course due to the development of a postinterventional pseudoaneurysm (aneurysm spurium) of the hepatic artery following a biopsy of the liver lesion. Interestingly, during surgery the parasitic mass appeared typical for cystic echinococcosis (CE) and revealed no infiltrations or extensive adhesions to adjacent structures as contrastingly expected from the preoperative imaging results of the abdomen. Nevertheless, the definite histology as well as referral evaluations [serology and polymerase chain reaction (PCR)] confirmed the diagnosis of AE. The patient has not been abroad for the last 20 years and at his farm he has been in constant contact to various animal species, including dogs and wild foxes. To our knowledge this is the first case of autochthonous AE infection in the federal state of Saxony, Germany.

CASE REPORT

In April 2010 a 76-year old farmer from Saxony was presented in the emergency department of a general district hospital with a severe abdominal pain under the suspicion of a biliary colic and pancreatitis, respectively. In the performed imaging of the abdomen a large cystic tumour in the left sided liver was detected. In the further course, suspecting a highly malignant atypical primary liver cancer, a biopsy for the presumed confirmation of the diagnosis was performed. After the intervention the patient was discharged in good clinical condition. Surprisingly, the histological findings from the tumour biopsy were consistent with the larval stage of *E. multilocularis*. Subsequent serological investigation by a referral laboratory for echinococcosis confirmed specific antibodies for AE in the patient's serum. Moreover, a pan-cestode 12S rRNA gene-PCR from the paraffin block was positive and sequencing of the amplicon revealed 100% identity with *E. multilocularis*. Therefore, an anti-infective drug treatment with albendazole was initiated.

Several days after liver biopsy the patient presented again with severe abdominal pain and jaundice. In the emergency CT scan of the abdomen the gross tumour revealed no progression. Though, a postjunctional pseudoaneurysm of a branch of the left hepatic artery in segment 4a in direct proximity to the tumour tissue was newly diagnosed. A subsequent coil-embolization of the aneurysm was successfully performed.

One month later, in May 2010, the patient was referred to our centre as a potential candidate for abdominal surgery. A thorough examination of patient data and history files revealed that the farmer has not been abroad in the last few decades. In his farm he has been in a constant contact and exposure to numerous domestic and wild animals, including dogs and foxes. The patient had neither B-symptoms nor further major ailments and was in good general condition. The blood tests revealed normal findings for alpha-1-fetoprotein, carbohydrate-antigen 19-9 and carcinoembryonic antigen. Preoperatively, due to pronounced cholestasis and hyperbilirubinaemia, an endoscopic retrograde cholangiography (ERC) with stenting of the main bile duct was performed. Clinical imaging prior to surgery revealed that the echinococcal lesion infiltrated liver segments 2 and 3 and showed a maximum diameter of 13.5 cm (Figure 1A and B). In addition, CT suggested the presence of adhesions in the

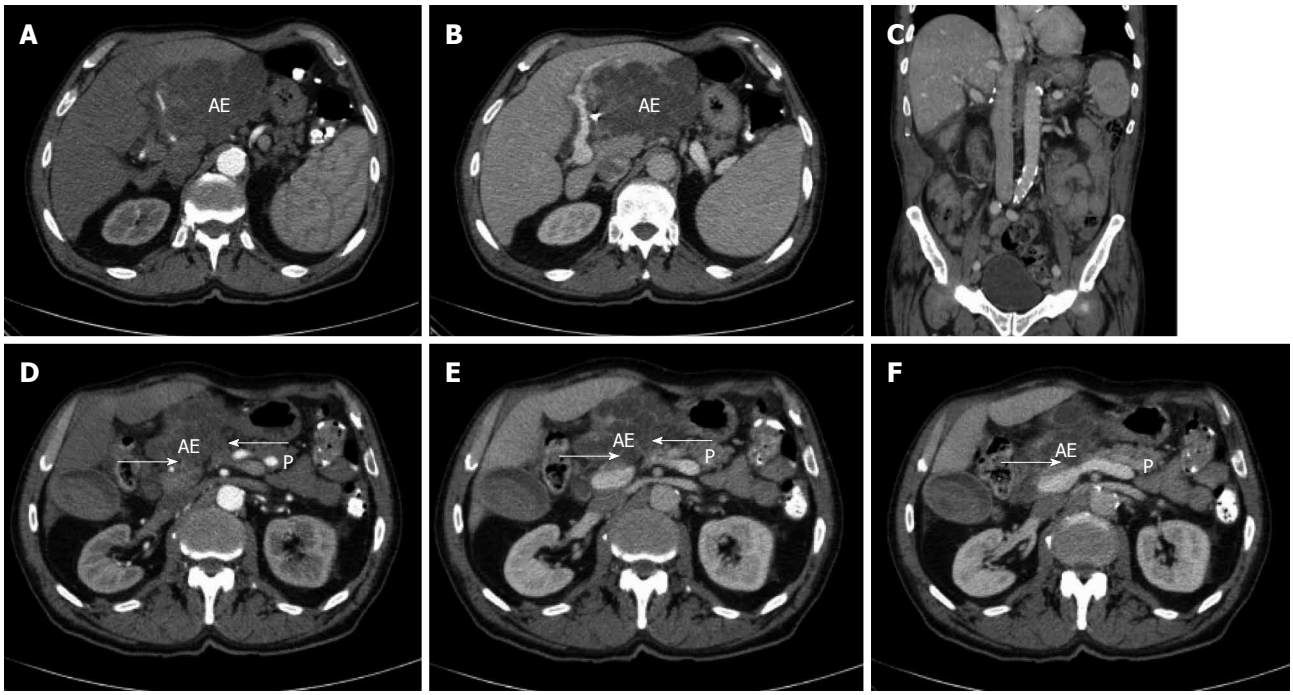

Figure 1 Radiological findings prior and after curative resection. A, B: Computed tomography of the abdomen displaying an extended tumour manifestation prior to resection; C: Computed tomography of the abdomen following extended left hemihepatectomy; D-F: Computed tomography of the abdomen prior to resection. Arrows: possible extensive adhesions to adjacent pancreatic head and corpus. AE: Alveolar echinococcus tumour; P: Pancreas.

area of the diaphragm and cystic infiltration of the pancreatic head and corpus (Figure 1 D-F). The diagnostic evaluation revealed no further extrahepatic manifestations. Hence, additionally to extended left hemihepatectomy, we considered a concomitant resection of the pencreatic head and corpus.

During surgery the tumorous manifestation in the left lobe of the liver was confirmed but, surprisingly, no infiltrative growth to neighboring structures was detectable. Basically, we observed a gross tumor with multiple large cystic structures that varied in size, *i.e.,* a growth pattern that is rather typical for CE. Further exploration revealed no cystic adhesions to the diaphragm. After exploration of the bursa omentalis, no infiltration of the pancreas was notable either. No evidence for further extrahepatic tumorous dissemination or lymph node metastases was found. As the restriction of the tumour to the left liver lobe was confirmed, we affirmed the indication for extended left hemihepatectomy with curative intent. The situs was then suffused with cloths imbued in hypertonic (10%) Sodium chloride (NaCl) solution. Subsequently the isolation of the proper hepatic artery and the selective division of the left hepatic artery followed. After isolation of the main trunk of the portal vein and the left portal vein branch, the latter was divided. Then cholecystectomy was performed. After the left hepatic vein was divided, lobus caudatus (segment 1) was mobilized. Then, a liver resection was completed without any intraoperative complications. Parenchymal transection was performed using an ultrasonic dissection device (cavitron ultrasonic surgical aspirator, CUSA®). The caudate lobe did not appear to be infiltrated but to ensure an additional safety distance it was resected as well. The parenchymal resection was performed along the level of the middle hepatic vein in direction to the gall bladder bed and then caudally to the hepatic hilum. At the hilum the liver dissection diverged to the left and then ended in the parenchymal bridge leading to the caudate lobe. In the region of the hilum the left hepatic duct was isolated and then selectively divided. After removing the left liver lobe and the caudate lobe, the situs was rinsed with hypertonic NaCl solution. A T-Drain was inserted in the main bile duct for decompression and easy access cholangiography. During the postoperative stay in the intensive care unit the patient did not develop any significant complications.

Based on the finding of multiple large cystic formations of the tumorous lesion an additional histological evaluation was performed in a referral centre for pathology. There, the original diagnosis of hepatic AE was confirmed (Figure 2). In the postoperative course no signs of insufficient liver function were notable. The conclusive pathological result showed the parasitic tumour was entirely resected (Figures 1C and 3).

After a total postoperative hospitalization of 30 d the patient was dismissed. The anti-infective drug treatment with albendazole was maintained as long-term therapy. The follow up visit 6 and 12 mo after resection revealed normal liver function and no evidence for recurrent disease.

DISCUSSION

AE, caused by the larval (metacestode) stage of *E. multilocularis*, is found in the northern hemisphere and is a

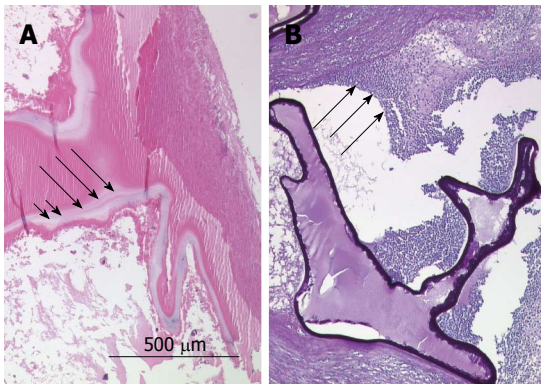

Figure 2 Referral evaluations for diagnosis of alveolar echinococcosis. A: The hematoxylin and eosin stain of paraffin sections displays the laminated layer as a narrow band (long arrows). The germinal layer is marked by short arrows; B: Periodic acid-Schiff (PAS) stain shows a strongly PAS-positive basophilic laminated layer displaying a bizarre narrow structural pattern. The long arrows indicate the typical severe inflammatory process associated with the characteristic tubular growth pattern of the parasite.

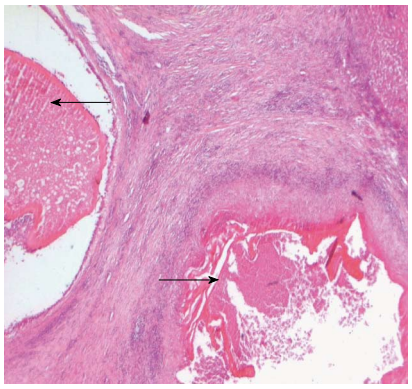

Figure 3 Histological findings after curative resection. Hematoxylin and eosin stain of paraffin sections displaying two daughter cysts containing no vital protoscoleces embedded in a larger lesion. Black arrows indicate avital protoscoleces.

potentially fatal disease. The parasite is transmitted to humans by eggs of the helminth shed into the environment by feces of foxes. Almost exclusively, the liver is affected[19]. Recent studies have shown that the endemic area of *E. multilocularis* is larger than previously known and has regionally expanded from rural to urban areas[20-22]. In addition, increasing fox populations are associated with higher infection risk in humans with a phase lag up to 20 years[15].

The macroscopic appearance of an echinococcal lesion is distinct in regard of its species and developmental stage. While parasitic masses of CE ordinarily display a typical structure of a single or multiple fluid-filled large unilocular cysts that can reach monstrous dimensions, AE preferentially exhibits metastasis and an infiltrative growth to adjacent host tissues with a spongy structure composed of numerous irregular small vesicles of several millimeters. Thus, the surgical therapy for hepatic AE conforms the operative principles established for malignant liver tumours, *i.e., in toto* removal of the tumour with additional safety distance and tumour free resection margins[4].

Chemotherapy with benzimidazoles is the backbone of the comprehensive treatment of AE and long-term anti-infective drug treatment has been established in many centres in Europe as well as in China[9]. In spite of remarkable improvement of long term patient survival after the introduction of anti-helmintic drug treatment, this therapeutic modality proved to be mainly parasitostatic. Therefore, surgical resection represents the therapy of choice for patients with operable lesions of AE.

In the present report we have described the first case of autochthonous infection with *E. multilocularis* in our federal state of Saxony, Germany. The tumour masses affected liver segments 2 and 3 and the patient received a curative extended left hemihepatectomy. Due to a suspected liver malignancy an interventional biopsy of the lesion was preoperatively performed. Usually, such a diagnostic step is considered a contraindication in cases of AE because of the risk of abdominal seeding and anaphylaxis. Fortunately, the postinterventional iatrogenic pseudoaneurysm of the left hepatic artery could be treated with success. Interestingly, the parasitic mass showed a macroscopic pattern that appeared typical for CE which is caused by the larval stage of the dog tapeworm, *Echinococcus granulosus* (*E. granulosus*). The cause of this phenomenon remains for the most part unknown but has been reported occasionally in historic reports. Dual infection with *E. granulosus* and *E. multilocularis* could have also been possible. Indeed, concomitant infections with both echinococcal species have been reported in the literature but in the present case the definite histology, PCR results, serology, and immunohistology for specific structural proteins were all clearly positive for *E. multilocularis* only[23]. Additionally, a major distinguishing factor between *E. granulosus* cysts and *E. multilocularis* is the presence of an adventitial layer around the *E. granulosus* metacestode. The histological evaluations did not detect such a structure in the present case. Furthermore, despite the expected extensive adhesions to the diaphragm and pancreas seen by preoperative imaging, no such condition or infiltration to neighboring structures could be confirmed, showing current limitations of modern imaging techniques. All together this data indicate that in the current era diagnosis as well as assessment of extent of local disease still remain a challenge for clinicians.

Recent investigations suggest that AE remains underreported and human infection can also occur in regions with low overall parasite prevalence. Case reports from regions remote from the areas of high prevalence may be strong hints of new areas at risk[18,24]. In addition to the high prevalence rates for AE in the southern geographic regions of Germany, recent data suggest growing numbers of AE cases in neighboring European countries, such as the Czech Republic. Some of these cases indicate an autochthonous character of the infection[25,26]. Thus, a possible enlargement of fox populations in the last decades as well as migration of infected animals might have

been a possible source for infection in the current case.

In conclusion, we have here described the first autochthonous infection with *E. multilocularis* in Saxony, Germany, providing the first evidence for a new geographical area at risk for the acquisition of AE. Albeit, curatively treated with an extended left hemihepatectomy the disease presented with uncommon findings and had an eventful course, constituting a challenge for clinicians.

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CASE REPORT

A white opaque substance-positive gastric hyperplastic polyp with dysplasia

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Abstract

The endoscopic findings of gastric hyperplastic polyps (HPs) with dysplasia have not been well-defined, and the clinical significance of these lesions, including their malignant potential, is unclear. In this report, we describe a case of a white opaque substance (WOS)-positive gastric HP with dysplasia. A 76-year-old woman was referred to our hospital for endoscopic resection of a gastric HP. Upper endoscopy revealed a 25-mm whitish and reddish polypoid lesion on the greater curvature in the lower third of the stomach. The whitish part was diagnosed as a WOS using conventional and magnifying endoscopy with narrow band imaging. An examination of the biopsy specimen indicated that the lesion was a typical gastric HP. However, because of its color and the presence of a WOS, we suspected that this lesion was an atypical gastric HP. Therefore, we performed a polypectomy. Histopathologically, diffuse low- to high-grade dysplasia was found on the surface of the polyp. We performed immunohistochemical staining using a monoclonal antibody specific for adipophilin as a marker of lipid droplets (LDs). LDs were detected in approximately all of the neoplastic cells, especially in the surface epithelium of the intervening apical parts and were located in the subnuclear cytoplasm of the neoplastic cells. According to endoscopic and histopathological findings, the WOS-positive epithelium indicated dysplasia of the gastrointestinal phenotype, which could absorb lipids. The presence of a WOS in a gastric HP may be considered an endoscopic finding that is predictive of the neoplastic transformation of a gastric HP. We suggest that a WOS-positive gastric HP should be resected endoscopically to investigate its neoplastic transformation.

Key words: Gastric hyperplastic polyp with dysplasia; White opaque substance; Adipophilin; Lipid droplet; Narrow band imaging

Core tip: In this report, we present the first case of a white opaque substance (WOS)-positive gastric hyperplastic polyp (HP) with dysplasia. We performed immunohistochemical staining using a monoclonal antibody specific for adipophilin as a marker of lipid droplets. According to endoscopic and histopathological findings, the WOS-positive epithelium corresponded to the dysplasia in this lesion. The presence of a WOS in a gastric HP may be considered an endoscopic finding that is predictive of the neoplastic transformation of a gastric HP. We suggest that patients with a WOS-positive gastric HP should be treated by endoscopic resection to investigate the neoplastic transformation of the HP.

Ueyama H, Matsumoto K, Nagahara A, Gushima R, Hayashi T, Yao T, Watanabe S. A white opaque substance-positive gastric hyperplastic polyp with dysplasia. *World J Gastroenterol* 2013;

19(26): 4262-4266 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i26/4262.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i26.4262

INTRODUCTION

With the widespread use of digestive endoscopy in recent years, gastric polyps are now diagnosed more frequently and can be easily studied after a biopsy or polypectomy. Gastric hyperplastic polyps (HPs) are among the most common type of benign epithelial gastric polyps[1-6]. Gastric HPs are usually considered to be benign lesions similar to adenomas; however, neoplastic transformation can occur but rarely. Moreover, endoscopic findings of gastric HPs with dysplasia have not been well-defined, and the clinical significance of these lesions, including their malignant potential, is unclear. A white opaque substance (WOS) is a finding from magnifying endoscopy (ME) with narrow band imaging (NBI), which was first reported by Yao *et al*[7-9] to be a substance in the superficial area of gastric neoplasias that obscures the subepithelial microvascular architecture. However, the presence of a WOS in gastric lesions other than adenomas and adenocarcinomas has not been reported. We report a rare case of a WOS-positive gastric HP with dysplasia.

CASE REPORT

A 76-year-old woman was referred to our hospital for further investigation and treatment of a gastric HP. Excluding the existence of the gastric HP, she had no specific symptoms and the results of the physical examination were normal. Her medical history included hyperlipidemia and diabetes mellitus, and there was no family history of gastrointestinal polyposis. She had not undergone proton pump inhibitor therapy. An assessment of Immunoglobulin G antibodies and a histological examination were negative for *Helicobacter pylori* infection. Upper endoscopy revealed a 25-mm polypoid lesion on the greater curvature in the lower third of the stomach (Figure 1A). The entire lesion was reddish with scattered whitish areas. The whitish parts were determined to be a WOS using conventional endoscopy and ME with NBI (Figure 1). The WOS in the lesion was comprised of two morphological types (Figure 1C and D). One type had a symmetrical distribution of a regular dotted pattern (Figure 1C), and the other type had an asymmetrical distribution of an irregular speckled and linear pattern (Figure 1D). An examination of the biopsy specimen revealed findings that were typical of a gastric HP without dysplasia. However, we suspected that this lesion was an atypical gastric HP because of its color and the irregular distribution of the WOS. Therefore, we performed a polypectomy, which was without complications. Histopathologically, the findings for the entire lesion were typical of

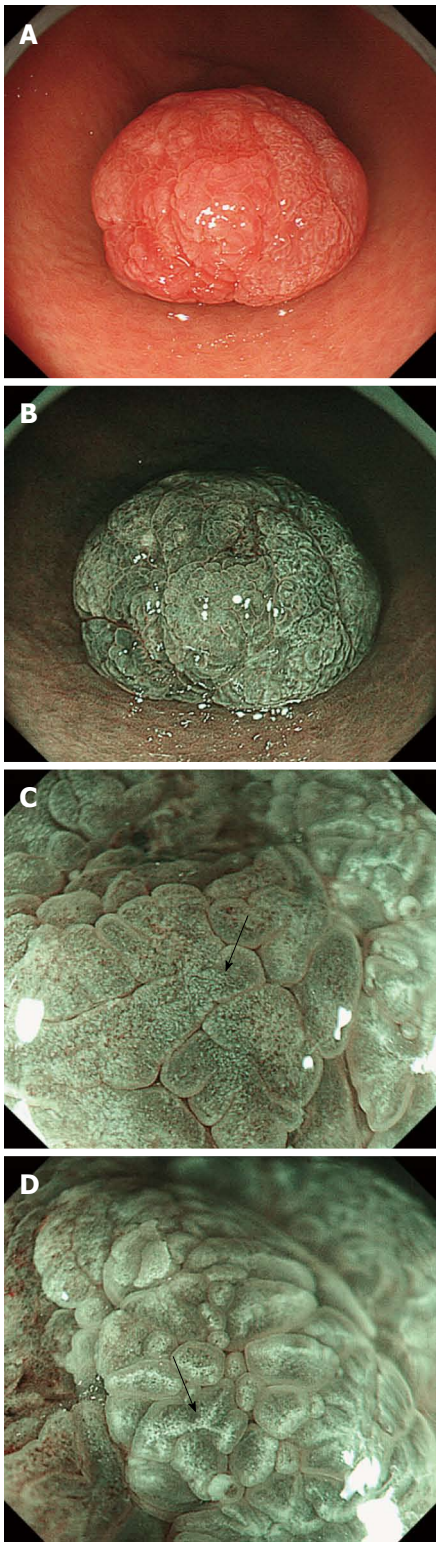

Figure 1 A white opaque substance-positive gastric hyperplastic polyp is shown on upper endoscopy. A: An endoscopic examination with a white light image revealed a 25-mm polypoid lesion on the greater curvature in the lower third of the stomach; B: A white opaque substance (WOS) was visualized on the surface of this lesion using conventional endoscopy and magnifying endoscopy (ME) with narrow band imaging (NBI); C: The ME with NBI findings. A regular dotted pattern of the WOS was distributed symmetrically (arrow); D: The ME with NBI findings. An irregular speckled and linear pattern was distributed asymmetrically (arrow).

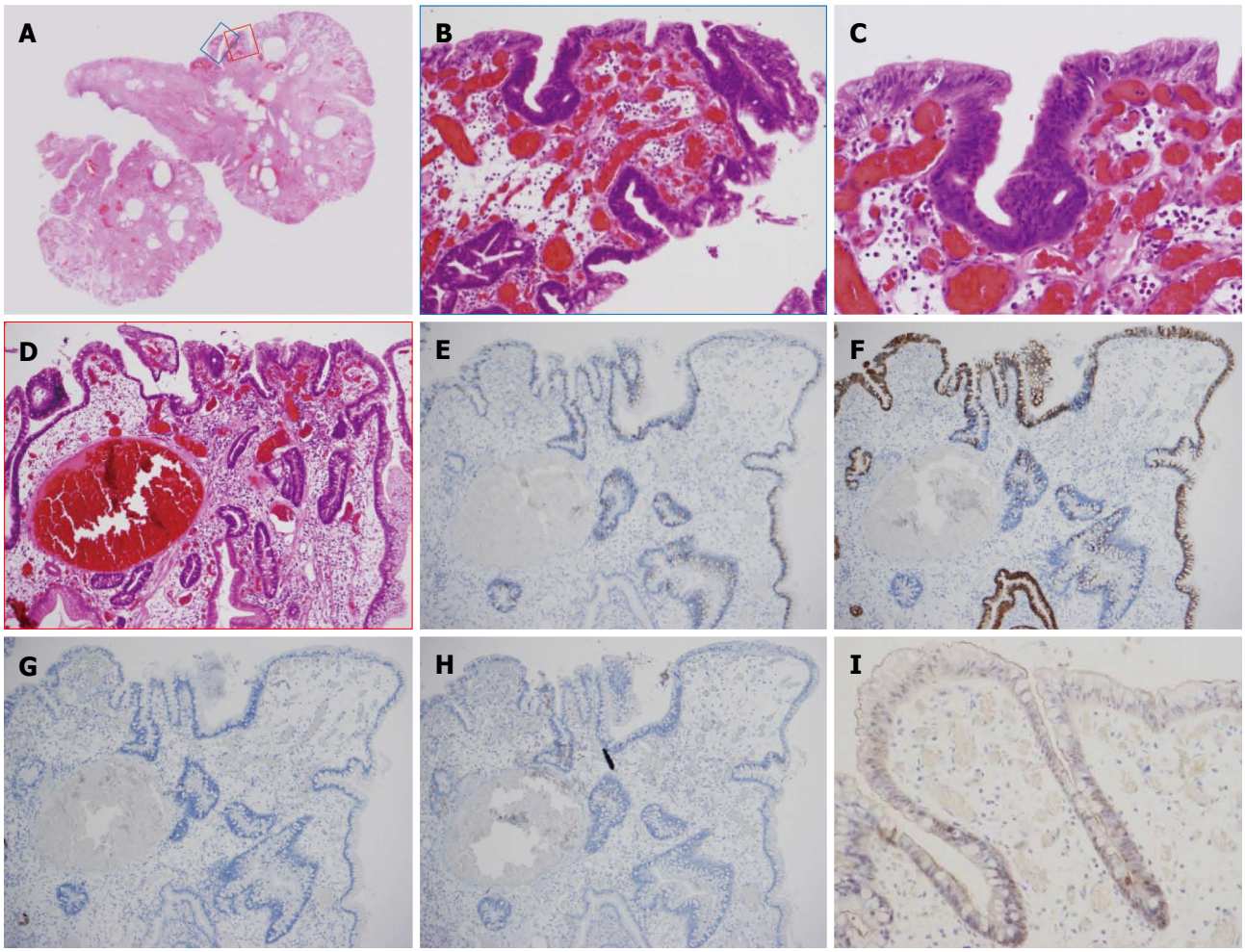

Figure 2 The resected specimen shows a gastric hyperplastic polyp with dysplasia. A-D: The histological examination of the resected specimens (hematoxylin and eosin stain). A: In the low power view, the findings for the entire lesion were typical of a gastric hyperplastic polyp; B, C: High-grade dysplasia was observed on the surface of the lesion; D: Low-grade dysplasia was observed on the surface of the lesion; E-I: The immunohistochemical examination. E: Mucin 2 (MUC2); F: MUC5AC; G: MUC6; H: CD10; I: Villin. The lesion had diffuse positivity for MUC5AC, focal positivity for MUC2 and villin, and negative staining for MUC6 and CD10.

a gastric HP, and diffuse low- to high-grade dysplasia was found on the surface of the lesion (Figure 2A-D). Immunohistochemically, the lesion had diffuse positivity for MUC5AC, focal positivity for mucin 2 (MUC2) and villin, and negative staining for MUC6 and CD10 (Figure 2E-I). This lesion was classified as having the gastrointestinal (GI) phenotype according to combinations of the expression of MUC2, MUC5AC, MUC6, CD10 and villin. The GI phenotype was detected in approximately all of the neoplastic cells, whereas an examination of the other cells indicated a gastric phenotype. The Ki-67 labeling index of dysplasia was slightly higher than that of a typical HP, and the positive cells were irregularly distributed. The overexpression of the p53 protein was not observed. In addition, adipophilin was detected in approximately all of the neoplastic cells, especially in the surface epithelium of the intervening apical parts and was located in the subnuclear cytoplasm of the neoplastic cells (Figure 3). This lesion was finally diagnosed as a WOS-positive gastric hyperplastic polyp with dysplasia. Surveillance endoscopy with biopsy specimens is planned for 6 mo after the endoscopic resection.

DISCUSSION

The endoscopic findings for gastric HP with dysplasia have not been well-defined. Typical HPs are markedly reddish polypoid lesions with a smooth surface, which occasionally has erosions. In this case, the entire lesion was reddish and was scattered with whitish areas, which differs from typical HPs. The whitish areas were determined to be a WOS using conventional and ME with NBI. Histopathologically, low- to high-grade dysplasia was diffusely present on the surface of the gastric HP. Adipophilin was detected in approximately all of the neoplastic cells, especially in the surface epithelium of the intervening apical parts. These findings suggested that the WOS-positive epithelium corresponded to the dysplasia in this lesion. Yao *et al*[10] reported that the hallmark of a WOS is the presence of lipid droplets (LDs) that accumulate in the superficial part of the epithelial neoplasia within the stomach. Using immunohistochemistry and immunoelectron microscopy, Ueo *et al*[11] found that the WOS resulted from an accumulation of LDs with adipophilin. These findings supported a relationship

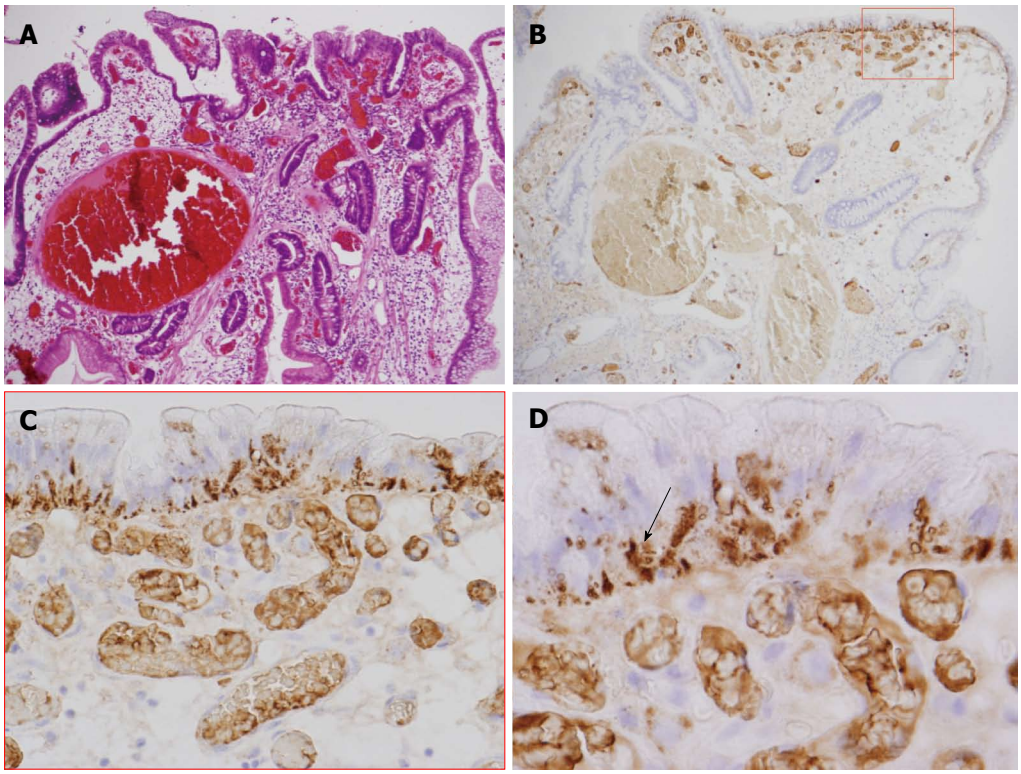

Figure 3 The immunohistochemical analysis indicates that dysplasia is positive for adipophilin. A: Low-grade dysplasia was observed on the surface of the lesion; B-D: The immunohistochemical examination of adipophilin. Adipophilin was detected in approximately all of the neoplastic cells, especially in the surface epithelium of the intervening apical parts and was located in the subnuclear cytoplasm of the neoplastic cells (arrow).

between the WOS and adipophilin in this case. In addition, the WOS in this lesion was comprised of two morphological types: one type with a symmetrical distribution of a regular dotted pattern and the other type with an asymmetrical distribution of an irregular speckled and linear pattern. We could not discriminate between these patterns pathologically. Yao *et al*[7] reported that the WOS in adenomas was regular and homogeneous, whereas the WOS in adenocarcinomas was irregular and speckled. In this case, we speculated that the findings for a WOS may be based on the differences in the shape, the intraepithelial and intracytoplasmic density and the distribution of the LDs between low-grade and high-grade dysplasia. In our case, most of the reddish area indicated low-grade dysplasia. Endoscopically, we determined that these areas were reddish because a slight accumulation of LDs may not allow these areas to be visualized as a WOS. We concluded that a WOS may be visualized only in the dysplastic areas of gastric HPs. The presence of a WOS in a gastric HP may be considered an endoscopic finding that is predictive of the neoplastic transformation of a gastric HP.

The neoplastic transformation of HPs has not been well-defined, and their clinical significance, including their malignant potential, is unclear. Kang *et al*[12] reported that the neoplastic transformation of gastric HPs was significantly associated with the postgastrectomy state and lesions that were 1 cm in diameter, pedunculated, and synchronous neoplastic lesion. Daibo *et al*[13] reported that cancer cells arose from the dysplastic area in HPs rather than directly from nondysplastic hyperplastic epithelium, which is consistent with the histogenesis of the malignant transformation of HPs. Endoscopic resection should be considered for these HPs to avoid the risk of missing HPs with neoplastic potential. In our case, the dysplasia was observed on the surface of the resected HP; however, an examination of the biopsy specimen indicated a typical HP without dysplasia. Regarding this discrepancy, we speculate that when the biopsy specimen was collected, a small sample was unintentionally taken from the part of the lesion that did not exhibit dysplasia. Using the biopsy specimen that was obtained, we could not clearly determine whether the lesion was a typical HP or an HP with low-grade dysplasia. Therefore, in gastric HPs, WOS positivity may be considered an endoscopic finding that indicates endoscopic resection.

The mechanism of the accumulation of LDs in gastric epithelium is unknown. Yao *et al*[10] proposed the following two possible mechanisms: the absorption hypothesis and the production hypothesis. In addition, they reported that the WOS in gastric neoplasms with an intestinal phenotype was caused by the accumulation of lipids and WOS-positive gastric neoplasms may be able to absorb lipids. Matsubara *et al*[14] demonstrated that the expression of adipophilin may be induced during the process of early colorectal carcinogenesis, which supports the production hypothesis that neoplastic cells synthesize LDs. In our case, the neoplastic cells that were

positive for adipophilin were of the GI phenotype. This finding suggests that the neoplastic transformation of gastric epithelium with a phenotypic change to the intestinal phenotype may require the ability to absorb lipids. However, further investigation is needed to elucidate the mechanism of LD accumulation.

In this report, we present the first case of a WOS-positive gastric HP with dysplasia. We suggest that patients with a WOS-positive gastric HP should be treated by endoscopic resection to investigate the neoplastic transformation of the HP.

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CASE REPORT

Unexpected endoscopic full-thickness resection of a duodenal neuroendocrine tumor

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Abstract

A 57-year-old man underwent endoscopy for investigation of a duodenal polyp. Endoscopy revealed a hemispheric submucosal tumor, about 5 mm in diameter, in the anterior wall of the duodenal bulb. Endoscopic biopsy disclosed a neuroendocrine tumor histologically, therefore endoscopic mucosal resection was conducted. The tumor was effectively and evenly elevated after injection of a mixture of 0.2% hyaluronic acid and glycerol at a ratio of 1:1 into the submucosal layer. A small amount of indigo-carmine dye was also added for coloration of injection fluid. The lesion was completely resected *en bloc* with a snare after submucosal fluid injection. Immediately, muscle-fiber-like tissues were identified in the marginal area of the resected defect above the blue-colored layer, which suggested perforation. The defect was completely closed with a total of 9 endoclips, and no symptoms associated with peritonitis appeared thereafter. Histologically, the horizontal and vertical margins of the resected specimen were free of tumor and muscularis propria was also seen in the resected specimen. Generally, endoscopic mucosal resection is considered to be theoretically successful if the mucosal defect is colored blue. The blue layer in this case, however, had been created by unplanned injection into the subserosal rather than the submucosal layer.

Key words: Endoscopic mucosal resection; Submucosal tumor; Neuroendocrine tumor; Hyaluronic acid; Perforation; Duodenum; Endoclip

Core tip: We herein report a case of endoscopic full-thickness resection of a duodenal neuroendocrine tumor after unplanned injection into the subserosal layer. Generally, large perforations require urgent salvage surgery and duodenal perforation is more serious than other sites of the gastrointestinal tract because of bile acid and pancreatic juice. In this case, we found the "mirror target sign" immediately, and repaired the defect endoscopically. Prompt recognition of this sign and rapid closing of the defect is important to minimize injury.

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INTRODUCTION

Endoscopic mucosal resection (EMR) technique has

been developed and widely performed to provide less invasive treatment for gastrointestinal tumors. However, perforation is perhaps one of the most unfavorable complications associated with EMR. The most effective way to avoid perforation is to maintain a sufficiently thick and long-lasting submucosal cushion by endoscopic fluid injection into the submucosal layer. Various solutions such as hypertonic saline, glycerin solution, and hyaluronic acid (HA) have been used, however, among those solutions, HA is preferred because of its higher viscosity[1]. Recently, EMR has been also applied for submucosal tumors, such as neuroendocrine tumors (NETs)[2-4]. We herein report a case of an endoscopic full-thickness resection of a duodenal NET after unplanned fluid injection into the subserosal layer.

CASE REPORT

An asymptomatic 57-year-old man underwent endoscopy for investigation of a duodenal polyp detected in a private full medical checkup at another hospital. Endoscopy revealed a whitish hemispheric submucosal tumor with a smooth surface, about 5 mm in diameter, in the anterior wall of the duodenal bulb (Figure 1). Endoscopic biopsy disclosed a NET histologically. Because endoscopic findings such as size, shape, and mobility of the lesion indicated the tumor existed in the submucosal layer, EMR was conducted for its removal.

The tumor was effectively and evenly elevated after endoscopic submucosal injection of a mixture of 0.2% HA and glycerol at a ratio of 1:1 with a small amount of indigo-carmine dye added for coloration. The lesion was completely resected *en bloc* with a snare after submucosal fluid injection. Immediately, muscle-fiber-like tissues were identified at the margin of the resected defect, which suggested perforation, although a blue-colored layer was detected in the resection defect (Figure 2). The defect was completely closed with a total of 9 endoclips (Figure 3). No pneumoperitoneum was detected during or after EMR and endoscopic closure. The muscle layer was involved in the underside of the resected specimen (Figure 4). Computed tomography performed after the procedure revealed a small amount of free air but no fluid collection in the retroperitoneal space. Though the patient was in the hospital for 5 d longer than planned, he was successfully treated conservatively with intravenous fluids including antibiotics for 4 d without oral intake, and no symptoms associated with peritonitis appeared. Histologically, the tumor was diagnosed as a NET grade (G) 1 limited within the submucosal layer without muscular or lymphovascular invasion. The horizontal and vertical margins of the resected specimen were free of NET and the muscularis propria (MP) was also seen in the resected specimen (Figure 5). The blue layer in the resection defect had been created by fluid injection into the subserosal layer rather than the intended submucosal layer. No local recurrence or metastasis was detected after a follow-up of 30 mo from EMR.

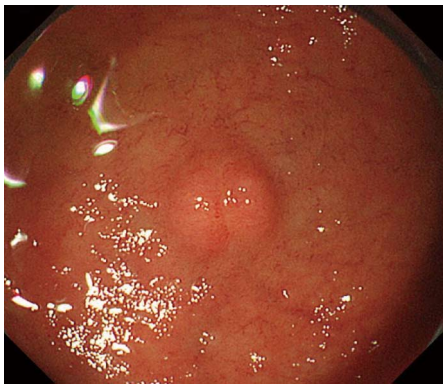

Figure 1 A neuroendocrine tumor, 5 mm in diameter, was detected in the duodenal bulb during endoscopy in an asymptomatic 57-year-old man.

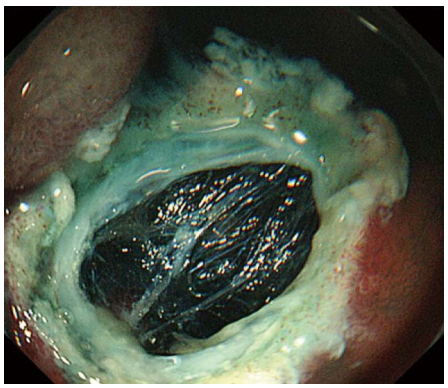

Figure 2 Although a blue-colored layer was identified in the resection defect, a small amount of a whitish layer was detected above the blue layer.

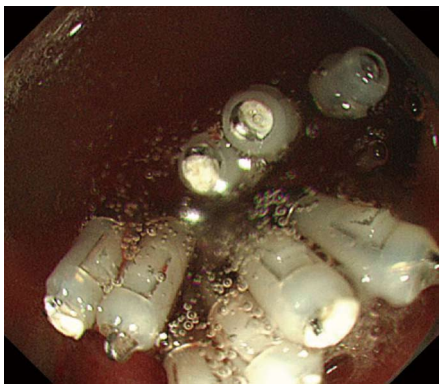

Figure 3 The defect was immediately closed with endoclips.

DISCUSSION

Duodenal NETs account for 2%-15% of gastrointestinal NETs, which are the most common type of NETs[5,6]. Some of them secret bioactive substances such as serotonin, histamine, and prostaglandin, and cause the following typical clinical presentations: cutaneous flushing, sweating, and gut hypermotility with diarrhea[7]. The metastasis rate of duodenal NETs is associated with tumor size. Duodenal NETs, which are located outside the peri-

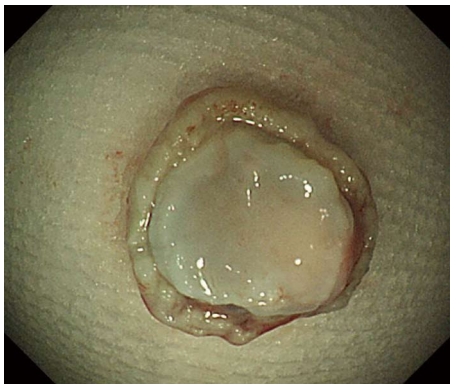

Figure 4 The muscle layer was clearly located on the underside of the resected specimen.

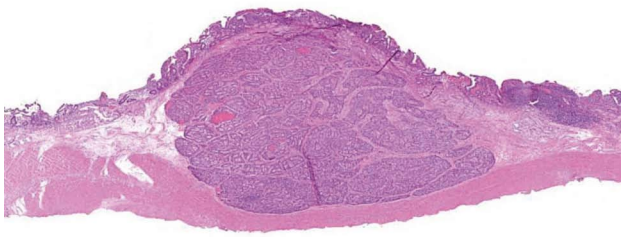

Figure 5 The muscularis propria was detected just beneath the submucosal layer (hematoxylin-eosin staining, loupe image).

ampullary region, 1 cm or smaller in size, and confined to submucosal layer, are considered to be good candidates for endoscopic resection[5,8,9]. Endoscopic resection of duodenal NETs, however, can be associated with a higher risk of perforation, as the bowel wall is thinner in the duodenum and deeper endoscopic resection is necessary because of the tumor localization.

During EMR, a sufficiently thick submucosal elevation created by appropriate fluid injection into submucosal layer is crucial for prevention of perforation, and a small amount of indigo-carmine dye is commonly added for coloration of the injected solution to help determine whether the resection defect is in the submucosal layer or muscular layer (blue or white). In the present case, the tumor was elevated easily, evenly, and sufficiently after fluid injection as if the solution was appropriately injected into the submucosal layer. In addition, as the resection defect was colored blue after resection, we initially thought that EMR was successful. After careful investigation of the defect, however, we detected a few whitish bundles suggesting the presence of MP above the blue layer, a so-called "mirror target sign". Therefore, we could recognize that the blue layer below the MP had been created by unplanned injection of fluid into the subserosal layer rather than the submucosal layer and concluded that our EMR had resulted in an unexpected full-thickness resection.

Both the "target sign" and "mirror target sign" were reported to help in identifying the endoscopic resection of the MP[10]. Surrounded by mucosa and submucosal tissue, the resected MP on the resected surface appears as the "target". In contrast, the resected defect consisted of 2 concentric rings, the inner ring seen as an area of exposed subserosal layer and the outer ring as the commonly encountered submucosal layer, which make the mirror image of the target sign. A similar case has been reported in an EMR during colonoscopy[11]. A detailed examination of the resection defect is important, but endoscopists should remember that the blue layer in the resection defect of EMR is not always the submucosal layer as unexpected subserosal injection can occur. Especially when high viscosity fluid such as HA is used during EMR, unexpected subserosal injection would raise the MP and result in an unexpected full-thickness resection. Retrospectively, the vertical approach of fluid injection in the thin duodenal wall would also explain this unexpected subserosal injection. We suggest that the injection needle could easily be misguided into the subserosal layer, as duodenal submucosal tumors occupy a significant space of the thinner submucosal layer of the duodenum. Furthermore, the thinner duodenal MP could be easily elevated by subserosal injection.

Generally, large perforations require urgent salvage surgery. Duodenal perforation related to endoscopic treatment is reported to require salvage surgery in as many as 43.6% of cases, and these perforations are more serious than those occurring at other sites of the gastrointestinal tract because of bile acid and pancreatic juice[12]. However, selected cases, especially those that can be totally repaired endoscopically, can be managed medically. In this case, prompt recognition of the potential perforation led to the successful endoscopic closure of the defect with endoclips, and moreover, subserosal fluid injection of HA also may have played some important role in sealing the defect. We believe both the clips and the subserosal fluid injection protected this patient against subsequent peritonitis and salvage surgery.

In conclusion, we herein report a case of endoscopic full-thickness resection of a duodenal NET after unplanned fluid injection into the subserosal layer. In this case, we found the "mirror target sign" immediately and repaired the defect endoscopically. A detailed examination of the resection defect regardless of its color, prompt recognition of signs of possible inappropriate resection, and immediate closure of the defect are important to minimize injury.

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4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.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]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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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

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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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